Cooperation between v-fos and v-rasHA Induces Autonomous Papillomas in Transgenic Epidermis but not Malignant Conversion

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Abstract

Transgenic mice have been previously established that express v-rasHA or v-fos exclusively in the epidermis by means of a targeting vector based on the human keratin 1 gene (HK1). Epidermal expression of v-rasHA (HK1.ras) or v-fos (HK1.fos) resulted in hyperplasia, hyperkeratosis, and later, in benign tumors. To assess the potential for oncogene cooperation in vivo mating experiments were performed. Resultant HK1.fos/ras mice exhibited an obvious increase in the severity of neonatal and juvenile preneoplastic phenotypes, together with the immediate onset of tumorigenesis as compared to single oncogene sibling controls. The HK1.fos/ras tumors grew aggressively and often compromised the animals by 10–12 weeks. However, tumors remained benign as determined by histotype and specific keratin markers. These data indicate that v-fos can cooperate with an initiating v-rasHA phenotype to generate autonomous papillomas, but additional events are required for malignant conversion.

Introduction

It is now well established that the multistage nature of carcinogenesis (1) proceeds via a series of distinctive changes at the molecular level which may involve the activation of oncogenes (2) or the inactivation of tumor suppressor/antioncogenes (3). Our understanding of the specific genetic or epigenetic changes in carcinogenesis has been greatly enhanced by the development of a variety of in vitro and in vivo model systems. One of the most successful in vivo experimental systems used to study multistage carcinogenesis to date has been the mouse skin model of chemical carcinogenesis, which identified the stages of initiation, promotion, and malignant conversion (4). This inherent multistage nature and the accessibility of the skin make this an attractive system to couple to the transgenic mouse approach to develop an animal model for multistage carcinogenesis. Furthermore, the ability to target gene expression to a specific tissue in transgenic mice has greatly enhanced the prospects of generating such an in vivo model. In particular, the targeting of known oncogenes (2), or mutated, dominant-negative antioncogenes (3), to a given tissue not only allows assessment of the role of oncogene/antioncogene action in tumor etiology but also allows mating experiments between individual transgenic strains to explore potential synergisms between genes (5).

Consistently it has been observed that particular classes of onco- genes can cooperate with each other to impart a progressive transformation both in vitro and in vivo (6). In the mouse skin system, transfection of v-fos or c-fos (activated by deregulated expression) into papilloma cell lines, which expressed a chemically activated rasHA oncogene, resulted in malignant conversion when grafted as part of a reconstructed skin onto nude mice (7). The direct cooperation between rasHA and fos in this assay was confirmed by infecting primary keratinocytes with replication defective (Psi II) retroviruses (8).

Coexpression of v-fos and v-rasHA in keratinocytes resulted in highly aggressive, metastatic squamous cell carcinomas, whereas expression of v-rasHA alone elicited benign papillomas (8). This result was in agreement with previous studies implicating v-rasHA activation as an early or initiating event in mouse skin (9, 10), producing benign tumors (papillomas), which activated fos expression could then convert (7, 8). This observation prompted the use of an epidermal targeting vector, derived from HK1, to produce transgenic mice expressing either v-rasHA (HK1.ras) or v-fos (HK1.fos) exclusively in the epidermis to exploit the roles of these oncogenes in vivo, in the background of a normal mouse (11, 12). Both genes successfully elicited epidermal phenotypes which were characterized by an initial preneoplastic hyperplasia and hyperkeratosis and later by the onset of benign tumor formation. Tumorigenesis was associated with a wound promotion stimulus (11, 12), which for rasHA often led to a regressing tumor (11). This suggested the necessity for secondary events before a tumor could achieve autonomous growth or malignancy. To test this hypothesis, mating experiments were performed and we now report that transgenic mice expressing both v-rasHA and v-fos (HK1.fos/ras) exhibited a greater severity in preneoplastic phenotypes and the early appearance of tumors which grew rapidly but did not convert to malignancy. Thus, rasHA and fos can cooperate in this transgenic mouse model to produce papillomas with an autonomous growth phenotype; however, malignant conversion requires additional oncogene/tumor suppressor gene involvement.

Materials and Methods

Generation and Identification of HK1.fos/ras Transgenic Mice.

The constructs used to target expression of v-rasHA and v-fos is shown in Fig. 1A and its design and targeting characteristics have been described previously (11, 12). Sequences encoding either v-rasHA (1) or the FBI/R chimeric form of v-fos (12) were inserted into the polylinker using BamHI and CiaI sites, creating the HK1.ras and HK1.fos transgenes, respectively. Transgenic mice were created using standard techniques as described previously (11, 12). Following detailed characterization with respect to expression levels (mRNA and protein) and phenotypes (11, 12), homozygous and heterozygous transgenic mice representative of specific HK1.ras and HK1.fos phenotypic characteristics were mated. Three independent rounds of matings were performed over a 1-year period using F1–F3 progeny from HK1.ras lines 1276 (mild phenotype, few tumors), 1203 (moderate phenotype, sporadic tumors), and 1622 (severe phenotype) and from HK1.fos lines 488 (moderate phenotype) and 443 (mild phenotype). Although the HK1.fos/ras coexpressors could be distinguished by phenotypic appearance from either HK1.ras- or HK1.fos-bearing animals, all mice were subjected to PCR analysis of tail DNA as described previously (11, 12) using oligos 1 and 2 (Fig. 1A) specific for either the v-rasHA or v-fos sequences: v-rasHA 1.5'-GGATCCGATAGAATACACGGTGC-3'; v-rasHA 2.5'-ATC- GATCCAGGACAGACACTTGAAC-3'; v-fos 1.5'-GGATACCGTTGATCT- CCGGTTTC-3'; v-fos 2.5'-CATGATGTTCTGATCAGGACAGCACACTTGCA-3'.

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2 To whom requests for reprints should be addressed, at Departments of Cell Biology and Dermatology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.
3 The abbreviations used are: HK1, human K1 keratin gene; RT/PCR, reverse transcriptase/PCR analysis; HK1.ras, HK1 vector containing v-rasHA; HK1.fos, HK1 vector containing FBI/R v-fos; HK1.ras/HK1.fos, HK1.ras and HK1.fos coexpressing mice; PCR, polymerase chain reaction; cDNA, complementary DNA.
Preparation and Analysis of RNA. Mouse epidermis prepared from 1-5-day-old neonates as described (13), together with surgically removed tumors, were routinely stored in liquid nitrogen prior to total RNA isolation using the RNeasy (Biotex Labs, Houston, TX) protocol developed by Chomczynski and Sacchi (14). Transgene expression was confirmed by Northern and RT/PCR analysis. Northern analysis was performed as described (11, 12) using a 32P-labeled 3' non-coding vector specific probe which identified a 1.3-kilobase HKI.ras- and 1.6-kilobase HKI.fos-specific band, respectively. To rapidly assess the large numbers of HKI.fos/ras tumors generated for expression of both transgenes, RT/PCR was used with BRL preamplification system (Bethesda, MD). Following manufacturers' instructions, cDNA was generated using 5 μg of total RNA and 200 ng of a HKI 3' non-coding specific oligo sequence [oligo 3 (Fig. 1A), 5'-ATCGACCTCGGTCTTGCC-3']. The resultant cDNA was amplified by PCR as described (11, 12) in a reaction mix containing both the 3' oligo 2-specific for either v-rasHa or v-fos (Fig. 1A) and oligo 4 (5'-CCCCGGGTTCGA ATTTGCCTCCTTCATTC-3') specific for vector sequences 5' to the intron (Fig. 1A) to allow for assessment of contaminating DNA in RNA samples.

Histology and Immunofluorescence. Routine histology was performed on epidermis and tumors fixed in Carnoy's solution (chloroform/acetic acid/ethanol 3:1:6 v/v) at 4°C overnight and transferred to 95% ethanol prior to being embedded in paraffin, sectioned, and stained with hematoxylin and eosin. At sacrifice, tissue samples were also embedded in OCT (Miles) and stored at -70°C prior to being sectioned. Frozen sections (5–7 μm) were analyzed for expression of mouse keratins K1, K13, and K14. Monospecific antikeratin antibodies (15, 16) from two species were used in double-staining immunofluorescence reactions. Sections were incubated with rabbit anti-mouse K1 (dilution, 1:500), guinea pig anti-K14 (1:500), rabbit anti-mouse K13 (1:500), or guinea pig anti-K13 (1:500) overnight as described (11, 12).

Results and Discussion

The HKI.ras lines 1276 (mild), 1203 (moderate), and 1622 (severe) were mated to males or females of the 488 (moderate) and 493 (mild) HKI.fos lines (11, 12). Regardless of the phenotypic severity of the parental lines or transgene expression levels (11, 12), essentially identical results were obtained for each mating. A total of 40 HKI.fos, 70 HKI.ras, and 59 HKI.fos/ras mice were generated from these matings. The genotypes of all mice were confirmed by PCR analysis of tail-tip DNA using oligos 1 and 2 (Fig. 1A) specific for either v-rasHa or v-fos (data not shown). Control siblings expressing HKI.fos alone or HKI.ras alone were typical representatives of their founder lines (11, 12). HKI.fos/ras coexpressors were immediately obvious since they displayed a greater severity in the rasHa-associated phenotypes (11) of a severely wrinkled skin (hyperplasia), smaller size, and hyperkeratosis (Fig. 2A). In the HKI.ras alone transgenic mice this phenotype typically peaked at approximately 10–12 days of age and then regressed (11). Conversely this hyperplastic/hyperkeratotic phenotype persisted throughout the lifespan of the HKI.fos/ras animals (Fig. 2A), albeit to a lesser extent after 10–14 days. Due to this marked increase in phenotypic severity, very few 1622 × 488 HKI.fos/ras siblings survived, a result similar to that observed previously in 1622 homozygotes, where the resultant massive hyperkeratosis in this severe HKI.ras phenotype often rendered the juvenile animals immobile (11). This aspect of lethality also occasionally occurred for other HKI.ras and HKI.fos matings, but at a much lower frequency, and once beyond the initial 10–14 days, HKI.fos/ras mice were generally viable.

The onset of tumorigenesis in HKI.fos/ras mice was early, with small lesions appearing in the axilla or inguinal areas by 18 days in 100% of animals. By 6 weeks (Fig. 2B) these lesions had grown rapidly but remained pedunculated, and numerous other lesions appeared over the entire surface of the mouse. HKI.fos control siblings were free of tumors for 6–7 months or longer; however, eventually tumors appeared in the axilla and inguinal areas. HKI.ras controls did not exhibit tumors until 10–12 weeks. A limited number of tumors developed and these exhibited slow growth and a high tendency to regress. These observations are identical to those previously reported for the HKI.ras or HKI.fos parental lines (11, 12). By 10–12 weeks HKI.fos/ras tumors became overlarge and collectively became greater than 10% of the animal body mass. This necessitated sacrifice of most animals; however, animals resulting from mating strains expressing...
the mildest parental HK1.ras or HK1.fos phenotypes (1276 × 493) produced less aggressive tumors and allowed the majority of animals to survive. To date (up to 1 year), no gross indications of malignant conversion have been observed.

The histology of numerous aggressive lesions showed that these tumors remained benign squamous papillomas with large areas of dysplasia but no areas of malignancy or carcinoma in situ (Fig. 2, C and D). To date, this histotype has remained even in older papillomas of the 1276 × 493 cross. Northern analysis (data not shown) of epidermal and tumor RNA showed that transgene expression levels in HK1.fos/ras mice exhibited no obvious changes compared to parental and sibling HK1.ras or HK1.fos animals (11, 12). To confirm transgene expression rapidly in the large number of tumors generated, RT/PCR was used and typical results are shown in Fig. 1B. The slight increase in band size of the amplified HK1.ras and HK1.fos cDNA compared to the HK1.fos and HK1.ras plasmid controls is due to the use of oligo 4 versus oligo 1 as the 5' oligo to PCR across the intron and rules out DNA contamination in RNA samples. All HK1.fos/ras papillomas tested to date, expressed both HK1.fos and HK1.ras mRNA (Fig. 1B, Lanes 7–14). The histological diagnosis of the HK1.fos/ras tumors, although consistent with the gross appearance of the tumors, was also confirmed by the expression of characteristic keratin markers. All tumors analyzed by double label immunofluorescence continued to express mouse K1 (Fig. 3A), which is often lost in mice on conversion to malignancy (15), and exhibited only focal K13 expression (Fig. 3B), indicative of a late stage but benign papilloma (16).

These results show that ras114 and fos can cooperate in papillomagenesis to achieve an autonomous growth phenotype but appear to require additional oncogene/antioncogene involvement to become malignant in this transgenic mouse model. This is consistent with previous studies utilizing transgenic mice in which breeding separate strains expressing myc and ras114 resulted in clonal malignant tumors, indicating that two cooperating oncogenes are insufficient for complete carcinogenesis (5). However, our current results contrast with a similar analysis of v-fos and v-ras114 cooperation in epidermal carcinogenesis using replication-defective (Psi II) viral infection of primary keratinocytes and grafting onto a nude mouse skin which resulted in malignant conversion (8). This discrepancy is likely to center on the inherent differences between the experimental systems. In particular, the unavoidable destruction of the physical tissue barriers and disruption of hormonal/homeostasis mechanisms due to the severe wounding at the graft site, coupled to the minimal immune competence of nude mice, may all have contributed to the malignant progression of v-fos/v-ras-expressing keratinocytes. Moreover, the genome of infected keratinocytes may have been exposed to multiple insertional mutations from viral integration, any one of which could have contributed to progression.

Our data to date point to a requirement of multiple complimentary events for complete carcinogenesis. It appears that ras114 activation can be an initiation event (11), producing immediate preneoplastic phenotypes (hyperplasia/hyperkeratosis) and predominantly regressing papillomas, whereas fos deregulation in our model produces phenotypes that require a long latency period and depend on a wound stimulus (12). Together, our data suggest that fos amplifies the ras114-induced phenotypes leading to the rapid development of autonomous papillomas. The actual mechanism whereby this is achieved is unknown, but it is likely to center on anomalies in the signaling pathway.
Our mice develop preneoplastic hyperplasia and regression prone papillomas, transgenic mice act as a specific model for epidermal carcinogenesis. Moreover, not only do our mice create types of tumors with predictable kinetics, coupled to the accessibility of the skin, represents an ideal opportunity to test novel therapeutic approaches, including gene therapy. However, only do our transgenic mice act as a specific model for epidermal carcinogenesis via \( \text{ras}^{\text{Hk1.fos}} \) activation, which culminates in further anomalous transcriptional control of target genes by the activated \( \text{v-fos} \). Furthermore, the observation that \( \text{fos} \) deregulation appears to amplify the \( \text{ras}^{\text{Hk1.fos}} \) phenotypes may be of significance in view of the fact that 12-O-tetradecanoylphorbol-13-acetate induces \( \text{fos} \) expression in vivo. Therefore, it may be that, in essence, constitutive \( \text{fos} \) expression acts downstream of \( \text{ras}^{\text{Hk1.fos}} \) activation on a mechanistic pathway similar to that of 12-O-tetradecanoylphorbol-13-acetate promotion.

The next logical step is to identify the tertiary cooperative events which would allow conversion of the HK1.fos/ras autonomous papillomas. Mouse chemical carcinogenesis studies have associated the trisomy of the mutated \( \text{ras}^{\text{Hk1.fos}} \) allele and the subsequent loss of the normal allele with progression (18, 19). Also, duplication of chromosome 6 (20) and the loss of a putative tumor suppressor gene distal to the \( \text{ras}^{\text{Hk1.fos}} \) locus on chromosome 7 (18, 21) appear important in papilloma progression. Therefore, the presence of the endogenous normal \( \text{c-ras}^{\text{Hk1.fos}} \) alleles may impart a normalizing function antagonistic to the \( \text{ras}^{\text{Hk1.fos}} \) cooperation in a null \( \text{p53} \) background.

Finally, the ability to target gene expression exclusively to the epidermis and generate animals expressing single or multiple oncogenes has produced an easily accessible, transgenic mouse model which appears unique among similar models in that it closely mimics the discrete stages involved in human carcinogenesis (1). For instance, our mice develop preneoplastic hyperplasia and regression prone papillomas when expressing a single oncogene, autonomous papillomas with double oncogenes and exhibit a very low frequency of spontaneous malignant conversion occurring in old (>16 months) HK1.ras and HK1.fos mice. Thus, the genetic predisposition to develop discrete types of tumors with predictable kinetics, coupled to the accessibility of the skin, represents an ideal opportunity to test novel therapeutic approaches, including gene therapy. Moreover, not only do our transgenic mice act as a specific model for epidermal carcinogenesis but they may also serve as a general epithelial model to study molecular carcinogenesis in vivo.

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4 D. A. Greenhalgh and D. R. Roop, unpublished observations.
ras<sup>TM</sup> AND fos COOPERATION IN TRANSGENIC EPIDERMIS


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