Strain-dependent differences in malignant conversion of mouse skin tumors is an inherent property of the epidermal keratinocyte

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Introduction

Mouse models have become increasingly valuable to study determinants for cancer susceptibility (1). The genetic purity of mouse strains, the elucidation of the complete mouse genome and the wide variation in susceptibility to cancer induction at specific organ sites among inbred strains provide a particularly rich background for identifying susceptibility and resistance determinants. The proof of principle for this approach has been strongly supported with the identification of specific genes determining cancer susceptibility in mice that also contribute to cancer pathogenesis in human patients. This approach has identified the phospholipase A2 gene (Pla2g2) as a modifier of intestinal neoplasia in the mouse and gastric neoplasia in the human (1,2). The protein tyrosine phosphatase receptor 1 gene (Ptprf) was identified as a factor responsible for mouse strain differences in susceptibility to colon carcinogenesis and is positioned in a locus frequently altered in several human tumors including colon cancer (3). Recently, a skin tumor susceptibility locus on mouse chromosome 2 was identified as Aurora 2 kinase (sk6), and a variant of the human homolog that is frequently amplified in colon tumors (STK15) was shown to transform cultured cells (4).

A widely studied mouse model for susceptibility and resistance determinants has been the induction of squamous tumors on mouse skin by chemical carcinogens and tumor promoters (5-7). This model has a number of advantages for susceptibility studies in that tumors are superficial, multiple and display reproducible phenotypic markers as they progress through predictable pre-malignant and malignant stages (8). Breeding strategies have produced strains with high overall tumor susceptibility or resistance confined to the skin epithelium (9) or with particular sensitivity to develop benign (squamous papillomas) or malignant (squamous carcinomas) tumors (10,11). This has resulted in the identification of independent or interacting susceptibility and resistance loci that modify papilloma or carcinoma development and survival of tumor-bearing animals (6,7,12,13).

Among the interesting inbred mouse strains studied, the FVB/N strain is widely used for production of transgenic mice. This strain was originally derived from outbred Swiss mice that were homozygous for the Fv-1a allele and thus, sensitive to infection with the B strain of Friend leukemia virus (14). An important feature of FVB/N mice is their susceptibility to carcinogenesis at several organ sites including spontaneous lung tumors (15) and induced colon (16) and mammary gland tumors (17). Studies addressing the susceptibility to chemically induced skin tumors in FVB/N mice revealed that this strain is moderately susceptible to papilloma formation, but the benign tumors are unusually prone to undergo malignant conversion (18). Furthermore, transgenic mice that express human papillomavirus type 16 (HPV-16) E6 and E7 oncogenes in skin develop squamous cell carcinomas while BALB/c, SENCAR and C57BL/6 mice with the same
transgene cassette develop only a few benign tumors (19). It is not clear if this unusual propensity for rapid and high frequency pre-malignant progression in the FVB/N phenotype is determined by systemic or target tissue factors, but the chemical and viral induction of skin tumors provide an opportunity to distinguish these considerations.

Previous studies have suggested that susceptibility to early events in skin tumor formation reside in the target tissue. Thus, skin from papilloma-susceptible SENCAR mice retained sensitivity to papilloma induction when grafted to nude mice and treated with carcinogens while skin from the resistant BALB/c strain retained resistance (20). Further, in vitro studies indicated that the target keratinocytes themselves determine this sensitivity. In an in vitro model where only initiated keratinocytes form colonies, the number of colonies derived from SENCAR skin with the parental strains, separate groups were promoted with 2 or 5 μg TPA (18). Conversion frequency was calculated by dividing the final carcinoma number by the maximum number of papillomas present in each group.

Cell culture and malignant conversion assay

Primary cultures of epidermal keratinocytes were prepared from newborn mice by the trypsin digestion method (26). Cultures of normal keratinocytes and HPV-immortalized cell lines were maintained in calcium-free Eagles Minimal Essential Medium (EMEM) supplemented with 8% chexol-treated fetal bovine serum (Gemini Bioproducts, Woodland, CA), and the final calcium concentration was adjusted to 0.05 mM. Primary dermal fibroblasts were prepared by digestion of mouse dermis with collagenase (26), and fibroblasts were maintained in EMEM with 1.4 mM calcium for 1 week prior to grafting. Cell number was determined using a Coulter Counter. Malignant conversion in vitro was quantified as described previously (24). Briefly, keratinocytes cultured from the skin of newborn mice from different strains were initiated by introduction of a H-ras oncogene by a replication defective retrovirus. This produces a population of cells that is phenotypically and genotypically analogous to papillomas. After 1 week in 0.05 mM calcium medium, cultures were treated with 13.6 μM N-methyl-12-nitro-N-nitroso-quinuclidine (MNNG) or vehicle (0.1% ethanol) for 1 h. After 48 h two dishes from each group were harvested to determine cell survival as a consequence of MNNG treatment (this determines cells at risk for conversion). The remaining cultures were maintained an additional week in 0.05 mM calcium medium and then shifted to 0.5 mM calcium medium for 4 weeks to select for expanding colonies that are calcium resistant. Fixed and rhodamine stained colonies >0.5 cm were counted and corrected for initial toxicity of MNNG treatment to determine corrected colonies per dish. At least 10 dishes were counted for each strain.

Retrovirus infection and immortalization assay

Primary mouse keratinocytes were inoculated into 60 mm dishes (1 × 10^6 cells/dish) in EMEM. After 24 h, cultures were infected with recombinant retroviruses encoding HPV-16 E6 and E7 genes (25) by replacing the culture medium with supernatant from retrovirus-producing cells for 3 h (27). Keratinocytes were infected with retroviruses encoding the empty vector as a control. Twenty-four hours after infection, cultures were trypsinized, re-plated into 3 × 60 mm dishes, and treated for 2 days with medium containing 200 μg/ml G418 to kill uninfected cells. After G418 selection, cultures were maintained for 5–6 weeks to allow colonies to form from the surviving cells. Subsequently, cultures were treated with 3% neutral-buffered formalin for 30 min, rinsed three times in PBS, and stained with Giemsa to visualize colonies. In some experiments selected colonies were picked with cloning pipettes and cells were subcultured to determine whether the cells were immortal. All of the colonies from a single 1 × 60 mm dish were pooled and subcultured to establish a monoclonal cell line.

Skin grafts

Immortal keratinocyte cell lines that had been subcultured 6–12 times were used for grafting experiments. Keratinocytes were grafted to BALB/c-derived athymic nude mice (Frederick Cancer Research and Development Center, Frederick, MD) that were between 2 and 3 months old. HPV-16-immortalized mouse keratinocytes (5 × 10^6 cells) were combined with primary mouse fibroblasts (5 × 10^5 cells) and the cell suspension (100 μl) was inoculated beneath a silicon grafting chamber on the dorsal skin of nude mice as described (28). Briefly, a small (1 cm diameter) circle of dorsal skin was removed and a silicon chamber was placed over the graft site. The suspension of keratinocytes and fibroblasts was inoculated into the chamber and allowed to establish a graft for 1 week. The chamber was removed subsequently, and the graft site was observed at weekly intervals. The dimension of tumors was measured using calipers. Mice were killed when any tumor dimension was >1 cm.

RT-PCR

Subconfluent cultures of mouse keratinocytes were lysed with Trizol (Invitrogen, Carlsbad, CA), and RNA was purified by extraction with chloroform and precipitation in isopropanol. Residual DNA was removed by precipitation in 2.5 M lithium chloride or by digestion with RNAs-free DNase (Sigma-Aldrich, St Louis, MO). For cDNA synthesis, 5 μg of total RNA was reverse transcribed for 1 h using the Superscript RTII first strand synthesis kit (Invitrogen). RT-reactions were diluted 2-3 fold for PCR, and PCR amplifications (20, 25 and 30 cycles) were performed in a volume of 50 μl using Platinum Plus PCR Supermix (Invitrogen) and primers for HPV-16 E6/E7 (forward, gacccagaaagttaccacag and reverse, tcatagtgtgcccattaacag). PCR products were analyzed on agarose gels.

Materials and methods

Tumor induction studies

Groups of 30 outbred SENCAR, BALB/c, C57BL/6 and FVB/N mice were initiated by treating with 25 μg DMBA/0.2 ml acetone at zero time and pretreated by treating with 2 μg 12-O-tetradecanoyl-phorbol-13-ace late (TPA)/0.2 ml acetone once per week for 20 weeks. Papillomas were counted every 2 weeks, and the papilloma incidence and multiplicity were calculated. SENCAR mice were maintained without further treatment for an additional 30 weeks. In order to promote a higher yield of papillomas in BALB/c, C57BL/6 and FVB/N mice, the weekly dose of TPA was increased to 5 μg from week 21 to 50. Animals were killed when suspected carcinomas were >1 cm in size or animals exhibited failing health, and tumors were examined histologically to confirm malignancy. Conversion frequency was calculated by dividing the final carcinoma number by the maximum number of papillomas present at weeks 44–48 in each group.

In order to determine whether the high frequency of malignant conversion in FVB/N mice is a dominant trait, skin tumor induction was compared in inbred SENCAR/Pt, FVB/N and (FVB/N × SENCAR/Pt) F1 mice. To induce skin tumors in these mice, the initiating dose of DMBA was 5 μg/0.2 ml acetone, and the once weekly TPA dose was either 2 μg for SENCAR/Pt and one F1 group or 5 μg for FVB/N and the other F1 group. The parental strains could not be compared at the same dose of TPA as 3 μg induced toxicity in SENCAR/Pt mice and 2 μg was sub-optimal for FVB/N mice. To compare F1 mice with the parental strains, separate groups were promoted with 2 or 5 μg TPA (18).
were separated on 1.4% agarose gels and detected by staining with ethidium bromide and UV transillumination.

Apoptosis
The susceptibility of HPV-immortalized cell lines to apoptosis was measured after three different experimental treatments including (i) maintaining cells in EMEM supplemented with 0.1% fetal bovine serum for 24 h, (ii) treatment with 0.08 mM adriamycin for 16 h or (iii) treatment with 100 mM etoposide for 16 h. The percentage of apoptotic cells was determined by adding 4',6'-diamidino-2-phenylindole (DAPI) at a final concentration of 5 μg/ml for 10 min and observing cultures by fluorescent microscopy. Nuclei that exhibited a fragmented morphology and stained brightly were graded as apoptotic.

Measurement of population doubling time
Cells were split at low density (1 × 10⁴ cells/dish) into 6 × 60 mm dishes. Cells that attached to the dish after 24 h were counted in three dishes. Trypan Blue was added to cells before the count to stain the non-viable cells. When the cells in the other 3 × 60 mm dishes were 70% confluent (~7 days), cell number was determined. The total number of population doublings was calculated from cell counts and divided by the number of days in culture to obtain population-doubling time.

Statistical analyses
Cell line data were analyzed statistically using Sigma Stat software (Systat Software, Point Richmond, CA). Analysis of variance and pair wise comparisons were performed at P < 0.05. Skin tumor induction data were analyzed by Fisher’s exact test with two-sided P values.

Results

FVB/N mice are very susceptible to malignant conversion after initiation-promotion in vivo and FVB/N keratinocytes are very susceptible to malignant conversion in vitro
Previous studies had indicated that the frequency of conversion of cutaneous papillomas to carcinomas was higher in FVB/N mice than other strains based on direct comparison after repeated DMBA treatment and historical data after initiation-promotion (18). Furthermore, FVB/N papillomas arising from promotion alone (without exogenous initiation) had a high conversion frequency. Figure 1 indicates that direct comparison of conversion frequencies for four strains of mice treated with initiation-promotion protocols indicate a 6-10-fold greater risk of conversion for papillomas on a FVB/N background than for other strains. Also evident is the complete resistance to conversion of C57BL/6 mice. The differences among SENCAR versus FVB/N are significant at P < 0.0001 and among BALB/c versus FVB/N significant at P < 0.013. Thus, papillomas arising on the FVB/N strain must be considered the ‘high risk’ type (29). Compatible with these in vivo data are results from the in vitro conversion assay (Table I) showing a very high frequency of calcium resistant foci in FVB/N-derived keratinocytes compared with the resistance of BALB/c and extreme resistance of C57BL/6 keratinocytes. The substantial number of spontaneous foci arising from FVB/N keratinocytes without MNNG treatment, reminiscent of the high conversion frequency of papillomas arising from treating non-initiated FVB/N skin with TPA, is notable. Furthermore, the high frequency of spontaneous conversion in FVB/N keratinocytes is suppressed in F₁ hybrids of C57BL/6 × FVB/N indicating this susceptibility trait is not dominant (Table I, experiment 2).

FVB/N keratinocytes are not more susceptible to immortalization by HPV-16 E6/E7 genes
We examined whether keratinocytes from different strains of mice varied in susceptibility to immortalization by HPV-16 E6 and E7 genes. Primary cultures were grown in 60 mm culture dishes (1 × 10⁵ cells/dish) and infected for 3 h with high titer

| Table I. Strain of origin of mouse keratinocytes determines sensitivity to spontaneous and induced malignant conversion in vitro |
|-----------------|----------|----------|----------|----------|
| Treatment       | FVB/N    | BALB/c   | C57BL/6  | C57BL/6 × FVB/N |
| Experiment 1    | Vehicle  | 0.1      | 8.6      | 0         | ND        |
|                  | MNNG (13.6 μM) | 3.3      | 41.4     | 0         | ND        |
| Experimenta 2    | None     | 1.0      | 11.3     | 0.1       | 0.5       |

*Primary keratinocytes from each mouse strain were initiated with the v-rasHv retrovirus, treated for 1 h with 0.1% ethanol or MNNG and selected in 0.5 mM calcium medium for 4 weeks. Cultures were fixed in formalin, stained with rhodamine, and foci were counted under a dissecting microscope. Toxicity from MNNG was similar in all groups. ND = not determined.
retroviruses encoding HPV-16 E6 and E7 genes plus the neomycin resistance gene. Controls consisted of using retroviruses that lacked the HPV genes, or mock infection (no retroviruses). Infected cultures were subcultured into 3 × 60 mm dishes, selected in medium containing G418, and maintained for 5–6 weeks to examine colony formation. Reproducibly, HPV-16 E6/E7 genes immortalized mouse keratinocytes. However, keratinocytes from BALB/c mice were immortalized at a lower efficiency than cells from other strains (Figure 2). To establish polyclonal HPV-immortalized cell lines, all colonies in a 60 mm dish were pooled and subcultured. In control experiments, cells infected with retroviruses that lacked the E6 and E7 genes did not form colonies that could be subcultured.

**FVB/N keratinocytes are more susceptible to malignant conversion after immortalization by HPV-16 E6/E7 genes**

HPV-immortalized keratinocyte cell lines from each strain of mouse were grafted to the dorsal skin of athymic nude mice. Grafting allowed the malignant potential of keratinocytes from different strains to be compared in the same host. A total of 200 mice received skin grafts and these animals were examined weekly for papillomas or carcinomas at the graft site. HPV-immortalized cell lines from SENCARA/Pt and FVB/N mice formed more tumors than cells from BALB/c or C57BL/6 mice (Figure 3, Table II). Differences in total tumor formation between FVB/N and SENCARA/Pt were not statistically significant (P > 0.05). However, the majority of tumors from FVB/N cells (78%) grew progressively and it was necessary to kill the animals. In contrast, only 46% of all tumors that arose from SENCARA/Pt cells grew progressively (Figure 3). The remaining tumors underwent partial or complete regression over the observation period of 12 months. Statistical analysis showed that the HPV-immortalized keratinocytes derived from FVB/N mice formed significantly more carcinomas than did cells from SENCARA/Pt mice (P < 0.05).

Since grafts contained both keratinocytes and fibroblasts, we examined whether tumor development was also influenced by fibroblasts of different strains. FVB/N immortal keratinocytes produced similar numbers of tumors regardless of whether they were grafted with fibroblasts from FVB/N (14 tumors/20 grafts), BALB/c (16/18) or SENCARA/Pt mice (34/69). The immortal BALB/c keratinocytes did not form more tumors when grafted with SENCARA/Pt (0/29), BALB/c (1/15) or FVB/N fibroblasts (0/10). These results suggest that the type of fibroblast did not influence tumorigenesis in this model.

Immortal keratinocytes from FVB/N or SENCARA/Pt mice formed tumors after 4–8 weeks. The tumors from BALB/c and C57BL/6 immortal keratinocytes arose later (19 and 32 weeks, respectively). The histology of tumors varied from well-differentiated squamous carcinoma to poorly differentiated. There was no significant correlation between tumor histology and strain of mouse. Histological analyses of lung, liver, spleen and kidney from tumor-bearing mice failed to detect metastasis of tumor cells.

**Immortal cell lines from different strains are not distinguished by growth parameters**

HPV-immortalized cell lines from each strain of mouse were compared in vitro for properties that might contribute to tumorigenesis. These properties included growth rate (population doubling time), susceptibility to apoptosis, and level of expression of HPV-16 E6 and E7 oncoproteins. The population doubling time varied between 30 and 41 h for different cell lines, and there were no strain-dependent differences (Table III). The susceptibility of HPV-immortalized cell lines to apoptosis was measured after three different experimental treatments including (i) maintaining cells in EMEM with reduced serum (0.1% fetal bovine serum) for 24 h, (ii) treatment with 0.08 μM adriamycin for 16 h or (iii) treatment with 100 μM etoposide for 16 h. The different immortal cell lines varied in sensitivity to apoptosis, but no strain was clearly more sensitive or resistant (Table III). The HPV-16 E6 and E7 oncoproteins are required for immortalization and malignant progression. While the level of E6 and E7 expression differed in specific cell lines (data not shown), these differences did not correlate with the tumorigenicity of a cell line from any specific strain. Thus, there was no difference in growth rate, susceptibility to apoptosis or expression of HPV genes in immortal cells from different strains of mice.

The frequency of malignant conversion of papillomas in F1 hybrids of FVB/N × SENCARA/Pt crosses reflects the frequency in the SENCARA/Pt parent

To determine if the sensitivity of the FVB/N genotype for malignant conversion is a dominant trait, FVB/N females were crossed with SENCARA/Pt males, and the parental and F1 mice were subjected to an initiation-promotion protocol.
This cross was chosen to assure that the F1 mice as well as the parents had a quantifiable papilloma yield, and the dose of TPA was chosen for each parent and both F1 groups to maximize papilloma induction (18). Figure 4 indicates that the final papilloma yield of the F1 animals was similar to the SENCARA/Pt parent at 2 \( \text{mg} \) TPA \((P \approx 0.43)\) and even higher than the SENCARA/Pt parent when 5 \( \text{mg} \) TPA was used for promotion. In contrast, the final papilloma yield of the F1 animals at 5 \( \text{mg} \) TPA was substantially higher than the FVB/N parent \((P < 0.0001)\). Nevertheless, when the carcinoma incidence was determined and the conversion frequency was calculated, the frequency was similar to the SENCARA/Pt parent in both F1 groups and substantially lower than the FVB/N parent. This indicates that the determinants for enhanced pre-malignant progression and malignant conversion in the FVB/N strain are subject to suppression by the SENCARA/Pt genotype, a finding consistent with the suppression detected in the F1 cross between C57/BL6 x FVB/N in the in vitro conversion assay.

**Discussion**

The clonal evolution of mouse skin keratinocytes through a series of predictable phenotypic stages toward squamous cell...
carcinomas as a consequence of topical treatment with initiating and promoting agents, exposure to ultraviolet light or transformation with oncogenic viruses has been a powerful model in defining the biology of carcinogenesis. The association of reproducible genetic and biochemical changes with these stages has contributed important information about the mechanism. The exceptional variability among mouse strains in susceptibility to these transforming protocols combined with confirmatory studies in genetically modified or hybrid crossed mice has also yielded insights into the genetic determinants of predisposition to cancer development that will undoubtedly have translational implications for identifying modifiers of human cancer risk (1,2,4,30,31). These latter studies have yielded more than a dozen loci exhibiting epistatic interactions that contribute to the development of pre-malignant papillomas (5–7,13).

It is now clear that a series of distinct loci determine the risk for progression from benign to malignant phenotypes in this model system. For example, interspecies crosses between Mus musculus and Mus spretus have indicated that loci on chromosomes 5 and 7 contribute to susceptibility to carcinoma development while a site on chromosome 6 confers resistance (12,13). The highly selected Car-S and Car-R mouse strains, derived from extensive intercrossing of eight inbred mouse strains and selection for sensitivity (S) or resistance (R) to skin papilloma formation, have paradoxically opposite sensitivity to the frequency of malignant conversion (11). These studies have yielded more than a dozen loci exhibiting epistatic interactions that contribute to the development of pre-malignant papillomas (5–7,13).

Strains not is a dominant trait. This result contrasts with the dominance of cancer risk associated with the F1 offspring of crosses between SSIN and SENCAR mice where susceptibility to malignant conversion was restored to the SSIN haplotype (32). Together these results suggest additional complexity in the genetic control of cancer predisposition.

Host factors that work together with or contrary to predisposition characteristics of the target cell undoubtedly contributed to this complexity. Previous studies have indicated that the inflammatory response associated with initiation-promotion studies is genetically determined, and mice have been bred to enhance or diminish this response (33). Inbreeding for enhanced acute inflammation diminishes both the papilloma and carcinoma yield, yet treatment with anti-inflammatory agents is also clearly chemopreventive in the skin model (34). The answer to this paradox may reside in the type of inflammatory response involved in a stage-specific manner. Thus, γδ T cells protect against malignant conversion of chemically induced skin tumors (35), and infiltration of mast cells in pre-malignant lesions enhances angiogenesis and malignant conversion of HPV-16-induced cutaneous neoplasms (36). Systemic cytokines may also contribute to pre-malignant progression as suggested by studies showing that elevated circulating TNF-α is associated with the rapid de novo appearance of squamous cell carcinomas on carcinogen-treated skin of transgenic mice over-expressing PKCε in the epidermis (37). The extent to which any of these responses contributes to cancer susceptibility in inbred strains remains to be established. While tumor stromal cells other than inflammatory cells can contribute to the tumor phenotype in several cancer models (38), our results suggest that normal dermal fibroblasts from either sensitive or resistant strains do not contribute to tumor outcome in HPV-16-immortalized keratinocytes.

While our studies do not reveal the precise factors responsible for the intrinsic sensitivity or resistance of keratinocytes for pre-malignant progression, they do provide some clues and a foundation for further analysis at the cellular level. From the HPV-16 studies, the frequency of immortalization of keratinocytes among the strains did not correlate with susceptibility to malignant conversion. Thus, immortalization is not the rate-limiting step in this model. This is consistent with the fact that HPV-16 E6 and E7 genes cooperate to immortalize keratinocytes with high efficiency, and that these immortal cells initially maintain a fairly normal pattern of differentiation when examined in organotypic culture or after grafting to nude mice.
(25,27). Thus, immortalization by HPV may represent an early step in pre-malignant progression. Nor could the growth rate of immortalized cells, susceptibility to apoptosis or expression of HPV-16 E6 and E7 oncoproteins explain the susceptibility to malignant conversion. Since the E6 and E7 oncoproteins would inactivate the p53 and Rb pathways in keratinocytes from all strains, it seems unlikely that these pathways are determinant for the strain differences. However, it should be noted that malignant progression is much accelerated in skin carcinogenesis studies performed on p53 null mice (39,40). The strain-associated susceptibility or resistance to MNNG-induced malignant conversion in the calcium-resistance assay suggests that carcinogen metabolism is not a determinant as MNNG is a direct acting mutagen. Other changes intrinsic to the target cells that have been associated with pre-malignant progression in the skin model include integrins, cadherins, the cytokine GROα, AP-1 transcription factors and the TGFβ signaling pathway (41–45). Using keratinocytes from inbred strains that vary substantially in susceptibility and retain this characteristic in vitro should be helpful in defining the underlying basis for these susceptibility differences.

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References


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