Immunogenetic Influences on the Initiation Stage of the Cutaneous Chemical Carcinogenesis Pathway

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Abstract

While it is generally agreed that environmental exposure to solar radiation and to certain classes of chemicals are the major causes of nonmelanoma skin cancer, it is also believed that genetic polymorphisms regulating immunological responses are important determinants of individual susceptibility to skin cancer. However, little is known about their interactions with the chemical carcinogenesis pathway prior to the actual development of tumors. This issue was examined by comparing susceptibility to skin cancer in C3H/HeN and C3H/HeJ mice, two strains that differ only at the lipopolysaccharide genetic locus, which serves as a regulator of a number of immunological activities. When subjected to a two-stage cutaneous tumorigenesis protocol, C3H/HeJ mice, which have a mutation at the lipopolysaccharide genetic locus that renders them deficient in their capacity to produce cytokines and to activate macrophages, developed nearly three times as many tumors as did C3H/HeN mice, which do not have this mutation. Epidermal DNA activate macrophages, developed nearly three times as many tumors as did C3H/HeN mice, which do not have this mutation. Epidermal DNA binding of 7,12-dimethylbenz(a)anthracene, an index of tumor initiation, was also significantly greater in C3H/HeN than in C3H/HeJ mice. Immunological activities regulated by the lipopolysaccharide genetic locus thus confer resistance to DMBA-induced cutaneous tumorigenesis in mice and are associated with changes that occur early in the tumorigenesis pathway, prior to the development of tumors.

Introduction

Although immunological factors play an extremely important role in controlling the growth and metastasis of tumors to remote sites (1), relatively little is known about interactions between the immune system and the carcinogenesis pathway at stages prior to the actual development of tumors. One strategy that has been used to assess the participation of the immune system in the pathogenesis of other diseases has been to compare the onset, progression, or severity of those diseases in inbred strains of mice that differ in their immunological capabilities (2–5). We have used this approach to evaluate whether there are immunological influences on early stages in the cutaneous carcinogenesis pathway. We compared the tumorigenic and tumor-initiating activities of the polyaromatic hydrocarbon carcinogen DMBA in C3H/HeN and C3H/HeJ mice. These two strains differ only at the Lps locus, a gene which lies on chromosome 4 of the mouse and which controls a number of immunological activities (6). C3H/HeJ, which carry the mutated Lps allele, respond poorly to the bacterial product LPS.

As a consequence, biosynthesis and release of TNF-α and of other cytokines and macrophage activation to a number of activating agents are deficient in this strain (6). C3H/HeN mice, on the other hand, carry the Lps allele and respond normally to LPS by synthesizing and releasing substantial quantities of cytokines and by activating macrophages in a normal manner. Our studies indicate that C3H/HeJ mice, which carry the mutated allele, exhibit both enhanced tumor and DMBA-DNA adduct formation, providing evidence that the Lps locus plays an important role in protecting against DMBA-induced skin tumorigenesis at a very early stage in the pathway.

Materials and Methods

Animals and Chemicals. Female C3H/HeN mice were obtained from Charles River Breeding Laboratories (Kensington, NY). Female C3H/HeJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). Preliminary studies confirmed previous results that both strains possess the high-affinity aryl hydrocarbon hydroxylase receptor in that they were highly inducible for aryl hydrocarbon hydroxylase activity by skin application of CYP1A1 inducers. All mice were 6 weeks of age when started on the study. DMBA was purchased from Aldrich Chemical Co. (Milwaukee, WI). TPA and d,l-ornithine were obtained from Sigma Chemical Co. (St. Louis, MO). [3H]DMBA (specific activity, 97.4 Ci/mmol) and d,l-[1-14C]ornithine (specific activity, 58 Ci/mmol) were purchased from New England Nuclear (Chicago, IL). All chemicals were of the highest purity commercially available.

Skin Tumorigenesis. Panels of mice (20 mice/panel) were subjected to a standard two-stage cutaneous chemical tumorigenesis protocol, using DMBA as the tumor initiator and TPA as the promoting agent (7). One hundred μg of DMBA (0.1% w/v in acetone) were applied to dorsal skin that had been shaved and treated with a depilatory the day before. One week later, 40 nmol of TPA were applied biweekly to the area of skin that had previously been treated with DMBA. Animals were examined weekly for the presence of tumors. Only tumors that had a diameter of 1 mm or greater and persisted for 2 weeks or longer were counted.

Assessment of DMBA-DNA Adduct Formation. Animals were treated with a single dose of 100 mcg [3H]DMBA (5 mmol in 0.1 ml acetone). At various times thereafter (as indicated in "Results"), animals were sacrificed by cervical dislocation. Skin was removed, epidermal homogenates were prepared, and DNA was purified using procedures that have been described previously (8). Isolated, epidermal DNA was digested with RNase A (1000 units/ml), washed three times in acetone, dried under nitrogen, and dissolved in 0.1 m sodium chloride (pH 7.0). The yield was estimated by measuring its absorption at 260 nm. The purity of isolated DNA was assessed by the absorbance ratios A260:A280 ≥ 1.98 and A260:A320 ≥ 2.21. An aliquot of isolated DNA was counted on a Packard Tri-Carb 460 CD liquid scintillation spectrometer to determine the amount of [3H]DMBA bound to epidermal DNA.

TPA Induction of ODC Activity. The kinetics of TPA-induced dermal ODC activity was assessed by applying 40 nmol of TPA topically to the skin of mice. At various times thereafter animals were sacrificed, epidermal 100,000 X g supernatant fractions were prepared,
and ODC activity was determined by measuring $^{14}$CO$_2$ production from $d_L$-$[^{14}$C]ornithine as described earlier (9).

Results

DMBA-induced Skin Tumorigenesis. The role that genes within the *Lps* locus play in cutaneous chemical tumorigenesis was evaluated by comparing the development of cutaneous tumors in panels of C3H/HeN and C3H/HeJ mice subjected to the identical DMBA initiation, TPA promotion protocol. As shown in Fig. 1, the number of tumors per mouse was much greater in C3H/HeJ mice than in C3H/HeN mice. This was significant at the $P < 0.001$ level using Fisher's exact test. In C3H/HeJ mice, the latency for tumor development was 5 weeks, whereas it was 11 weeks for C3H/HeN mice. A similar trend was observed when the data were evaluated as the percentage of mice with tumors (Fig. 2). By 15 weeks on protocol, 72% fewer adducts were observed. Tumors from both strains were found to be squamous papillomas.

$[^{3}$H]DMBA Binding to Epidermal DNA. Carcinogen-DNA adduct formation has been shown to correlate quite closely with subsequent tumorigenesis by chemical carcinogens, and quantitation of adduct formation has been utilized as an index of the susceptibility to tumor initiation (10, 11). C3H/HeN and C3H/HeJ mice were therefore treated with topically applied $[^{3}$H]-DMBA, and adduct formation to epidermal DNA was determined (Table 1). Significantly less $[^{3}$H]DMBA binding to epidermal DNA occurred in C3H/HeN compared to C3H/HeJ mice. This was evident 1 day after $[^{3}$H]DMBA application and was progressive. After 24 h there were 18% fewer detectable DMBA-DNA adducts in the epidermis of C3H/HeN mice, and by 20 days, 72% fewer adducts were observed.

![Fig. 1.](image1.png) Comparison of the number of tumors per mouse in C3H/HeN and C3H/HeJ mice subjected to the same DMBA-induced skin tumorigenesis protocol. The number of tumors per mouse was plotted as a function of the number of weeks on the test.

Table 1: Extent and persistence of topically applied $[^{3}$H]DMBA binding to epidermal DNA in vivo in C3H/HeN and C3H/HeJ mice

<table>
<thead>
<tr>
<th>Days after DMBA treatment</th>
<th>Covalent binding (pmol/mg DNA)</th>
<th>% fewer DMBA-DNA adducts in C3H/HeN compared to C3H/HeJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C3H/HeJ*</td>
<td>C3H/HeN*</td>
</tr>
<tr>
<td>1</td>
<td>141.0 ± 6.3</td>
<td>177.0 ± 2.4</td>
</tr>
<tr>
<td>5</td>
<td>90.0 ± 4.2</td>
<td>60.0 ± 1.3</td>
</tr>
<tr>
<td>10</td>
<td>25.6 ± 1.1</td>
<td>8.6 ± 0.5</td>
</tr>
<tr>
<td>20</td>
<td>15.0 ± 0.8</td>
<td>4.2 ± 0.3</td>
</tr>
</tbody>
</table>

* Mean ± SEM of four individual values.

Induction of Epidermal Ornithine Decarboxylase Activity by TPA. It is recognized that the induction of epidermal ODC by cutaneous application of tumor promoters is a necessary, though not sufficient, index of the tumor promotion stage of carcinogenesis (Ref. 9 and references therein). Experiments were therefore conducted to determine whether epidermal ODC activity differed in C3H/HeN and C3H/HeJ mice following topical application of TPA. The skin of each strain was painted with 40 nmol of TPA, and at various times thereafter skin was removed and assayed for epidermal ODC activity. The kinetics of TPA-induced epidermal ODC activity were similar in the two strains (Fig. 3). This, in combination with the $[^{3}$H]DMBA binding data, provide strong evidence that differences in susceptibility to DMBA-induced skin tumor formation in these animals relate to differences at the tumor initiation stage of tumorigenesis.

Discussion

The induction of cancer is a multifactorial process that remains only partially defined. While the role of exposure to environmental carcinogens is now indisputable, it is clear that there are a number of other determinants that can influence tumor susceptibility. Our data provide evidence that factors regulated by the *Lps* locus play an important role in controlling skin tumor development in experimental animals. These are likely to be immunological in nature, since the *Lps* locus controls the synthesis and production of cytokines, including TNF-α, and participates in the activation of macrophages.
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Fig. 3. Induction of epidermal ODC activity by TPA in C3H/HeN and C3H/HeJ mice. Animals were treated with TPA, and ODC activity was assessed as described in “Materials and Methods.” Each data point represents the mean ± SEM of four mice.

However, other, as yet unidentified nonimmunological differences between these two strains could also be involved. The conclusion that the Lps locus plays a role in skin tumor development is based on the observation that C3H/HeN and C3H/HeJ mice differed substantially in their capacity to generate cutaneous tumors in response to topical application of the prototypic polyaromatic hydrocarbon carcinogen DMBA. Carcinogen-DNA adduct formation was also significantly greater in C3H/HeN mice, indicating that susceptibility to DMBA-induced tumor formation was likely due primarily to differences that occur early in the tumorigenesis pathway, during tumor initiation. Taken together, these results are consistent with the hypothesis that the efficiency of the host immune response in removing adducts formed by topical application of chemical carcinogens shortly after the time of exposure has a critical impact on tumor susceptibility. In other words, attempts are made by the host immune system to minimize the adverse effects of carcinogenic chemicals almost immediately after exposure rather than waiting until tumors have already developed.

The finding that C3H/HeJ mice were more susceptible to chemical-induced skin tumorigenesis than were C3H/HeN mice was somewhat surprising in light of what has been predicted for UV radiation-induced skin tumors (5). In C3H/HeN mice, low doses of UV radiation create a milieu within the skin in which suppression of selected cell-mediated immune responses can occur (12, 13). In contrast, treatment of C3H/HeJ mice with similar doses of UV radiation evokes no immunosuppressive response (5). Since local UV-induced alterations in cell-mediated immunity are thought to play an important role in the immunopathogenesis of UV-induced skin cancer (1), it has been proposed that the genetic defect at the Lps locus in C3H/HeJ mice enhances resistance to this type of skin cancer (5). However, it should be noted that no experiments have been conducted subjecting C3H/HeN and C3H/HeJ mice to a UV-induced photocarcinogenesis protocol to test this hypothesis.

Our studies have not addressed differences in the capacity of C3H/HeN and C3H/HeJ mice to destroy tumors once they have already developed. Although there are no data regarding the growth of transplanted tumors in LPS-nonresponsive mice compared to LPS-responsive mice, macrophages from C3H/HeN mice do have an enhanced capacity to kill tumor cells in vitro as compared to macrophages from C3H/HeJ mice (14).

However, even if such growth-inhibitory effects on DMBA-induced tumors are observed in vivo, it does not diminish the importance of the effects of the Lps locus on tumor initiation. It is well established that the formation of DMBA adducts with DNA is a necessary precondition for the development of skin cancer and that the level of adducts correlates closely with tumorigenicity (10, 11).

Although activation of the Lps locus has clearly been associated with endotoxin administration, one implication of our results is that other mediators besides LPS influence the activation of this genetic locus. This observation is not without precedent, since other environmental chemicals, many of which are tumorigenic, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (15), polybrominated biphenyls (16), and mycotoxins (17), have been associated with endotoxin hypersensitivity. Lps locus-induced production of tumor necrosis factor has been identified as mediating many of the immunotoxic effects of these agents, although its effect on tumorigenic activity has not been investigated. This could mean that, in addition to its role in the host response to Gram-negative bacteria, the Lps locus may play an important role that protects against tumor induction.

The Lps gene controls a number of immunological activities (6). Thus, an issue of utmost importance is identification of the precise activity governed by the Lps locus that inhibits the tumor initiation phase of the chemical tumorigenesis pathway. Animals treated with immunotoxins develop a “wasting syndrome” similar to that observed following TNF-α administration to animals (15). TNF-α is also known to be a potent cytolytic and cytostatic agent for neoplastic cells (18, 19). It has been shown to be produced by keratinocytes (20), to have a proinflammatory influence on the skin (21), and to regulate keratinocyte proliferation (22). It is thus possible that TNF-α production by keratinocytes or other cells exposed to DMBA could protect animals against tumor induction by selective cytolytic or cytostatic effects on DMBA-initiated cells. In this scenario, C3H/HeJ mice would develop an excessive number of tumors because of an inherent deficiency in TNF-α production.

An alternative explanation for our findings may relate to differences in the capacity of these two strains of mice to mount a cell-mediated immune response against DMBA-initiated cells. In previous studies, we have found that when DMBA is applied to the skin of C3H/HeN mice, it initiates a vigorous contact hypersensitivity response (23). Although the role between contact hypersensitivity to DMBA and DMBA carcinogenesis is unclear, we have also found that the magnitude of DMBA contact hypersensitivity is greater in C3H/HeN than in C3H/HeJ mice (5). Quantitative and/or qualitative differences in the cutaneous inflammatory infiltrate that develops following DMBA administration could diminish the number of immunocompetent cells with cytolytic activity for DMBA-initiated keratinocytes in C3H/HeJ mice and thereby alter the neoplastic effect.

Nonmelanoma skin cancer is the most common type of human malignancy. While environmental exposure is ultimately responsible for the development of such neoplasms, immunological factors also play a prominent role. Using murine cutaneous tumorigenesis as a model, we have demonstrated that genetic differences at the Lps locus, a gene which controls a variety of immunological activities, can influence susceptibility to skin cancer development and that such changes are associated with differences that occur very early in the tumorigenesis pathway.

5 C. A. Elmets, unpublished data.
pathway. The importance of these observations rests upon the concept that the ultimate development of a tumor in response to a carcinogenic chemical, while clearly requiring DNA modification and oncogene expression, may also be influenced by the efficiency by which these adducts are identified and removed by the host immune system.

References

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