A Dominant Negative c-jun Specifically Blocks Okadaic Acid-induced Skin Tumor Promotion

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ABSTRACT

Okadaic acid (OA) is a prototypical non-phorbol ester skin tumor-promoting agent that works by inhibiting protein phosphatases, leading to an increase in protein phosphorylation. Increased protein phosphorylation can lead to stimulated signaling through various signal transduction pathways. One or more of the pathways affected by OA leads to increased signaling via the activator protein 1 (AP-1) transcription factor. Because AP-1 signaling has been shown to be required for skin tumor promotion by phorbol ester, studies were undertaken to determine whether AP-1 signaling is also required for 7,12-dimethylbenz(a)anthracene (DMBA)-initiated/OA-promoted skin tumorigenesis. Transgenic mice expressing a dominant negative c-jun (TAM-67) controlled by the keratin 14 promoter in ICR mice were used to determine the effects of OA on AP-1 signaling. By crossing the TAM-67 mice with mice expressing an AP-1-responsive luciferase, it was shown that TAM-67 decreases AP-1 activation in response to OA treatment by 95%. After 7,12-dimethylbenz(a)anthracene initiation, the TAM-67 mice and nontransgenic littermates were promoted with twice weekly applications of OA. These experiments showed that TAM-67 expression decreased tumor multiplicity by 90%. Additional experiments with TAM-67 mice showed that the hyperplastic response to OA is not impaired in these mice, nor were there differences in OA-induced transcription of various genes known to be AP-1 responsive under other conditions. This result suggests that only a subset of AP-1-regulated genes is targeted by TAM-67 when it prevents tumor promotion by OA. A determination of the mechanism by which TAM-67 can block tumor promotion without affecting hyperplasia will be important.

INTRODUCTION

OA is a non-phorbol ester tumor promoter. OA is a diarrheic shell fish toxin isolated from black sponges (1). The OA family of toxins includes microcystin-LR, a hepatotoxin. Microcystin is a potent liver tumor-promoting agent in rats (2). Microcystin in contaminated drinking water may be the cause of elevated rates of primary liver cancer in some areas of China (2). Microcystin-LR has been shown to mediate tumor promotion via inhibition of protein phosphatase type 1 and type 2A (3). Similarly, OA acts by inhibiting protein phosphatases 1, 2A, and 3, which leads to an increase of the phosphorylated forms of such proteins as Cdc25, histone H1, phosphorylase kinase, PKA, PKB, PKC, IκB kinases, and cyclin-dependent kinases (4, 5). The absence of the dephosphorylation control of these proteins can lead to an increase in proliferation, aberrant mitosis, or growth arrest, depending on the cell type. It has been suggested that the effects of the OA class of tumor promoters are mediated in part by the transcription factor AP-1 (6).

The AP-1 transcription factor is a dimer consisting of 18 different combinations of Jun-Jun or Jun-Fos proteins. The Jun family of proteins includes c-Jun, JunB, and JunD. The Fos family of proteins includes c-Fos, FosB, Fra-1, and Fra-2 (7). AP-1 activation can affect proliferation, cell cycle, growth arrest, and apoptosis (8). In particular, AP-1 has been shown to regulate cyclin D1, p53, p21 (cip1/waf1), p19 (ARF), and p16 (7). Thus, the effect of AP-1 activation in some cases closely parallels treatment with OA. In the mouse transformed keratinocyte 308 cell line, the major components of the OA-activated AP-1 complex are JunD and FosB (8). OA has been shown to activate extracellular signal-regulated kinase 1/2, which in turn can phosphorylate and activate JunD and FosB in 308 cells (9). OA also induces expression of urokinase-type plasminogen activator and stromelysin-1 in vivo, both of which are known to be regulated by AP-1 (10). Thus, the transcription factor AP-1 plays a crucial role in the biological effects of OA.

Nonmelanoma skin cancer is often studied using the two-stage model of mouse skin carcinogenesis. In this model, a single dose of an initiating agent is used, followed by multiple doses of a tumor-promoting agent. This results in formation of benign papillomas. The initiating agent for these studies is often DMBA. Chronic exposure to tumor-promoting agents such as phorbol esters results in the development of papillomas. The archetypical phorbol ester is TPA. TPA has been found to activate the AP-1 family of transcription factors. Thus, TPA induces gene transcription that eventually leads to the formation of benign tumors (11).

There is evidence that AP-1 contributes to all of the stages of skin tumor promotion/progression. The role of AP-1 in skin tumor promotion/progression was first suggested by experiments in JB6 cells in which promotion-sensitive (P- cells) but not promotion-resistant (P+ cells) were shown to have elevated AP-1 activity in response to agents known to cause transformation of P+ cells (12). The importance of this activity has been demonstrated using AP-1 inhibitors, dominant negative c-jun, or all-trans-retinoic acid (13) to block transformation by TPA or epidermal growth factor. The evidence that AP-1 plays a role in maintenance of the malignant phenotype comes from studies of cell systems. In one model, Domann et al. studied the papilloma-forming cell line 308 and a malignant variant, 10Gy5 (14). In this model, they found that the malignant cells had constitutive elevation of AP-1 binding and transactivation. This was associated with an increase in expression of c-jun and c-fos. The elevated AP-1 activity was also associated with increased expression of the MMPs stromelysin-1 and urokinase-type plasminogen activator. Further evidence for the role of AP-1 in tumor formation comes from studies of the mechanism of action of phenolic antioxidants. This work showed that the phenolic antioxidant BHQ induced expression of members of the fos family that have low transactivation potential. This work showed that AP-1 complexes induced by BHQ could block activation of AP-1 activity by TPA, thus providing a possible mechanism of BHQ activity by decreasing the transactivation potential of AP-1 complexes (15).

One way to study the role of specific proteins in carcinogenesis is
to create a dominant negative. Brown et al. (16) created an NH₂-terminal deletion of c-jun, removing the transactivating domain. This protein, called TAM-67, inhibits AP-1-induced transcription when expressed in cells (16). Whereas the wild-type c-Jun can transform normal rat embryo cells in cooperation with an activated c-Ha-ras, TAM-67 cannot (16). Expression of TAM-67 significantly decreased the transformation of JB6 cells after treatment with tumor-promoting compounds such as TPA and epidermal growth factor (13). In addition, stable expression of TAM-67 in 308 cells was shown to block TPA-induced Matrigel invasion (17). TAM-67 expression in malignant cells blocked tumor formation upon s.c. injection into athymic nude mice (18). These results demonstrate that TAM-67 can influence important events in tumorigenesis.

To directly test the importance of AP-1 in skin tumor promotion in vivo, Young et al. (19) created a transgenic mouse that expresses TAM-67 in the skin driven by the K14 promoter. Transgenic TAM-67 mice developed significantly fewer tumors when treated with a DMBA/TPA protocol for skin tumor promotion. It would therefore be of interest to determine whether the requirement for AP-1 activation extends to other tumorigenesis protocols. To address this issue, we have bred K14-TAM-67 mice onto an ICR background, a strain of mouse susceptible to OA-induced skin tumor promotion. We have treated these mice with a DMBA initiation, OA promotion protocol. We found that these mice developed fewer tumors than their wild-type littermates, demonstrating that TAM-67 specifically inhibits skin tumor promotion. These results support the notion that tumor promoter-induced AP-1 activity is important for skin tumor promotion by an environmentally relevant agent and can thus be considered as a relevant molecular target for cancer prevention.

MATERIALS AND METHODS

Breeding of Transgenic Mouse Lines. K14-TAM-67 (19) and AP-1-Luc (20) mice were obtained from Dr. Colburn (National Cancer Institute-Frederick) on a DBA/2 genetic background. Both of these transgenes were bred onto the ICR genetic background by crosses with ICR mice from Harlan Sprague Dawley (Indianapolis, IN). After generation N6, these mice were used in experiments. Offspring carrying the K14-TAM-67 transgene and/or AP-1 Luc transgene were identified by PCR analysis of tail DNA using the following primers: K14 primers (GenBank accession number U11076; 1693–1717 and 2205–2181 bp) with H-Ras primers 7817 (ccacccccattaacgtgtgacgagcc) and 7817 (gctagccataggtggctcactgatg) or Luc primers gcggaatacttcgaaattg and 5'-aacatgctcagggaacagg-3'.

RNA Isolation. Tissues for RNA expression were harvested from animals after CO₂ asphyxiation. Full thickness dorsal skin was snap-frozen in liquid nitrogen. Epidermis was separated from the dermis as described previously (19, 21). For RT-PCR, 10–1.5-mm ear punches were harvested. All tissue was pulverized in liquid nitrogen with a mortar and pestle and immediately placed in extraction buffer, homogenized, and extracted with phenol-chloroform using a protocol.

RT-PCR. Two-tube RT-PCR was performed as described by the Qiagen protocol for Omniscript reverse transcriptase. Total RNA was primed using 200 ng of each antisense primer (5’-ggcctcatgatttattg-3' for mouse GAPDH and 5'-gtgataagggaatggttgg-3' for the human growth hormone incorporated with the TAM-67 transgene). The reverse transcription reaction was followed by 40 cycles of PCR. Sense primers were 5'-aagattgtcagcaatgcatcc-3' for GAPDH and 5'-aacatgctcagggaacagg-3' for TAM-67.

Assays of AP-1-Luc Activity. AP-1-Luc activity was measured in AP-1-Luc/K14-TAM-67 mice and in their AP-1-Luc/K14-TAM-67 siblings. Mice were treated with 100 µl of acetone on the left ear and 100 µl of OA (2 µg) on the right ear. After 24 h, six ear punches of 1.5 mm were collected. The Luc activity of these samples was determined. A BCA protein assay (Pierce, Rockford, IL) was then performed, and relative light units (RLU) was normalized to protein amount.

Two-stage Skin Carcinogenesis. Dorsal skins of 8-week-old mice were shaved, and 2 days later, a single dose of 100 µg (390 nmol) of DMBA (Sigma, St. Louis, MO) in 200 µl of acetone was applied. One week after initiation, mice were treated with 2 µg of OA (Alexis, San Diego, CA) in 200 µl of acetone twice a week for 20 weeks.

OA Induction of Hyperproliferation and AP-1 Activation. Four animals/group were shaved 2 days before treatment. A single dose of either acetone or OA (2 µg in 0.2 ml of acetone) was painted on dorsal skin. Samples were collected 48 h after the treatment. Five-µm sections were stained with H&E. OA-induced hyperproliferation was determined from H&E-stained slides. Hyperproliferation was determined by measuring the thickness of the epidermis. Three measurements were made at random from the surface of the epidermis, starting from the top of the basement membrane to the bottom of the stratum corneum.

Northern Blot Analysis of Gene Expression. Total epidermal RNA (15 µg) was size-fractionated on a formaldehyde-agarose gel and transferred to a nylon membrane. This was then sequentially probed for stromelysin-1, keratin 6 (K6), epidermal fatty acid-binding protein, and 75S as described previously (10). A phosphorimaging screen was then exposed to the blot overnight, and the results were scanned on a MD Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

To demonstrate that the K14-TAM-67 transgene was expressed in transgenic mice, RNA isolated from ear punches from mice identified as TAM-67 or TAM-67 was used for RT-PCR. The primers were located in TAM-67 and an exon of the human growth hormone gene 3' to the TAM-67 coding sequence. Thus, the primers amplified only the transgene. Control reactions for GAPDH were also run. This analysis indicated that the TAM-67 mice actively transcribed the TAM-67 transgene in the epidermis and that the TAM-67 littermates did not transcribe the transgene (Fig. 1A). It should be noted that in the absence of a cDNA template, the samples from TAM-67+ mice showed a band primed from contaminating DNA. In the presence of a cDNA template (in the TAM-7, +RT lane), the DNA band disappeared due to the relative abundance of cDNA template versus DNA contamination.

To determine the effects of TAM-67 on OA-induced AP-1 activity, K14-TAM-67+ transgenic mice were crossed with AP-1-Luc+ mice. The offspring, which were either Luc+/Tam- or Luc+/Tam+, were treated on the ears with 2 µg of OA in acetone or with acetone alone. Ear punch biopsies were taken 24 h later and harvested for protein. The Luc assay based on these protein samples (Fig. 1B) showed that the skin of these mice had a detectable basal AP-1 activity. There was a small, statistically insignificant difference in basal AP-1 activity between Luc+/K14-TAM-67 and Luc+/K14-TAM-67 mice. Upon treatment with OA, there was an 80-fold increase in AP-1 activity in Luc+/K14-TAM-67 mice, whereas in Luc+/K14-TAM-67 mice, AP-1 activity was only induced 8-fold. This is an inhibition of OA-induced AP-1 activity of >95%. Thus, TAM-67 expressed in the epidermis of ICR mice can block OA-induced AP-1 activity without affecting basal activity.

Having demonstrated that induction of AP-1 activity in the skin of K14-TAM-67 mice was inhibited, a long-term initiation/promotion skin carcinogenesis protocol was performed to determine the effects of inhibition of OA-induced AP-1 activity on skin tumor promotion mediated by OA. The TAM-67 mice and TAM-67 littermates were initiated with 100 µg (390 nmol) of DMBA in 0.2 ml of acetone. One week later, the mice were started on twice weekly treatments of 2 µg of OA in 0.2 ml of acetone applied to the dorsal skin. After 20 weeks of promotion, the experiment was stopped. Fig. 2 shows that the TAM-67+ mice developed fewer papillomas/mouse than the TAM-67 littermates, and in the few mice that developed tumors, the onset of papillomagenesis was delayed. There was an ~90% inhibition in OA-promoted papilloma response in the transgenic mice as compared...
ing agents induce hyperplasia of the skin. To determine whether TAM-67 had an effect on the hyperplastic response to OA, mice were treated on the dorsal skin with a single dose of OA (2 μg), and treated skin was harvested 48 h later (the time of maximal hyperplasia; data not shown). The results (Fig. 3) indicated that TAM-67 did not significantly inhibit the hyperplastic response to OA at the time of maximal induction of hyperplasia by this dose of OA.

Another important class of mediators of tumor development is the enzymes that degrade the extracellular matrix. Because OA has previously been shown to induce the MMP stromelysin-1 in vivo (10), this was used as an example of these enzymes. In addition, it is known that the promoter region of the murine stromelysin-1 gene contains an AP-1 binding site. Epidermal RNA was harvested 18 h after four mice were treated with either OA or acetone. These samples were then subjected to Northern blot analysis with a probe for murine stromelysin-1. This analysis (Fig. 4) indicated that OA induced stromelysin-1 mRNA in both TAM-67+/H11001 and TAM-67+/H11002 mice and that there was no apparent difference in the extent of induction with this dose and time of harvest.

Other genes known to be induced by OA (10) were examined by Northern blot analysis. As shown in Fig. 4, the transcription of

Fig. 1. A, TAM+ mice actively transcribe TAM-67, and TAM− littermates do not. The 1.5-mm skin punches were snap-frozen and then pulverized in liquid nitrogen. Total cellular RNA was isolated by dissolving tissue in guanidinium salt and phenol/chloroform extraction. Transcription of TAM-67 was determined by RT-PCR analysis of this RNA. The sizes of the various amplification products are indicated. Amplification of GAPDH was included as a positive control. RT, - reverse transcriptase. B, TAM-67 blocks AP-1-driven Luc activity in vivo. The ears from 10 ICR/AP-1 Luc+/K14-TAM-67+ and 10 ICR/AP-1 Luc+/K14-TAM-67− mice were treated with acetone (left ear) or with 2 μg of OA (right ear). Tissue was isolated 24 h later from 1.5-mm ear punches and lysed. Luciferase activity was determined on 25 μl of lysate. A BCA protein assay was then performed, and the Luc results were normalized to 30 μg of protein. □, acetone; ■, OA.

Fig. 2. TAM-67 transgenic mice have decreased tumor multiplicity in response to OA promotion. Sixteen TAM-67+ mice and 16 TAM-67− littermates were shaved and initiated with a single dose of 390 nmol of DMBA 2 days later. These mice were then primed, starting the following week, with twice weekly doses of 2 μg of OA in 200 μl of acetone. Papilloma development was monitored once a week. ▲, TAM−; □, TAM+.

Fig. 3. TAM-67 expression does not change OA-induced hyperplasia. TAM-67+ mice and TAM-67− littermates were treated on the dorsal skin with 2 μg of OA in 200 μl of acetone. Forty-eight h later, skin was harvested, fixed in buffered formalin, and embedded in paraffin, and sections were stained with H&E. Five-μm sections were then evaluated for epidermal thickness. □, acetone; ■, OA.

Fig. 4. Expression of TAM-67 does not change the expression of some OA-induced genes. TAM-67+ mice and TAM-67− littermates were treated on the dorsal skin with 2 μg of OA or acetone. Eighteen h later, full-thickness skin was snap-frozen, and epidermis was separated. Epidermis was then pulverized in liquid nitrogen. Total cellular RNA was isolated by dissolving tissue in guanidinium salt and phenol/chloroform extraction. After electrophoretic separation and capillary transfer to a GeneScreen nylon membrane, samples were probed for the indicated genes. The membrane was then exposed to a phosphorimaging screen, and the image was scanned by a MD-Storm PhosphorImager (Molecular Dynamics).
hyperproliferative K6 was induced by OA at 18 h. K6 is also known to have an AP-1 binding cis element in the gene promoter region (22). Additionally, the transcription of K6 was unaffected by expression of TAM-67 in the epidermis with this dose and time of harvest. The epidermal form of fatty acid-binding protein was also examined. These results (Fig. 4) show that this differentiation-associated protein was induced at 18 h and that TAM-67 did not change the induced or steady-state basal levels of expression of this gene with this dose and time of harvest.

**DISCUSSION**

The ICR strain of mice is especially sensitive to OA, a prototypical non-phorbol ester skin tumor-promoting agent that works by inhibiting protein phosphatases. Expression of dominant negative c-jun in the skin of these mice blocked OA-induced activation of AP-1 with little or no effect on induced hyperplasia. More importantly, expression of TAM-67 protected the mice from OA-induced tumorigenesis.

Interestingly, TAM-67 expression did not inhibit transcription of prospective AP-1-targeted genes activated by OA. Stromelysin-1, a MMP involved in degradation of the extracellular matrix, was similarly activated by OA in both TAM-67+ and TAM-67– mice. It was not surprising that OA induction of K6, a marker of hyperproliferation, was also unaffected by TAM-67 expression because TAM-67 expression does not block OA (Fig. 3) - TPA (19)-induced hyperproliferation. TAM-67 expression did not affect the expression of epidermal fatty acid-binding protein, an OA-induced gene thought to be associated with keratinocyte differentiation.

The transcriptional activity of AP-1 proteins is not limited to activation of the Jun-Jun or Jun-Fos complexes. Jun and Fos family members can interact with a variety of other proteins including members of the ATF family of cyclic AMP response element-binding proteins (23) and the p65 subunit of NF-kB (24). One possible explanation for the antitumorigenic activity of TAM-67 is interaction with other families of transcription factors. TAM-67, like c-Jun, interacts with p65. Furthermore, TAM-67 expression blocks TPA activation of NF-kB in JB6 cells and 308 murine keratinocytes, TNF-α activation of NF-kB in JB6 cells, and elevated NF-kB levels in human keratinocytes (17, 25, 26). Thus, it is possible that TAM-67 is inhibiting activation of NF-kB-regulated genes in the OA-treated skin. NF-kB is known to regulate several cytokines including TNF-α. Recently, it has been shown that TNF-α knockout mice have reduced papilloma formation in response to both DMBA/OA or DMBA/TPA (27). Thus, TNF-α knockout mice may be deficient in the same signaling pathways as K14-TAM-67 transgenic mice.

The signaling blocked by TAM-67 has been shown to be essential for chemical tumor promotion. This leads to the idea that a genetic reagent such as TAM-67 could be clinically efficacious in blocking tumor promotion. This idea is attractive because a rate-limiting step in tumor formation could be blocked in a population that had a known exposure to an initiating event by either chemical or physical carcinogens. Delivery of such an agent in an inducible form, such as the tetracycline induction system, could prove to be therapeutically useful in populations that are at high risk of developing various forms of cancer including skin cancer.

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