

Effect of Suppressive Oligodeoxynucleotides on the Development of Inflammation-Induced Papillomas

Hidekazu Ikeuchi, Takeshi Kinjo, and Dennis M. Klinman

Abstract

Inflammation contributes to the development of papillomas and squamous cell carcinomas in the well-established 7,12-dimethylbenz(*a*)anthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA) model of skin carcinogenesis. Synthetic oligonucleotides (ODN) containing repetitive TTAGGG motifs have been shown to block deleterious inflammatory reactions in murine models of autoimmunity, pneumonitis, and shock. This article examines whether treatment with suppressive (Sup) ODN can interfere with DMBA/TPA-induced inflammation, thereby reducing papilloma formation. Results indicate that Sup ODN block TPA-dependent skin hyperplasia, edema, and leukocytic infiltration. Sup ODN also inhibit the upregulation of genes encoding pro-oncogenic chemokines and other markers of inflammation including CXCL2, CCL2, COX-2, and ODC (ornithine decarboxylase). Of greatest import, Sup ODN reduce papilloma formation in a dose- and sequence-dependent manner. These findings suggest that Sup ODN may provide a novel means of preventing inflammation and associated oncogenesis. *Cancer Prev Res*; 4(5); 752–7. ©2011 AACR.

Introduction

Inflammatory processes are associated with the development and/or progression of nearly 1 in 5 cancers (1). Ongoing inflammation has pleomorphic effects, including aiding in the proliferation and survival of malignant cells, promoting angiogenesis, facilitating metastasis, and subverting the host's antitumor response. Limited data suggest that treatment with anti-inflammatory agents may reduce host susceptibility to cancer (1, 2).

One approach to downregulating proinflammatory responses is through the use of immunosuppressive oligonucleotides (Sup ODN). These ODN express repetitive TTAGGG motifs patterned after the immunosuppressive domains present in mammalian telomeres (3). Previous studies established that Sup ODN can downregulate injurious inflammatory reactions in diseases including arthritis, lupus, toxic shock, and silicosis (3–8).

This article presents a well-established murine skin cancer model (9) to examine whether Sup ODN can block the inflammation associated with carcinogenesis.

In this system, the "initiator" [7,12-dimethyl benz(*a*)anthracene (DMBA)] induces oncogenic transformation whereas the "promoter" [12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)] drives inflammation and tumorigenesis (9). Papillomas begin to appear 2 to 3 months after weekly TPA treatment, and a fraction of these undergo malignant transformation to form squamous cell carcinomas (SCC). This murine model shares important features of human multistage carcinogenesis, including well-defined oncogenic changes at the cellular level and the necessity for inflammation to support papilloma and subsequent carcinoma development (9). Early markers of disease in this model include the upregulation of proinflammatory cytokines and chemokines (including CXCL2 and CCL2). Carcinogenic transformation is preceded by the upregulation of ornithine decarboxylase (ODC) and COX-2 (10, 11). Thus, one approach to determining the effect of novel therapies is by monitored changes in the activation state of these genes. Results from this work indicate that Sup ODN reduce TPA-dependent inflammation and that this is associated with significantly decreased papilloma development.

Materials and Methods

Reagents

Phosphorothioate oligodeoxynucleotides (ODN) were synthesized at the CBER Core Facility (Bethesda, MD). The sequence of suppressive ODN A151 was "TTAGGGT-TAGGGTTAGGGTTAGGG" and of control ODN 1612 was "GCTAGATGTTAGCGT." Previous studies showed that the effect of suppressive ODN was sequence dependent but

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not length dependent and that the length of the control ODN did not impact their activity (3). DMBA and TPA were obtained from Sigma Aldrich.

Animals

Female CD-1 mice (5–6 weeks old) were obtained from the Charles River Laboratories and acclimatized for 1 week prior to use. All experiments were conducted under Animal Care and Use Committee approved protocols.

Induction of skin papillomas

A 2-stage skin carcinogenesis protocol was followed (9). Briefly, hair was shaved from the dorsum of the mice. Only those animals that were in the resting phase of their hair cycle were used in this study. Tumor induction was initiated by topical application of 50 μg of DMBA in 200 μL of acetone. Tumor growth was promoted by weekly topical applications of 2.5 μg of TPA in 200 μL of acetone starting 2 weeks after DMBA application and continuing for 16 weeks. ODNs were also administered topically in acetone. To ensure the absorption of topically administered agents, the stratum corneum was removed by "stripping" skin sites 5 times with Scotch Magic Tape 810 (3M) immediately before application.

Analysis of TPA-induced inflammation

CD-1 mice were shaved and treated with 2.5 μg of TPA in 200 μL acetone \pm 30 μg of ODN as described earlier. The animals were sacrificed 8 to 18 hours later. Eight-millimeter punch biopsy specimens were removed, weighed, embedded in paraffin, and stained. Infiltrating leukocytes were identified on sections stained with antimyeloperoxidase antibodies. Sections were analyzed using an Olympus IX50 microscope fitted with a digital camera. Cell numbers were determined using Image J software in 3 randomly selected sites/slide and the results were analyzed statistically.

Measurement of chemokine expression by RT-PCR

Skin biopsy specimens were homogenized in TRIzol reagent (Invitrogen) and mRNA was extracted using an RNeasy Mini kit (Qiagen). Reverse transcription was carried out using a QuantiTect Reverse Transcription kit (Qiagen). ODC, COX-2, and chemokine mRNA levels were examined using the Applied Biosystems StepOne Real-Time PCR System, in which primers obtained from the Gene Expression Assay Set (Applied Biosystems) were amplified using the TaqMan Gene Expression Master Mix Kit. Chemokine mRNA expression levels were then calculated by StepOne software (Applied Biosystems) after correction for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) expression independently for each sample.

Statistical analysis

All *in vivo* studies involving papilloma development used a minimum of 10 mice per group. Statistical significance was determined using Student's *t* test and 1-way ANOVA

followed by Scheffe's *F* test. A *P* value of less than 0.05 was considered to represent a statistically significant difference between-group means.

Results

Suppressive ODNs reduce TPA-induced skin inflammation

TPA administration causes the rapid onset of cutaneous edema accompanied by significant leukocyte infiltration (9, 12). To assess whether Sup ODN can block TPA-dependent inflammation, areas of skin to which TPA had been administered were treated with Sup or control ODN. These skin sections were then biopsied and examined histologically. As seen in Figure 1, both the edema and cellular infiltration induced by TPA were significantly reduced by Sup ODN treatment ($P < .05$). These effects were sequence specific, as control and CpG ODN had no effect (Fig. 1; data not shown).

Suppressive ODN prevent the development of DMBA/TPA-induced papillomas

Papillomas were elicited by painting the skin of 7-week-old CD1 mice once with DMBA and then weekly for 4 months with TPA. Papillomas first arose after approximately 10 weeks and increased in frequency and size thereafter (Fig. 2A).

To determine whether the reduction in inflammation found after Sup ODN treatment was associated with decreased papilloma development, ODN were coadministered topically with TPA. Both the number of mice that developed papillomas and the number of papillomas/animal were significantly reduced when Sup ODN was coadministered with TPA ($P < 0.01$, Fig. 2A). The same effect was observed when 300 μg of Sup ODN was administered by intraperitoneal injection within 1 hour of TPA treatment (Supplemental Fig. S1). In contrast, control ODN had no impact on papilloma formation (Fig. 2A).

Dose–response studies were conducted to verify the observation that Sup ODNs protected mice from papilloma development. As seen in Figure 2B, the highest dose of ODN administered (50 μg) significantly reduced the number of papillomas when compared with the lowest dose (1 μg , $P = 0.027$) whereas the intermediate dose (5 μg) had a detectable but more modest impact on papilloma frequency.

Additional parameters were examined to clarify the conditions under which Sup ODN inhibited papilloma development. Local administration of Sup ODN was highly effective when delivered with or after TPA administration ($P < 0.01$, Fig. 3A). The efficacy of Sup ODN was significantly reduced when administered prior to TPA, consistent with previous findings showing that Sup ODN have their greatest effect on activated cells (13). The initiation and duration of therapy also impacted efficacy. Uninterrupted treatment with Sup ODN throughout the period of TPA administration was most effective, reducing papilloma incidence by 90%–100% (Fig. 3). When Sup ODN delivery

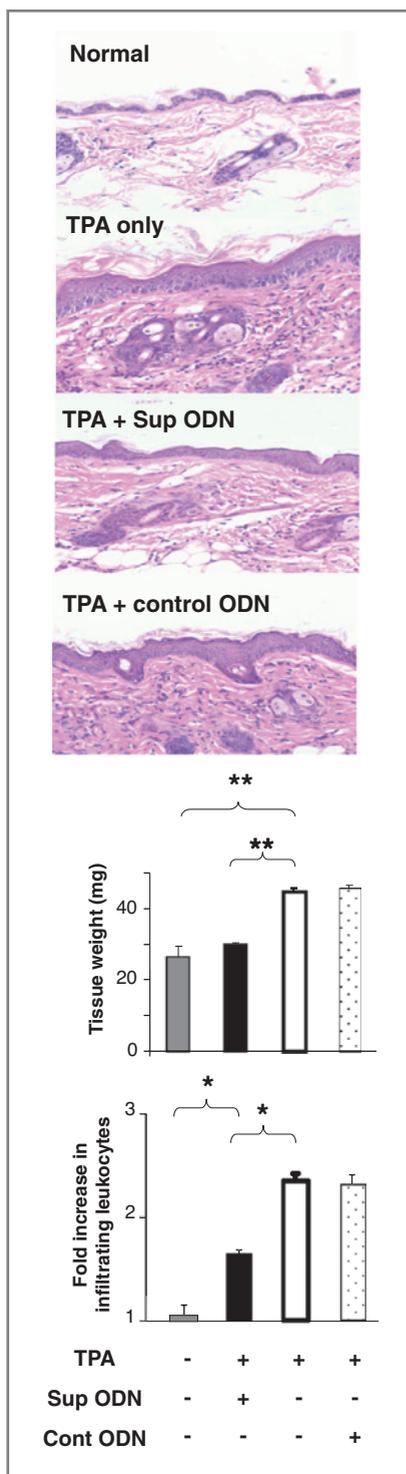


Figure 1. Effect of Sup ODN on TPA-induced skin inflammation. CD-1 mice were treated topically with 2.5 μ g of TPA \pm 50 μ g of ODN. Skin biopsy specimens were taken 16 hours later and weighed (to assess skin edema). Hematoxylin and eosin-stained tissue sections were used to measure skin thickness (left), whereas the fold difference in the number of infiltrating leukocytes was determined in 3 hpf per specimen by using Image J software. Results reflect the mean \pm SE from 3 independent experiments involving 5 independent mice per group per experiment. Note that TPA Rx also induced epidermal hyperplasia. **, $P < 0.01$, *; $P < 0.05$.

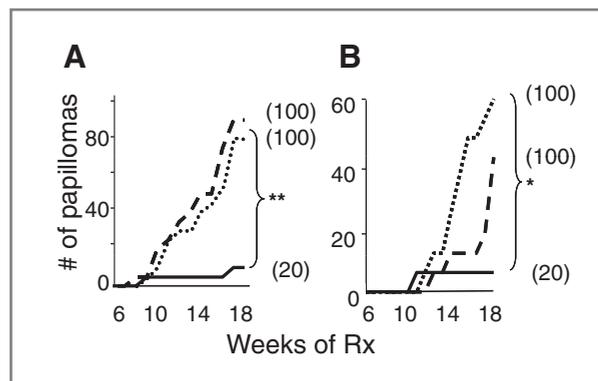


Figure 2. Effect of immunomodulatory ODN on DMBA/TPA-induced papillomas. CD-1 mice ($N = 10$ per group) were treated at 7 weeks of age with 50 μ g of DMBA, followed by weekly topical applications of 2.5 μ g of TPA for 4 months. A, the effect of coadministering 50 μ g of control (dashed line) or suppressive (solid line) ODN versus TPA alone (dotted line) on the cumulative number of papillomas is shown. B, the effect of coadministering 50 μ g (solid line), 5 μ g (dashed line), or 1 μ g (dotted line) of suppressive ODN with TPA is shown. In both panels, the percentage of mice per group that developed papillomas is shown in parenthesis. **, $P < 0.01$, *; $P < 0.05$.

was discontinued after 5 weeks, the onset of papilloma formation was significantly delayed ($P < 0.01$) but then began to increase. In contrast, no benefit was observed when the effects of TPA were unopposed by Sup ODN therapy for 6 weeks (Fig. 3B).

Effect of suppressive ODN on markers of inflammation and carcinogenesis

TPA reportedly triggers the upregulation of mRNA encoding proteins that promote tumor formation [such as ODC and prostaglandin endoperoxide synthase 2 (COX-2; refs. 14, 15)] and chemokines that promote inflammation (such as CXCL2 and CCL2; ref. 16). Consistent with previous findings, the level of mRNA encoding ODC, COX-2, CXCL2, and CCL2 all increase by more than 6-fold ($P < 0.05$) at sites treated with TPA (Fig. 4). The effect of Sup ODN on this TPA-dependent change in mRNA expression was monitored by QT-PCR. As seen in Figure 4, Sup ODN significantly reduced the expression of mediators associated with inflammation and carcinogenesis. This activity was sequence specific, as control ODN had no effect.

Discussion

There is considerable evidence that inflammation can contribute to the initiation and/or progression of cancer. Animal models show that mutagenized cells are better able to survive, proliferate, and metastasize when placed in an inflammatory environment (2), whereas epidemiologic studies show that the risk of developing cancer is significantly increased by chronic infection and/or inflammation (1). Mechanistically, inflammation has been shown to enhance angiogenesis, reduce tumor-specific immunity, and limit the activity of chemotherapy and radiation therapy (17, 18).

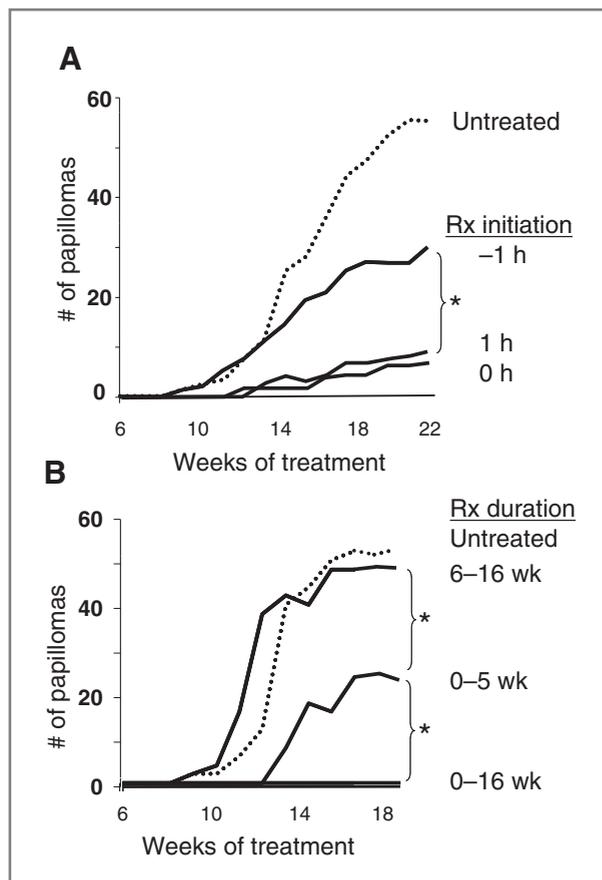


Figure 3. The timing and duration of Sup ODN administration effects papilloma development. CD-1 mice were treated with DMBA and TPA (dotted line) as described in Fig 2. A, 50 μ g of Sup ODN was administered before, after, or simultaneous with TPA. B, mice were treated with 50 μ g of Sup ODN for 0-5, 6-16, or 0-16 weeks. Data show the cumulative number of papillomas in 10 mice per group. *, $P < 0.05$.

Carcinogenesis in humans is a multistage process in which cells exposed to mutagens are subsequently stimulated to grow (and avoid physiologic control mechanisms) through interactions with their environment (9). A murine model that recapitulates important features of this multistage process utilizes DMBA to induce mutations in the *Ras* oncogene followed by TPA to promote inflammation and thus drive papilloma formation (9). This article examines the ability of immunosuppressive oligonucleotides to block TPA-induced inflammation and interrupt the carcinogenic process (manifest by a significant reduction in papilloma development). The Sup ODN used in this work contain 4 repeats of the suppressive TTAGGG motif present in mammalian telomeres (3). Previous studies showed that Sup ODN with 2 to 4 such motifs could prevent or delay both autoimmune and inflammatory diseases including lupus, arthritis, EAE, toxic shock, and silicosis (3-8). These effects were linked to the ability of Sup ODN to inhibit the ongoing activation of immune cells and concomitantly reduce the production of proinflammatory cytokines and chemokines (13, 19).

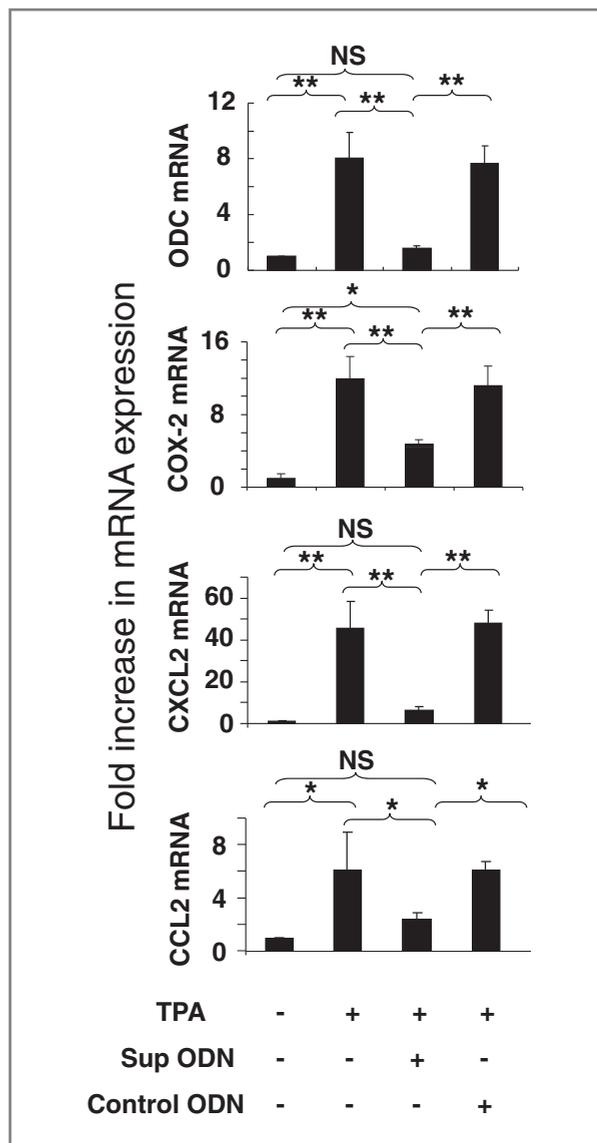


Figure 4. Effect of Sup ODN on TPA-induced mRNA upregulation. Full-thickness skin biopsy specimens were taken 8 hours after the topical administration of 2.5 μ g of TPA \pm 50 μ g of ODN. QT-PCR was carried out on mRNA isolated from 4 independent samples per treatment group. mRNA levels of the inflammatory mediators ODC and COX-2 mRNA and the inflammatory chemokines CXCL2 and CCL2 were monitored. Data show the relative changes in mRNA levels as determined by RT-PCR after correction for the housekeeping gene *GAPDH*. Results reflect the mean \pm SE from 3 independent experiments involving 4 independently studied tissue sections per group per experiment. **, $P < 0.01$; *, $P < 0.05$; NS; no significant difference.

Delivering Sup ODN throughout the period of TPA administration provided significant protection against papilloma development ($P < 0.01$, Figs. 2 and 3). ODN treatment was effective when delivered with, or after, TPA-induced inflammation, consistent with earlier studies showing that Sup ODN activity was maximal in the face of ongoing inflammation (13). This protection was dose dependent and sequence specific, as no

protection was conferred by control ODN lacking the TTAGGG motif (Figs. 1, 2, and 4). Optimal protection required that Sup ODN be administered throughout the 16-week period of TPA exposure (Fig. 3B). Discontinuing Sup ODN therapy after 5 weeks significantly delayed the onset but did not prevent the eventual development of papillomas (Fig. 3). Protection was not observed when treatment with Sup ODN was delayed until after TPA had been administered for 6 weeks. These outcomes are consistent with those observed in studies of murine lupus: early and continuous treatment with Sup ODN was most effective, and starting and then discontinuing therapy slowed but did not prevent disease whereas initiating therapy late had little effect on disease progression (4). Although the current study does not document a causative link between inflammation and tumor development, the results are consistent with a model in which repeated TPA administration maintains an inflammatory milieu that drives papilloma formation. Of interest, both processes were blocked by continuous Sup ODN treatment. In contrast, Sup ODN therapy was ineffective if delivered with DMBA (data not shown), suggesting that Sup ODN has no impact on tumor initiation.

To better understand the mechanism underlying these effects, the impact of Sup ODN on TPA-induced chemokine production was examined. Mice treated with TPA develop an inflammatory response characterized by skin edema, epithelial hyperplasia, and leukocyte infiltration (20). The proinflammatory cytokines CXCL2 and CCL2 help mediate these events and are linked to TPA-induced neutrophilic infiltration and papilloma development (21–23). Co-administering Sup ODN with TPA significantly ($P < 0.05$) reduced the expression of mRNA encoding these proteins, an effect that is both dose dependent and sequence specific (Fig. 4; data not shown).

If TPA administration is extended beyond 20 weeks, a fraction of the papillomas develop into SCCs (9). This process is preceded by a significant increase in the expres-

sion of ODC and COX-2 (10, 24). ODC helps regulate epithelial cell proliferation and provides an excellent marker of neoplastic transformation and tumor growth (10, 25, 26). COX-2 is associated with the appearance of skin tumors (11, 27), and its overexpression correlates with enhanced tumor invasiveness, increased angiogenesis, and antiapoptotic cellular responses via the production of prostaglandin E2 (11, 27, 28). Although the conversion of papillomas to SCC was not monitored in the current study, it is intriguing to note that Sup ODN significantly reduced the levels of ODC and COX-2 mRNA in TPA-treated skin ($P < 0.01$, Fig. 4).

Epidemiologic studies indicate that prolonged use of anti-inflammatory agents (such as aspirin and COX-2 inhibitors) may reduce the risk of colon, lung, esophageal, and stomach cancer (17, 29, 30). Murine models confirm that chronic administration of certain anti-inflammatory agents can decrease the frequency of chemically induced colon, bladder, lung, and skin cancer (31–37). This study is the first to show that a novel class of ODNs designed to selectively inhibit immune activation also prevent the development of inflammation-dependent cancer. These Sup ODNs are patterned after and recapitulate the anti-inflammatory activity of normal human telomeric DNA and thus are safe when administered repeatedly to mice and nonhuman primates (reviewed in ref. 38). Ongoing studies are directed toward determining the impact of Sup ODN in additional models of inflammation-induced cancer and clarify whether there is a causative link between their effect on inflammation and carcinogenesis.

Disclosure of Potential Conflicts of Interest

D.M. Klinman and members of his laboratory hold or have applied for patents concerning the activity of Suppressive ODN, including their use in preventing tumorigenesis. The rights to all such patents have been transferred to the U.S. government.

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