T-oligo Treatment Decreases Constitutive and UVB-induced COX-2 Levels through p53- and NFκB-dependent Repression of the COX-2 Promoter*\(\textsuperscript{5}\)

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Chronically irradiated murine skin and UV light-induced squamous cell carcinomas overexpress the inducible isozyme of cyclooxygenase (COX-2), and COX-2 inhibition reduces photocarcinogenesis in mice. We have reported previously that DNA oligonucleotides substantially homologous to the telomere\(\textsuperscript{3}\)-overhang (T-oligos) induce DNA repair capacity and multiple other cancer prevention responses, in part through up-regulation and activation of p53. To determine whether T-oligos affect COX-2 expression, human newborn keratinocytes and fibroblasts were pretreated with T-oligos or diluted alone for 24 h, UV-irradiated, and processed for Western blotting. In both cell types, T-oligos transcriptionally down-regulated base-line and UV light-induced COX-2 expression, coincident with p53 activation. In fibroblasts with wild type versus dominant negative p53 (p53\(\textsuperscript{WT}\) versus p53\(\textsuperscript{DN}\)), T-oligos decreased constitutive expression of a COX-2 reporter plasmid by >50%. We then examined NFκB, a known positive regulator of COX-2 transcription. In p53\(\textsuperscript{WT}\) but not in p53\(\textsuperscript{DN}\) fibroblasts and in human keratinocytes, T-oligos decreased readout of an NFκB promoter-driven reporter plasmid and decreased NFκB binding to DNA. After T-oligo treatment and subsequent UV irradiation, binding of the transcriptional co-activator protein p300 to NFκB was decreased, whereas binding of p300 to p53 was increased. Human skin explants provided with T-oligos had markedly decreased COX-2 immunostaining both at base-line and post-UV-light, coincident with increased p53 immunostaining. We conclude that T-oligos transcriptionally down-regulate COX-2 expression in human skin via activation and up-regulation of p53, at least in part by inhibiting NFκB transcriptional activation. Decreased COX-2 expression may contribute to the observed ability of T-oligos to reduce photocarcinogenesis.

Nonmelanoma skin cancer accounts for well over 1 million cases of human malignancy annually in the United States, and the incidence continues to rise (1–3). The major initiator and promoter of skin cancer is UVB radiation (4, 5). Among the contributing effects of UVB radiation on skin are the formation of cyclobutane-pyrimidine dimers and pyrimidine (6–4) photoproducts (6, 7), which lead to mutations in key regulatory genes (8), epidermal hyperplasia (9, 10) allowing for expansion of mutated clones (11), immunosuppression (12, 13), and inflammation (14, 15).

One way inflammation in particular is thought to affect carcinogenesis is by promoting epidermal hyperplasia and proliferation through production of cytokines and various second messengers such as prostaglandin E\(\textsubscript{2}\) (16). The major enzyme responsible for the UVB-induced prostaglandin synthesis is cyclooxygenase-2 (COX-2),\(\textsuperscript{5}\) the inducible isozyme of the cyclooxygenase enzyme (17) that carries out the rate-limiting step of prostaglandin and thromboxane production (18–20). COX-2 has been shown to be overexpressed in numerous human malignancies, including colon, lung, and breast cancers (21–24). In relation to skin cancer, UVB irradiation increases both mRNA and protein levels of COX-2 in human keratinocytes (25). Recent studies have shown increased COX-2 expression in human skin in response to acute UVB exposure as well as increased COX-2 expression in human and murine tumors that were induced by chronic UVB exposure (26, 27). Furthermore, specific inhibitors of COX-2 such as celecoxib have been shown not only to decrease tumorigenesis and increase tumor latency in hairless mice models (28) but also to decrease tumor growth in hairless mice with pre-existing UVB-induced tumors (29). In addition, COX-2-overexpressing transgenic mice have shown dramatic increase in predisposition to tumor development in tumor promotion studies (30).

Given the evidence implicating COX-2 in tumorigenesis and tumor maintenance, methods to decrease COX-2 levels in response to UVB irradiation are currently being investigated as a promising means of cancer prevention. Known inhibitors of COX-2 include estrogens, antioxidants, and p53 (31–33). The presence of active p53 in particular has been shown to decrease both the mRNA and protein levels of COX-2 in mouse embryo fibroblasts (33). In a study of head and neck squamous cell carcinomas (SCCs), tumors with mutated p53 showed higher COX-2 protein levels than tumors expressing wild type (WT) p53 (34). Evidence thus suggests that activating p53 and thereby reducing COX-2 expression in the absence of DNA damage might decrease photocarcinogenesis and inhibit growth of established tumors.

In mammalian cells, telomeres are tandem repeats of a short DNA sequence, 5’-TTAGGG-3’ and its complement that cap chromosome

\(\textsuperscript{5}\) The abbreviations used are: COX-2, cyclooxygenase-2; pTT, thymidine dinucleotide; WT, wild type; DN, dominant negative; SCC, squamous cell carcinoma; T-oligos, telomere homolog oligonucleotide (repeats of TTAGGG): EMSAs, electrophoretic mobility shift assays; TBPs, TATA-binding proteins; ATM, ataxia telangiectasia mutated.
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ends and form a large loop structure (35). The loop is held closed by an ~100–400-base single-stranded 3’-overhang that inserts into the proximal double-stranded telomere and is secured by binding proteins, particularly telomere repeat binding factor (TRF2) (36). Disruption of this loop structure by sequestration of the binding protein with a dominant negative construct (TRF2DN) leads to exposure of the 3’-overhang sequence (38–40) and hence initiate signaling for DNA damage-like responses without antecedent DNA damage.

T-oligos with 100% telomere homology, delivered at a sufficiently high dose, elicit predominantly “end point” cancer protective responses such as apoptosis or proliferative senescence that remove cells, particularly malignant cells, from the proliferative pool (40, 42, 43). However, it is also possible to provide cells with a less than maximal DNA damage signal by reducing the T-oligo dose and/or employing a shorter or less telomere-homologous oligonucleotide. Under these circumstances, it is possible to observe transient reversible cell cycle arrest (44), increased melanogenesis in pigment cells and intact skin (44–46), release of immunomodulatory cytokines from keratinocytes associated with abrogation of allergic contact sensitization or elicitation in intact skin (47, 48), enhanced rate and accuracy of DNA repair both in vitro and in vivo (49–51), and decreased mutation rate and tumor development in vivo (51). Moreover, malignant cells appear to undergo apoptosis or senescence more readily in response to a given T-oligo at a given dose than do their normal nontransformed cellular counterparts (42, 43).

In the present study, we show that treatment with either of two oligonucleotides with partial telomere homology decreases constitutive and UVB-induced COX-2 levels in cultured human fibroblasts, human skin explants, and intact murine skin. These responses are shown to occur at least in part through up-regulation and activation of p53, leading to transcriptional repression of COX-2 promoter activity. We propose that in addition to the previously reported protective DNA damage responses, treatment with T-oligos may also decrease the cutaneous inflammatory response through inhibition of COX-2 expression, a possible additional means of reducing photocarcinogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary human neonatal fibroblast and keratinocyte cultures were established as described (49, 50). Cells were incubated at 37 °C in 5% CO₂. Cell lines permanently (retroviral transfection) expressing WT p53 (R2FWT) or dominant negative p53 (R2FD) (52–54) were the generous gift from Dr. Jim Rheinwald (Department of Dermatology, Harvard Skin Disease Research Center, Harvard Medical School, Boston) and were maintained in R2F medium containing 42.5% Dulbecco’s modified Eagle’s medium, 42.5% F-12, 15% calf serum, and 0.1% epidermal growth factor at 37 °C in 5% CO₂.

Oligonucleotides—Previous experiments have shown that 100 μM of thymidine dinucleotide (pTT), representing one-third of the telomere repeat, and 40 μM of pGAGTATGAG (p9-mer), a 55% homologous sequence, are roughly bioequivalent concentrations for the elicitation of UV mimetic responses (49, 50, 55, 56), including p53 up-regulation and activation. Oligonucleotides (pTT and p9-mer) were synthesized with phosphodiester linkage by Midland Certified Reagent (Midland, TX) and diluted in H₂O to form a 2 mM stock. This stock solution was then diluted in the appropriate culture medium to 10 or 40 μM, respectively, and added to culture dishes for use in experiments. Cells and skin explants were provided T-oligos only once at time 0 and then harvested at intervals, according to the design of the specific experiment. All experiments were conducted using both pTT and p9-mer and gave identical results with either T-oligo.

UVB Irradiation—After 48 h of incubation in medium containing pTT, p9-mer, or diluent alone, cells or skin explants were placed in phosphate-buffered saline and irradiated through the plastic culture dish cover by using a solar simulator (Spectral Energy Corp., Westwood, NJ). The 1-kilowatt xenon arc lamp (XMN-1000-21; Optical Radiation Corp., Azusa, CA) irradiance was adjusted to 5 × 10⁻³ watts/cm², and dishes were exposed to 15 ml/cm² as measured with a research radiometer fitted with a UV light probe at 285 ± 5 nm (model IL1700 A; International Light, Newburyport, MA) (56, 57), a protocol that exposes cells to a spectrum of light resembling terrestrial sunlight (58). Sham-irradiated cultures were handled identically, except that they were shielded with aluminum foil during irradiation. After irradiation, cells were given fresh medium lacking T-oligos.

Western Blot Analysis—Total cellular proteins were collected as described previously (46). Concentrations were determined by the Bio-Rad method, and 50 μg of protein were run in each lane on a 10% denaturing SDS-polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane. Antibody reactions were performed with the following antibodies: phospho-p53Ser¹⁵ (Cell Signaling Technology, Beverly, MA), p53 DO-1, COX-2, NFkB/p65, and actin (all from Santa Cruz Biotechnology, Santa Cruz, CA). Western blot analysis was then performed as described (46).

Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays (EMSAs) using consensus p53 and NFkB oligonucleotides (Santa Cruz Biotechnology) and 5 μg of nuclear protein from variously treated cells were carried out as described previously (59). Reactions were electrophoresed on 5% nondenaturing polyacrylamide gels, dried, and processed for autoradiography. For competition experiments, 50–100-fold excess of unlabeled DNA were added to the reaction 20 min before the addition of radiolabeled probe.

Transfection Studies—Constructs containing the full-length pPBES2 (−1432/+59) human Cox-2 promoter, a deletion construct of pPBES2 (−327/+59), and an NFkB binding region site-specific mutant of pPBES2 (−327/+59) attached to a luciferase reporter (60, 61) or an NFκB reporter plasmid (Promega Corp., Madison, WI) were employed. The pGL2 vector used for cloning the reporter construct was obtained from Promega (pGL2-Basic, Promega Corp., Madison, WI) and was used as an empty vector control. A plasmid containing Renilla luciferase (pRL-CMV, Promega Corp., Madison, WI) was co-transfected as a control for transfection efficiency. R2FWT and R2FD cells were plated in 35-mm tissue culture dishes and incubated in R2F medium overnight to reach 50–60% confluence the next day. Cells were then transfected using the Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Two μg of plasmid DNA were co-transfected with 0.15 μg of the Renilla luciferase plasmid in each dish. After 4–6 h of transfection, cells were supplemented either with R2F medium alone (diluent) or R2F medium with 40 μM p9-mer or 100 μM pTT. Cells were then incubated for 24 and 48 h at 37 °C in 5% CO₂ before harvesting for dual-luciferase assay. Promoter activity was then assayed using a dual-luciferase reporter assay system (Promega Corp., Madison, WI) according to the manufacturer’s protocol. Firefly luciferase values were corrected for transfection efficiency according to Renilla luciferase values.
and revealed minimal differences in transfection efficiency among dishes. Luciferase activity was then expressed as percent of diluent value, setting diluent as 100%.

**Immunoprecipitation and Immunoblotting**—Cell lysates were pre-cleared with protein G-Sepharose beads for 2 h at 4 °C. Then 100 μg of total cell proteins were incubated with 2 μg of monoclonal p300 antibodies (GeneTex Inc., San Antonio, TX), followed by the addition of protein G-Sepharose beads. The immunoprecipitated products were then subjected to SDS-PAGE and Western blot analysis as described (62). After transferring proteins to nitrocellulose membrane antibody reactions were performed with NFκB (p65) antibodies and p53 (DO-1) (both from Santa Cruz Biotechnology).

**Human Skin Explant Studies**—Human skin fragments from healthy donors (aged 56 ± 15 years, mean ± S.D.) were brought to the laboratory within 30 min after excision during plastic or facial reconstructive surgery. After removing subcutaneous fat and deep dermis, skin was cut into 5 × 5-mm squares and placed in 60-mm tissue culture dishes. Paired skin explants were then incubated in either medium alone or medium supplemented with 100 μM pTT or 40 μM p9-mer for 24 h. Medium consisted of Dulbecco’s modified Eagle’s medium with 10% calf serum plus KBM-2 with growth factors (50/50 v/v). The skin explants were then irradiated with a single dose of 30 mJ/cm² UVB. One set was sham-irradiated as a negative control. For each treatment, one explant was harvested immediately after UVB irradiation. The dishes were then re-fed with fresh medium lacking T-oligos, and explants were harvested at 6, 18, and 24 h after UVB irradiation. Harvested skin was snap-frozen at −80 °C in OCT medium for later processing.

**Immunohistochemistry and Immunofluorescence**—Snap-frozen human skin explants were processed for staining by cutting 4–6-μm sections and fixing them in acetone for 10 min at −20 °C. COX-2 staining was performed using the Ultravision Detection System (TQ-015-HA, Labvision Corp., Fremont, CA) according to manufacturer’s protocol. Primary antibodies used included anti-COX-2 (Santa Cruz Biotechnology), human anti-p53 DO-7 (DakoCytomation, Carpinteria, CA), and anti-phospho-p53Ser15 (Cell Signaling Technology, Beverly, MA). For the p53 DO-7 and p53Ser15 stainings, sections were blocked in 10% goat normal serum in Tris-buffered saline for 15 min at room temperature and then incubated with primary antibody overnight at 4 °C. Sections were then washed in Tris-buffered saline three times for 5 min each before being incubated with the appropriate fluorescein isothiocyanate-labeled secondary antibody at 37 °C for 45 min. Finally, sections were washed as before and mounted with Vectashield mounting medium containing 4’,6-diamidino-2-phenylindole to visualize nuclei and were stored at −20 °C. We delineated ~10-μm × 1-mm areas using computer-assisted image analysis and counted p53Total and p53Ser15 (+) nuclei in the epidermis. For each time point we analyzed an average of three randomly selected visual fields of p53Total and p53Ser15 stained epidermis from three to five donors per treatment condition. To avoid bias all counts were done by a single investigator for whom all samples were blinded by another investigator.

**Statistical Analysis**—Difference in protein expression, Cox-2 promoter activity, and p53Total and p53Ser15 (+) nuclei in T-oligo versus control-treated samples were analyzed by the analysis of variance post hoc analysis using the StatView statistical program (SAS Institute, Cary, NC). Groups were considered different when p < 0.05 (50).

**RESULTS**

**T-oligo Pretreatment Down-regulates Baseline and UVB-induced COX-2 Protein Levels That Coincide with Up-regulation and Activation of p53 Levels**—The effect of T-oligo pretreatment on constitutive and UV light-induced levels of COX-2 and p53 was examined by Western blot analysis (Fig. 1), and results were confirmed by densitometric analysis of the blots (Fig. S1). In diluent-treated sham-irradiated cells, the constitutive p53Total and p53Ser15 levels were negligible at 8 h and moderately increased 24 h later (Fig. 1a and Fig. S1, a and b), consistent with approaching confluence of the cultures.6 Reciprocally, COX-2 was constitutively expressed in diluent-treated sham-irradiated samples at 8 h and decreased by 24 h (Fig. 1a and Fig. S1c). In comparison, in T-oligo-treated sham-irradiated cells, COX-2 protein levels were strikingly lower at 8 h and virtually undetectable at 24 h, time points 56 and 72 h after T-oligo supplementation (Fig. 1a and Fig. S1c), suggesting that T-oligo treatment for 48 h down-regulates constitutive COX-2 levels in human fibroblasts. These decreases in COX-2 protein level were

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6 V. Marwaha, B. A. Gilchrest, and D. A. Goukassian, unpublished observations.
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inversely to the increases in both p53\textsuperscript{total} and p53\textsuperscript{Ser15} in T-oligo-treated sham-irradiated cells (Fig. 1a and Fig. S1, a and b).

UV irradiation up-regulated p53\textsuperscript{total} and p53\textsuperscript{Ser15} levels by 8 h and through 24 h and up-regulated COX-2 within 24 h, as reported previously (25, 50, 55), in diluent-treated cells (Fig. 1b and Fig. S1, d–f). T-oligo-treated cells also showed UV light-induced increases in p53\textsuperscript{total} and p53\textsuperscript{Ser15}, with both sham- and UV light-induced levels markedly higher than in diluent-treated controls, as expected (55). As also expected if active p53 negatively regulates COX-2 levels, in T-oligo-treated cells COX-2 was virtually undetectable by 24 h after UVB (Fig. 1b and Fig. S1f), the time of maximal UV light-induced COX-2 protein expression reported by others (27, 63). After UV irradiation, maximal p53 induction and activation are reported to occur at ~2–24 h (depending on UV light dose) (64). Because T-oligo pretreatment appeared to accelerate the time of peak UV light induction and activation of p53 as well as to increase the magnitude of p53 induction and activation (Fig. 1), we examined a more detailed time course, harvesting fibroblasts pretreated with T-oligo or diluent alone for 48 h immediately after UV irradiation and after 4, 6, 8, 16, and 24 h (Fig. S2). Consistent with our interpretation of the experiment shown in Fig. 1, diluent-pretreated cells showed peak phospho-p53\textsuperscript{Ser15} induction at 6–8 h, with a return to baseline by 24 h, whereas T-oligo-pretreated cells had ~85% higher phospho-p53\textsuperscript{Ser15} levels immediately post-irradiation and had tripled this induction within 4 h of an ~3-fold increase that gradually declined through 24 h (Fig. S2). Total p53, in contrast to activated p53, showed similar patterns in both T-oligo and diluent pretreated fibroblasts with substantial inductions by 4 h of post-UV light that declined only slightly by 24 h. However, in diluent-pretreated cells, p53\textsuperscript{total} levels were far lower (~4-fold) of p53\textsuperscript{total} levels in T-oligo-pretreated cells (Fig. S2). In combination, these data are consistent with a delayed direct effect of T-oligos on COX-2 expression, such as altered transcription rate subsequently reflected in protein levels. Alternatively or in addition, this may indicate involvement of an subsequent event downstream of p53 activation, such as p53-mediated inhibition of a known positive COX-2 transcriptional regulator, such as NFkB, NF-IL6, or AP1 (65–67).

T-oligo Pretreatment Down-regulates COX-2 through p53-dependent Repression of COX-2 Promoter—To evaluate further the involvement of p53 in T-oligo-induced down-regulation of COX-2 expression, we performed transient transfection studies using two isogenic fibroblast cell lines, one that expresses WT p53 (R2FWT) and one that is permanently inactivated by R2FDD cells. We then evaluated the effect of T-oligos on NFkB-driven transcription by transfecting R2FWT and R2FDD cells with an NFkB reporter plasmid. In cells expressing p53\textsuperscript{WT}, within 24 h T-oligo treatment decreased NFkB-driven transcription by ~35%, whereas in p53\textsuperscript{DN} cells T-oligo treatment had no inhibitory effect on NFkB-driven transcription (Fig. 2a). We next transfected these cells with the COX-2 reporter plasmid. In R2FWT cells, treatment with T-oligos decreased COX-2 transcription by more than 50%, whereas in R2FDD cells T-oligo treatment had virtually no effect (Fig. 2e). In combination, these results demonstrate that functional p53 is required for T-oligo-induced repression of the COX-2 promoter and suggest that the effect may be mediated at least in part through NFkB.

As shown by Western blot analysis and quantification by densitometry, R2FDD cells expressed higher constitutive levels of p53\textsuperscript{total} and p53\textsuperscript{Ser15} than R2FWT cells (~6- and 40-fold, respectively), as expected (54), and showed virtually no T-oligo-induced up-regulation of either p53\textsuperscript{total} or p53\textsuperscript{Ser15} (Fig. 2a and Fig. S3a), whereas in R2FWT cells by 24 h T-oligo induced a more than 5-fold increase in p53\textsuperscript{Ser15} and a 37% increase in p53\textsuperscript{total} levels (Fig. 2a and Fig. S3b).

To verify that the p53 status of the cell lines had not changed over time in culture, we next evaluated T-oligo-induced p53 DNA binding activity. EMSA showed that in T-oligo-treated R2FDD cells, consensus sequence binding was minimal and did not increase over time, whereas in contrast binding in R2FWT cells was far higher as early as 8 h and was maximal by 24 h (Fig. 2b). These data confirm the reported p53 status in the R2FWT versus R2FDD cells.

The transcription factor NFkB is a known positive regulator of COX-2 gene expression (68). Because recent reports indicate that activation of p53 by various stimuli inhibits NFkB activity (69, 70), we next evaluated NFkB DNA binding to its consensus sequence after T-oligo treatment. In both R2FWT and R2FDD cells that received T-oligos in fresh medium, NFkB binding decreased between 1.5 and 8 h and then increased again by 16 h (Fig. 2c), consistent with the known serum-mediated bi-phasic increase in NFkB activity (71–73). However, by 24 h, the time of maximal p53 activation in R2FWT cells (Fig. 2b), there was a marked decrease in NFkB binding activity compared with R2FDD cells (Fig. 2c), suggesting that p53 activation inhibited NFkB DNA binding activity after T-oligo treatment.

To examine if T-oligo-induced p53-mediated repression of the COX-2 promoter depends on inhibition of NFkB transcriptional activity, we evaluated the effect of T-oligo treatment on NFkB-driven transcription by transfecting R2FWT and R2FDD cells with the COX-2 gene promoter with a site-specific mutation of the NFkB binding region (see diagram in Fig. S4a). In R2FWT cells transfected with WT COX-2 promoter, treatment with T-oligos decreased COX-2 transcription by more than 70%, whereas in R2FWT cells transfected with the mutated COX-2 promoter, T-oligo treatment had no inhibitory effect (Fig. S4b). In R2FDD cells, transfected with either WT or NFkB mutant COX-2 plasmid, T-oligo treatment had no effect (data not shown). These data demonstrate direct involvement of NFkB in T-oligo-induced p53-mediated repression of COX-2 promoter.

T-oligo Pretreatment Down-regulates Base-line and UVB-induced COX-2 Protein Levels in Human Keratinocytes, Coinciding with Activation of p53, p53-dependent Inhibition of NFkB Activity, and NFkB-dependent Transactivation—Because keratinocytes are a primary target for UVB damage and are the cells that give rise to UV light-induced actinic keratoses and SCC, we also examined the effect of T-oligos on primary human keratinocytes. Keratinocytes were pretreated with either T-oligos or sham alone for 48 h and then sham or UV light-irradiated. Cells were then placed in medium without T-oligos and harvested for analysis of COX-2 protein levels and p53 DNA binding activity 8 and 24 h after irradiation. As shown by Western blot analysis (Fig. 3a) and quantified by densitometry (Fig. S5) in sham-irradiated keratinocytes, constitutive COX-2 expression was low at 8 and 24 h and similar in diluent-treated versus T-oligo-treated cells. As reported previously (27, 74), UV irradiation markedly up-regulated COX-2 within 24 h in diluent-treated control cells (Fig. 3b and Fig. S5). However, as expected if T-oligos negatively regulate COX-2 in keratinocytes as well as in fibroblasts, COX-2 levels in T-oligo-treated keratinocytes were reduced by >50% at both 8 and 24 h (Fig. 3b and Fig. S5), the time of maximal UV light-induced COX-2 protein expression reported by others in this cell type (27, 74).

To substantiate further the involvement of p53 in T-oligo-induced down-regulation of COX-2 expression in human keratinocytes, we next evaluated T-oligo-mediated changes in p53 DNA binding activity using the protein from the same samples that was used to evaluate COX-2 protein levels in keratinocytes. At 8 and 24 h, EMSA showed minimal p53 DNA binding activity in T-oligo-treated or diluent-treated sham-irradiated cells, similar to the negative control (Fig. 3c, lanes 7–10 versus 2). As expected, UV irradiation increased p53 binding activity in dilu-
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ent-treated keratinocytes between 8 and 24 h (Fig. 3c, lanes 3 versus 5). However, in T-oligo-treated UV light-irradiated keratinocytes, p53 binding activity was increased 4-fold as early as 8 h after UVB compared with the levels in diluent-treated UV light-irradiated cells (Fig. 3c, lanes 4 versus 5), although by 24 h p53 DNA binding activity was less and was comparable in diluent- versus T-oligo-treated UV light-irradiated samples (Fig. 3c, lanes 6 versus 5). These data confirm an inverse relationship between p53 activity and COX-2 levels in human keratinocytes, as well as in fibroblasts.

Because NFκB is a known positive regulator of COX-2 transcription and we found that T-oligo treatment inhibits NFκB DNA binding activity in fibroblasts, experiments were performed to confirm this presumptive mechanism for indirect T-oligo-mediated p53-dependent inhibition of COX-2 transcription in keratinocytes. We reasoned that a possible limiting factor for the transcriptional activity of both p53 and NFκB may be the binding to the transcriptional co-activator protein p300 (70, 75). To address this possibility, we pretreated human keratinocytes with diluent or T-oligo for 24 h. Cells were then UV light-irradiated with 15 mJ/cm² followed by incubation in medium without T-oligos for 8 and 24 h and harvested for immunoprecipitation with anti-p300 antibodies followed by Western blot analysis for NFκB/p65 and p53 (Fig. 3d). In diluent-treated UV light-irradiated keratinocytes, NFκB/p65 binding to p300 increased markedly between 8 and 24 h after UV irradiation (Fig. 3d, lanes 1 versus 3). In contrast, in T-oligo-pre-treated UV light-irradiated keratinocytes, NFκB binding to p300 was decreased relative to control as early as 8 h, and by 24 h NFκB binding to p300 was virtually undetectable (Fig. 3d, lanes 2 versus 4). These data strongly suggest that T-oligo treatment decreases NFκB transcriptional activity via reduction of NFκB binding to its transcriptional co-activator p300 protein. Consistent with this interpretation, the amount of p53 binding to p300 was strikingly increased in T-oligo-treated versus control cells at both 8 and 24 h (Fig. 3d, lanes 1 and 3 versus 2 and 4, respectively). These data are consistent with an indirect mechanism of T-oligo-mediated p53-dependent inhibition of NFκB transcriptional...
activity in which activated p53 successfully competes with NFκB for binding to their common co-activator, p300.

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**FIGURE 3.** T-oligo pretreatment activates p53 and down-regulates constitutive and UV light-induced COX-2 expression in keratinocytes. Human newborn keratinocytes were treated as in Fig. 1. 

**a**, paired dishes were then sham-irradiated and harvested after 8 and 24 h for evaluation of COX-2 and actin (loading control) protein levels. These experiments were repeated two times with similar results. 

**b**, paired dishes were then UVB-irradiated with 15 mJ/cm², harvested, and processed as for a. At 8 h COX-2 expression was comparable in diluent-treated and T-oligo-treated samples. In UV light-irradiated control samples, COX-2 expression increased by 8 h with a further increase at 24 h, whereas in T-oligo-treated UV light-irradiated cells, COX-2 expression was significantly down-regulated throughout the 24 h. Similar results were obtained in three independent experiments.

**c**, cells lysates from the same samples that were used to evaluate COX-2 protein levels in keratinocytes in a were used to evaluate T-oligo-mediated changes in p53 DNA binding activity in EMSA. Specificity of binding is confirmed by significant reduction of the band in the control lane using 50X excess of cold probe and protein lysate of keratinocytes treated with T-oligos for 8 h (lane 2 versus lane 4).

**d**, cell lysates from the same samples that were used to evaluate COX-2 protein levels and p53 binding activity in keratinocytes in b and c (only T-oligo-treated UV light-irradiated) were used for immunoprecipitation (IP) with anti-p300 antibodies followed by Western blot analysis for NFκB/p65 and p53. T-oligo treatment decreased binding of NFκB to p300 by 8 h and was virtually undetectable by 24 h, whereas in T-oligo-treated UV light-irradiated keratinocytes p53 binding to p300 was increased already by 8 h and was even greater by 24 h.

**FIGURE 4.** T-oligo treatment decreases constitutive and UVB-induced COX-2 levels in human skin explants. Human skin explants (10 per donor, 5 for each T-oligo) were prepared from otherwise discarded normal adult skin and treated immediately as described under "Experimental Procedures." Untreated nonirradiated skin showed constant low COX-2 expression over the 48-h experiment. 

**a**, in paired explants harvested after 24 h, T-oligos (pTT 100 µM; p9-mer data not shown) modestly decreased low base-line levels of COX-2 expression in the suprabasal epidermis. 

**b**, in paired explants harvested after T-oligo treatment and subsequent UVB irradiation, T-oligos strikingly decreased UVB-induced levels of COX-2 expression in the suprabasal epidermis as early as 18 h and continued through 24 h after irradiation. Specificity of COX-2 antibody staining was confirmed by staining sections of nontreated and diluent/T-oligo-treated and UVB-irradiated samples with mixed keratin human monoclonal mouse anti-human antibodies (DakoCytomation, Carpinteria, CA). No difference in epidermal staining pattern between nontreated and treated and then UV light-irradiated samples was detected, suggesting specificity of COX-2 antibody staining (Fig. 56, a and b). Dermal epidermal junction is indicated by the dashed line; ×200 magnification, all panels.
increase in COX-2 immunostaining by 16 h that diminished slightly but persisted through 24 h (Fig. 4b).

**T-oligo-induced Inhibition of COX-2 Expression in Human Skin Is Associated with Up-regulation and Activation of p53 Protein**—To confirm our *in vitro* findings of an inverse relationship between T-oligo-induced decreases in constitutive and UV light-induced COX-2 levels and increases in p53 level and activity, adjacent sections of the same human tissue that were used for COX-2 immunostaining were processed for both p53<sub>total</sub> and p53<sub>Ser15</sub> immunofluorescent staining. In T-oligo-treated versus vehicle-treated skin after 24 h, there was a >100% increase in the number of constitutively p53<sub>total</sub> positive (+) nuclei (26 ± 4 versus 12 ± 2, *p < 0.009*) (Fig. 5, a and c). In UV light-irradiated samples by 6 h the numbers of p53<sub>total</sub> (+) nuclei in T-oligo-treated samples were increased more than 3-fold above vehicle controls (38 ± 5 versus 12 ± 6, *p < 0.004*), and more than 10-fold above sham-irradiated samples, whereas the modest and variable increase in UV light-irradiated control samples remained insignificant (Fig. 5, b and d). By 24 h, the numbers of p53<sub>total</sub> (+) nuclei were similar in T-oligo-versus dilluent-treated UV light-irradiated skin samples (29 ± 4 versus 26 ± 5, *p = 0.6*) and more than 20-fold higher in both cases than in the sham-irradiated controls (Fig. 5, b and d).

T-oligo treatment minimally increased the number of nuclei with detectable constitutive p53<sub>Ser15</sub> levels (0.3 ± 0.2 versus 0.5 ± 0.2, vehicle versus T-oligo, *p = 0.09*) (Fig. 6, a and c). However, compared with vehicle-treated UV light-irradiated skin, there was a modest but statistically significant increase in the number of p53<sub>Ser15</sub> (+) nuclei in T-oligo-treated UV light-irradiated skin samples at 6 and 24 h (6 h, 10.3 ± 0.8 versus 14 ± 0.6, *p < 0.001*; and 24 h, 8.5 ± 0.9 versus 13 ± 0.6, vehicle versus T-oligo, *p < 0.001*) as well as striking and highly significant increases above sham-irradiated untreated samples in both cases (Fig. 5, b and d). Taken together with the data of COX-2 immunostaining (Fig. 4b), these results establish an *in vivo* relevance of the cause-and-effect inverse relationship between increased p53 levels and activity and decreased COX-2 levels in T-oligo-treated human fibroblasts and keratinocytes (Fig. 1, a and b, and Fig. 3, a–c).

**DISCUSSION**

UV irradiation is the major environmental carcinogen for human skin (77, 78), initiating and promoting development of both melanoma and nonmelanoma skin cancers (79–81). Mechanisms that contribute to UV light-induced mutagenesis and carcinogenesis include inactivation of tumor suppressor genes and/or activation of oncogenes (82–84), events that may also lead to clonal expansion of affected cells (85–89). However, in recent years a great deal of evidence has emerged suggesting that UV light-induced inflammation also plays an important role in tumor promotion and progression (26, 90–93). In murine models of skin carcinogenesis, it has been shown that administration of nonsteroidal anti-inflammatory drugs, especially selective COX-2 inhibitors, reduces the prevalence and multiplicity of UV light-induced neoplasms (74, 92–95), strongly implying direct involvement of COX-2 in cutaneous carcinogenesis.

The present study demonstrates that topical application of telomere 3’-overhang homolog DNA oligonucleotides, collectively termed T-oligos, inhibits UV light-induced up-regulation of COX-2 *in vitro* and ex
Multidrug resistance gene (MDR1), and B-cell lymphoma gene-2 (Bcl2).

Many genes are reported to be negatively regulated by p53. These proteins (TBPs) by preventing binding of TBPs to the TATA motif (98).

It was suggested that p53 suppresses gene expression by interfering with formation of the transcription preinitiation complex with TATA-binding proteins (TBPs) by preventing binding of TBPs to the TATA motif (98). Many genes are reported to be negatively regulated by p53. These include c-Fos, c-Jun, c-Myc, IL-6, heat shock protein 70 gene (HSP70), multidrug resistance gene (MDR1), and B-cell lymphoma gene-2 (Bcl2) (98–103).

We demonstrate that T-oligos decrease COX-2 levels at least in part by decreasing COX-2 transcription, an effect that is dependent on p53 activity, not on total p53 levels. It is known that p53 suppresses a variety of promoters that contain one or more TATA boxes (96, 97). It was suggested that p53 suppresses gene expression by interfering with formation of the transcription preinitiation complex with TATA-binding proteins (TBPs) by preventing binding of TBPs to the TATA motif (98). Many genes are reported to be negatively regulated by p53. These include c-Fos, c-Jun, c-Myc, IL-6, heat shock protein 70 gene (HSP70), multidrug resistance gene (MDR1), and B-cell lymphoma gene-2 (Bcl2) (98–103).

Relevant to p53-dependent regulation of COX-2, it was shown that p53 inhibits the formation of complexes between TBPs and the murine and human COX-2 promoters in a cell-free system (33). The same authors also reported that wild type but not temperature-sensitive mutant p53 competed with TATA-binding proteins for binding to the mouse and human COX-2 promoters over a 100–bp segment surrounding the transcription initiation start on COX-2 promoter (33). Accordingly, to test the hypothesis that T-oligo-mediated p53-dependent suppression of COX-2 depends on repression of COX-2 promoter activity by activated p53, we tested T-oligo effect in p53WT versus p53DN cells. In cells expressing p53WT, treatment with T-oligos decreased constitutive COX-2 promoter-driven transcription of a reporter plasmid by 54%, whereas in p53DN cells T-oligo treatment had virtually no effect on COX-2 promoter activity, demonstrating that functional p53 is required for T-oligo-induced repression of the COX-2 promoter. Our findings may also resolve apparently conflicting data in the literature concerning regulation of COX-2 gene expression by p53, as investigators reporting positive p53 regulation of COX-2 in various mutant and tumor-derived cell lines correlated only the level of expression of the two proteins and did not assess p53 activity (104).

We demonstrate that T-oligos decrease COX-2 levels at least in part by decreasing COX-2 transcription, an effect that is dependent on p53 activity, not on total p53 levels. It is known that p53 suppresses a variety of promoters that contain one or more TATA boxes (96, 97). It was suggested that p53 suppresses gene expression by interfering with formation of the transcription preinitiation complex with TATA-binding proteins (TBPs) by preventing binding of TBPs to the TATA motif (98). Many genes are reported to be negatively regulated by p53. These include c-Fos, c-Jun, c-Myc, IL-6, heat shock protein 70 gene (HSP70), multidrug resistance gene (MDR1), and B-cell lymphoma gene-2 (Bcl2) (98–103).
E2F1 may be of particular relevance to our findings, as there is evidence suggesting E2F1-mediated inhibition of NFκB in various cell types (107, 108). Indeed, Tanaka et al. (107) showed that endogenous E2F1 competes with NFκB/p50 for binding to the p65 subunit of NFκB and that this physical interaction of E2F1/p65 inhibits NFκB transcriptional activity. Additionally, Phillips et al. (109) have shown in Saos2 cells lacking p53 that E2F1-induced inhibition of NFκB nuclear translocation and activity is mediated by the abrogation of TRAF-2 protein and inhibition of IkB kinase phosphorylation. It will be of interest to further investigate, specifically in cells with nonfunctional p53, such as the majority of UV-light-induced skin neoplasms, whether T-oligo-mediated E2F1-dependent inhibition of NFκB in the absence of functional p53 may also inhibit COX-2 expression.

To date, attention has been directed toward inhibition of COX-2 enzyme activity, and much less attention has been given to modulation of COX-2 protein levels that are constitutively increased in many tumor cells (110). However, compared with young skin, there is an age-associated increase in constitutive and UV-light-induced prostaglandin E2 production and COX-2 expression in human skin (26). The resulting chronic low grade inflammation may have crucial pathophysiologic implications for many aging processes, such as loss of collagen, and may contribute to the development and progression of age-associated diseases, including malignancies. Indeed, experimental COX-2 overexpression has been also reported to increase tumorigenesis in a variety of organ systems, including colon, lung, prostate, breast cancer, urinary bladder, pancreas, and liver (76, 111–115), in addition to skin (26–29). COX-2 overexpression is also increasingly implicated in the process of tumor angiogenesis (neo-vascularization) through increasing vascular endothelial growth factor levels as well as producing prostaglandins and thromboxanes that promote endothelial cell migration (18).

We conclude that T-oligo treatment beneficially affects multiple contributors to carcinogenesis; T-oligo transiently inhibit cell proliferation (38–41, 44–51, 55, 56), enhance DNA repair capacity (49–51), and decrease mutagenesis and photocarcinogenesis (51), as well as induce apoptosis and senescence of malignant cells (38, 40, 43). In the present report we demonstrate that T-oligos also decrease constitutive and UV light-induced levels of COX-2, an inflammatory mediator strongly implicated in the processes of photocarcinogenesis, chemical and spontaneous carcinogenesis, and tumor angiogenesis (116–118). Furthermore, we suggest that T-oligos may also combat the tendency for chronic low grade inflammation that accompanies aging (26).

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T-oligo Treatment Decreases Constitutive and UVB-induced COX-2 Levels through p53- and NF κB-dependent Repression of the COX-2 Promoter

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