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Reduction of UV-induced skin tumors in hairless mice by selective COX-2 inhibition

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UV light is a complete carcinogen, inducing both basal and squamous cell skin cancers. The work described uses the selective COX-2 inhibitor celecoxib to examine the efficacy of COX-2 inhibition in the reduction of UV light-induced skin tumor formation in hairless mice. UVA-340 sun lamps were chosen as a light source that effectively mimics the solar UVA and UVB spectrum. Hairless mice were irradiated for 5 days a week for a total dose of 2.62 J/cm^2. When 90% of the animals had at least one tumor, the mice were divided into two groups so that the tumor number and multiplicity were the same ($P < 0.31$). Half of the mice were then fed a diet containing 1500 p.p.m. celecoxib. Tumor number, multiplicity and size were then observed for the next 10 weeks. Ninety-five percent of the tumors formed were histopathologically evaluated as squamous cell carcinoma. COX-2 expression and activity were increased in tumors. After 10 weeks, the difference in tumor number and multiplicity in the drug-treated group was 56% of UV controls ($P < 0.001$). The results show that the orally administered selective COX-2 inhibitor celecoxib prevents new tumor formation after the onset of photocarcinogenesis and suggest that treatment with celecoxib may be very useful in preventing UV-induced skin tumors in humans.

Introduction

UV light has been well documented as a complete carcinogen responsible for initiation and promotion of both basal and squamous cell carcinomas (1). Tumors produced by exposure to UV light constitute nearly 50% of cancers diagnosed in the USA today (2). Approximately 90% of the 900 000–1 200 000 new cases of skin cancer each year are attributable to UV light irradiation (3). UV light is defined as those wavelengths between 200 and 400 nm, termed UVA (320–400), UVB (290–320) and UVC (200–290). UVC is filtered out by the ozone layer of the Earth's atmosphere, so that it has little biological relevance in skin tumor formation (4). Of those wavelengths reaching the Earth's surface, the most effective in causing squamous cell carcinoma are wavelengths within the UVB range. However, previous studies have clearly demonstrated the capacity for UVA (320–400) to induce oxidative stress, also associated with photocarcinogenesis (5,6). Moreover, on the surface of the Earth, UVA wavelengths are the most abundant in the solar spectrum. Therefore, the role of UVA wavelengths in skin tumorigenesis due to chronic sun exposure must also be considered. The work described here was performed using a light source including both UVA and UVB to mimic the UV emission of the sun as closely as possible and make extrapolation to human photocarcinogenesis easier.

A number of contributing mechanisms to UV-induced skin tumorigenesis have been defined (7,8). However, recent work has suggested that UV-induced prostaglandin synthesis may also be a significant contributing factor. Prostaglandin synthesis occurs via the coordinate action of a phospholipase that liberates arachidonic acid from membrane phospholipids and a cyclooxygenase (COX) which converts the free arachidonic acid to prostaglandins (9,10). Two isoforms of COX have been described which share 60% homology, COX-1 and COX-2. COX-2 is highly regulated and induced by inflammation while COX-1 is considered primarily a housekeeping form (9). UV exposure of the skin is known to induce prostaglandin production (11,12). This occurs both by increasing synthesis and activity of cytosolic phospholipase A_2 and induction of COX-2. The acute up-regulation of COX-2 by UV radiation suggests that it may contribute to photocarcinogenesis in the same way that COX-2 has recently been shown to contribute to colon cancer (13,14).

In patients with familial adenomatous polyposis, treatment with the non-steroidal anti-inflammatory drug Sulindac has been shown to significantly reduce colon cancer (13). The relationship of this finding to COX-2 has been demonstrated in studies of mice expressing the mutant *Apc716* gene, responsible for intestinal polyposis in mice (15). In these studies, the colon cancer phenotype associated with deletion of *Apc716* was reduced 7-fold in animals in which the COX-2 gene was knocked out (15). These data suggest that there may be a beneficial effect of non-steroidal anti-inflammatory drug treatment in prevention of squamous cell carcinoma of the skin. Evidence supporting the potential involvement of COX-2 in human actinic keratosis and squamous cell carcinoma of the skin was recently obtained (11). Increased COX-2 staining in lesional skin has been shown by immunohistochemistry and acute UV light has been shown to induce synthesis of COX-2 in human epidermis by western blot (11). Thus, it seems likely that available therapeutic agents could be highly beneficial in reducing the incidence of skin cancer in humans. The studies described below were done to address this question. The anti-inflammatory agent used in this study was celecoxib (Celebrex™), a selective COX-2 inhibitor. Celecoxib is a diaryl-substituted pyrazole that has been shown to inhibit prostaglandin synthesis primarily via inhibition of COX-2 and at therapeutic concentrations in humans it does not inhibit the COX-1 isozyme (16). In this study, we observed a beneficial effect of celecoxib in skin tumor formation and progression induced by chronic broad band UV (UVA + UVB) exposure in hairless mice.

Materials and methods

Mice and the COX-2 selective inhibitor

Inbred hairless female mice of the Skh:HR-1 albino strain were purchased from Charles River Laboratory, Wilmington, MA. Mice were housed four per cage.

Abbreviations: COX, cyclooxygenase; PGE_2, prostaglandin E_2.

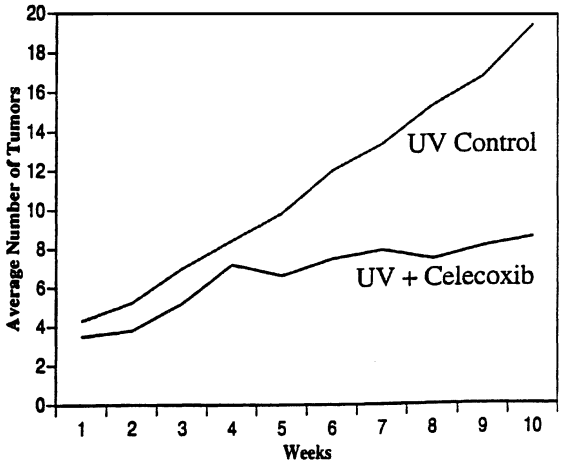

Fig. 1. Effect of celecoxib on tumor multiplicity. Tumor multiplicity in control, UV and UV + celecoxib mice was measured for 10 weeks after administration of celecoxib was begun. Irradiated mice were divided into two groups with an equal tumor burden when 90% of the mice had at least one tumor present. Feeding of 1500 p.p.m. celecoxib was begun at time + 0 on the graph, 6 weeks after the last UV exposure. The average number of tumors present each week are shown. No tumors were present in control animals not receiving UV light. The difference between UV + celecoxib- and UV-treated animals was calculated at weekly intervals using Student's *t*-test. Weeks 1–4, $P < 0.31$; at week 10, $P < 0.001$.

This strain was selected for study because it is the standard model for UV photocarcinogenesis work (16). Celecoxib [SC-58635, 4-(5-(4-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl)bezenesulfonamide] was supplied in a standard mouse diet by Searle (St Louis, MO). After completion of the irradiation protocol, mice were fed control diet or identical chow into which celecoxib had been incorporated so that the final concentration of drug was 1500 p.p.m. Body weight was monitored every week and the amount of food consumed documented to ensure that their ingestion of drug was appropriate.

UV irradiation

The UV light source was provided by a bank of four UVAsun-340 sun lamps which have irradiance between 295 and 390 nm (Q-Panel Lab Products, Cleveland, OH). This light source was selected because it mimics natural sun exposure better than FS20 lamps and can be easily administered over the large surface area required for mouse colony irradiation. The mice were irradiated for 5 days/week beginning at a dose of 0.035 J/m^2 UVB and 11.34 J/m^2 UVA, as measured by an IL1700 meter (International Light, Newburyport, MA). A SED 400 probe was used to determine irradiance in the UVA range and a SED 240 probe was used for measurement of UVB. The initial dose of light was chosen because it represents 70% of an edema dose and is equivalent to 30 min of noonday sun in Rochester, NY, in the autumn. The dose of light was increased by 10% each week to a dose of 0.069 J/cm^2. The final cumulative dose was 2.62 J/cm^2 UVB.

Tumor measurement

Papilloma and tumor incidence was documented by counting papillomas or frank carcinomas and measuring their size at weekly intervals. Digital photographs at a fixed distance were taken weekly. Animals losing 15% of their body weight during the protocol or whose tumors became infected were sacrificed. The histology of the tumors was documented in tumors harvested from animals sacrificed at the end of the experimental protocol.

Immunohistochemistry

Tissues were paraffin embedded and cut into 4 μm sections onto positively charged slides (Superfrost/Plus; Fisher Scientific, Pittsburgh, PA). Sections were then deparaffinized and permeabilized. Samples were incubated with COX-2 antibody (anti-PGHS-1 monoclonal antibody; PG27; Oxford, MI) overnight at 4°C. This antibody was diluted 1:400 in phosphate-buffered saline–blocking buffer. Control sections were incubated with normal rabbit serum diluted to the same concentration as the antibody being studied. Sections were detected with a streptavidin–biotin affinity system (Ominitags Plus; Shandon-Lipshaw, Pittsburgh, PA) and visualized with diaminobenzidine (Dako Corp., Carpinteria, CA), which reacts with peroxidase to give a brown reaction product. Slides were counterstained in hematoxylin-1 (Richard-Allan Scientific, Kalamazoo, MI). Pictures were taken under a Nikon photomicroscope for histochemistry (18).

Microsome activity and inhibitors

Mice were sacrificed by CO_2 inhalation according to institutional protocols and skin samples were immediately frozen on dry ice. The samples were pulverized after freezing in liquid nitrogen. The powder was suspended in a 50 mM Tris–HCl, 0.25% sucrose, pH 8.3, buffer then homogenized in a Dounce homogenizer. Samples were then centrifuged at 14 000 *g* at 4°C for 20 min. The supernatant was transferred to a fresh tube and centrifuged at 100 000 *g* at 4°C for 20 min to pellet microsomes. The pellet was resuspended in 50 mM potassium phosphate buffer, pH 7.4, with 1 mM phenol. Specific inhibitor studies were performed by incubating 60 ng of microsomal protein for 15 min at room temperature with inhibitors provided by Dr Karen Seibert (Searle Corporation, St Louis, MO). SC58560 (0.1 μM) was used in experiments examining inhibition of COX-1 activity; SC58236 (1 μM) was used to examine inhibition of COX-2. These concentrations were based on previously determined IC_{50} values for recombinant human enzyme of 0.0048 and 0.009 for SC58560 and SC58236, respectively. After preincubation with selective inhibitors, 30 μM arachidonic acid was added and samples were incubated for an additional 15 min at 37°C. Preliminary experiments revealed that this concentration of arachidonic acid saturated the enzyme. The reactions were then quenched with 10 mg/ml indomethacin (Sigma, St Louis, MO). The prostaglandin E_2 (PGE_2) content of the samples was analyzed by ELISA (Caymen Chemical, Ann Arbor, MI). PGE_2 detected (428 ± 267 pg) in unstimulated samples is subtracted from values shown in Figure 4.

ELISA

The quantity of PGE_2 and thromboxane B_2 (TXB_2) in supernatants was determined by ELISA using specific antibody available from Caymen Chemical according to the manufacturer's instructions. The limit of detection is 30 pg/μl for PGE_2. The antibody recognizes PGE_2 well, but cross-reacts with PGE_1 poorly (18%). The cross-reactivity with other prostanoids is <0.01%.

Statistical analysis

Tumor multiplicity, tumor volume and body weights were compared between the control group and the celecoxib diet group. Tumor multiplicity, expressed as the mean number of tumors per animal, was analyzed by the unpaired *t*-test.

Results

Irradiation and treatment of mice with celecoxib

The experimental protocol selected was designed to mimic as much as possible the clinical setting in which intervention for prevention of human squamous cell carcinoma might occur. A light source which emits both UVA and UVB was selected because human carcinoma occurs in the presence of the full UV spectrum. A protocol in which drug treatment was not initiated until after tumor formation began was also chosen to mimic the behavior of many patients who have been diagnosed with skin cancer. Thus, drug treatment was begun after UV irradiation ended and tumor formation began.

Twenty-six animals were included in both the non-irradiated control and the UV groups. Irradiation was begun using a dose of light which was 70% of the dose of light needed to produce edema in preliminary experiments. The UV group was exposed to a total of 2.62 J/cm^2 UVB. Animals were irradiated for a total of 13 weeks, at which time UV irradiation was discontinued. When 90% of the animals in the irradiated group had at least one tumor, the mice were divided into two groups so that the tumor number and multiplicity were approximately the same ($P < 0.31$). Starting at week 19, half of the animals were fed the drug, as were half of the non-irradiated control mice. The tumors started most frequently on the lower back and neck of the UV-irradiated animals. No tumors were observed in non-UV-exposed areas. In addition to the appearance of tumors, some thickening of the skin occurred in irradiated animals. No tumors were observed in the animals which were not exposed to light at any time during the protocol, whether they were administered the drug or not. Throughout the protocol, there were no notable differences in food intake nor were there significant decreases in body weight as a result of drug administration.

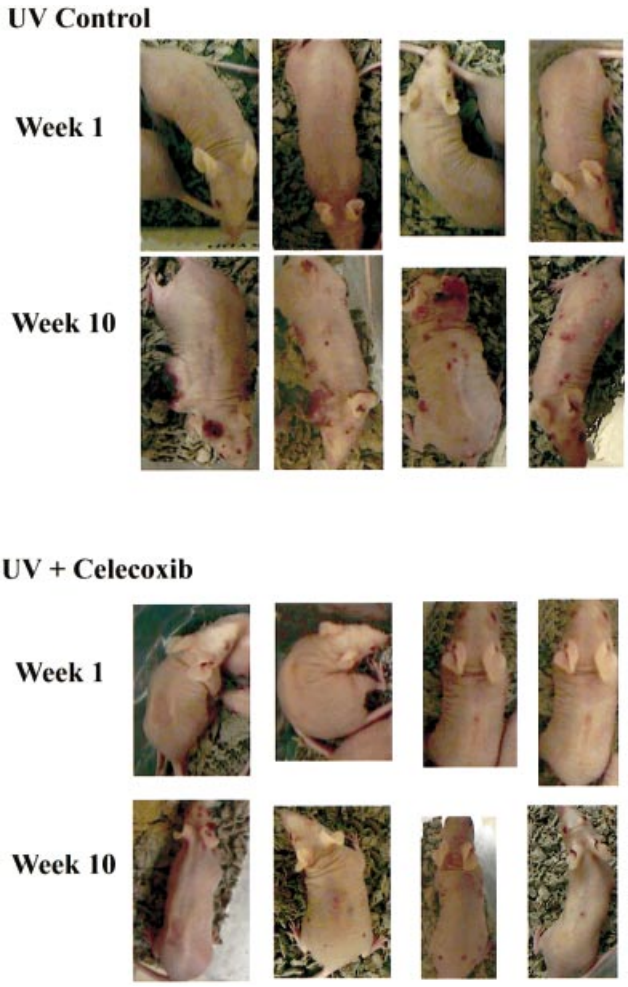

Fig. 2. Tumor growth in UV- versus UV + celecoxib-treated animals. The top row in each group of photographs shows the appearance of each animal at the initiation of celecoxib feeding. The same mouse is shown 10 weeks later underneath its baseline picture. The animals in the top panels were not treated, while those in the bottom panels received 1500 p.p.m. celecoxib for 10 weeks.

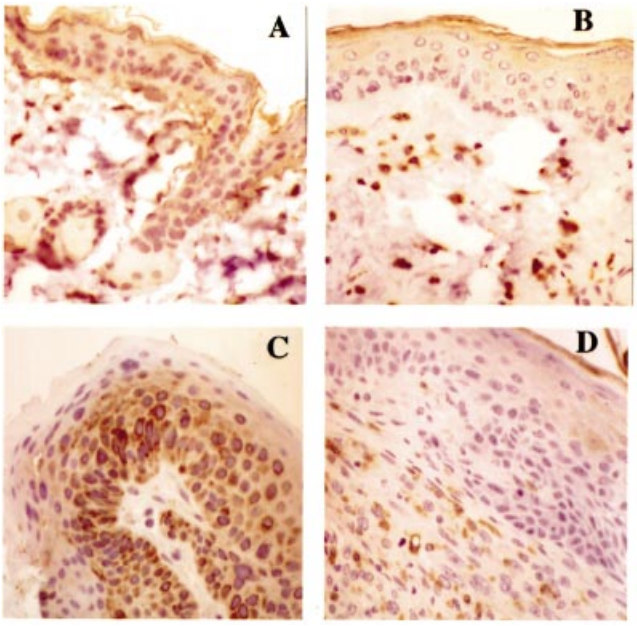

Fig. 3. Immunohistochemistry staining of COX-2 in mouse skin samples. Skin samples were stained for COX-2 at the end of the study protocol. COX-2 staining gives a brown reaction product. (**A**) Control animal (no UV exposure); (**B**) UV, last irradiated 16 weeks prior to study; (**C**) papilloma from irradiated mouse; (**D**) squamous cell carcinoma from irradiated mouse.

Table I. Average tumor volume at week 10

Group	UV	UV + celecoxib
Number of tumors	19.2	8.54
Tumor size (mm^3)	5.09 ± 0.67	1.45 ± 1.99
Tumor weight (g)	3.52 ± 0.36	1.25 ± 0.13

Effect of celecoxib on tumor number and growth

The tumor multiplicity (number of tumors per mouse) was recorded weekly. For the first 4 weeks after beginning celecoxib in the diet, no difference in the tumor multiplicity between the two groups was observed ($P < 0.31$). However, beginning 4 weeks after initiating celecoxib treatment, a slower increase in the number of tumors present was observed. This trend continued over the course of the experiment; by the end of week 10, celecoxib inhibited the tumor multiplicity in the drug-treated group significantly compared with the control group ($P < 0.001$; Figure 1). Despite the pronounced effect on the number of new tumors, there was no observable effect on the growth of the tumors already present. Tumors present on the animals were measured weekly and their rate of growth calculated. When growth of tumors in celecoxib-treated animals was compared with that of animals not receiving treatment, no difference in the rate of growth of these tumors was found with drug treatment. There was also no involution of tumors or papillomas observed. Ten weeks after initiating drug treatment, the tumor burden in the control group was sufficiently large that the experiment was ended. The effect of celecoxib on tumor burden was sufficiently strong that the changes were readily evident clinically (Figure 2).

Even a highly selective inhibitor of COX-2 such as celecoxib may have inhibitory effects on COX-1 at very high serum concentrations. To validate that celecoxib was acting primarily to inhibit COX-2, blood levels were obtained from treated animals. The area under the curve was 2.12 ± 0.73 μg/ml, a level of celecoxib consistent with inhibition of COX-2 but little inhibition of COX-1. Lack of inhibition of COX-1 was further confirmed by measuring thromboxane synthesis induced by calcium ionophore stimulation of platelets obtained from treated mice. No suppression of this COX-1-mediated thromboxane synthesis was observed (8.0 ± 3.0 ng versus 11.0 ± 3.0 ng).

Tumor morphology and immunohistochemical distribution of COX-2

Squamous cell carcinoma is reported to be the most common type of tumor induced by UV irradiation in hairless mice (17). At the end of the celecoxib treatment interval, all animals were killed and 20 large tumors submitted for pathological diagnosis. Analysis of the tumors revealed that >95% were squamous cell carcinoma. A significant proportion (30%) showed an aggressive spindle cell morphology; the remainder ranged from well to moderately differentiated tumors. One B cell lymphoma was also found. Tumor weight in each group was measured in addition to tumor size. Both tumor size and weight were significantly less in treated animals (Table I). Immunohistochemistry was done on samples of skin obtained from non-irradiated control animals, previously irradiated mice (killed 16 weeks after the last dose of

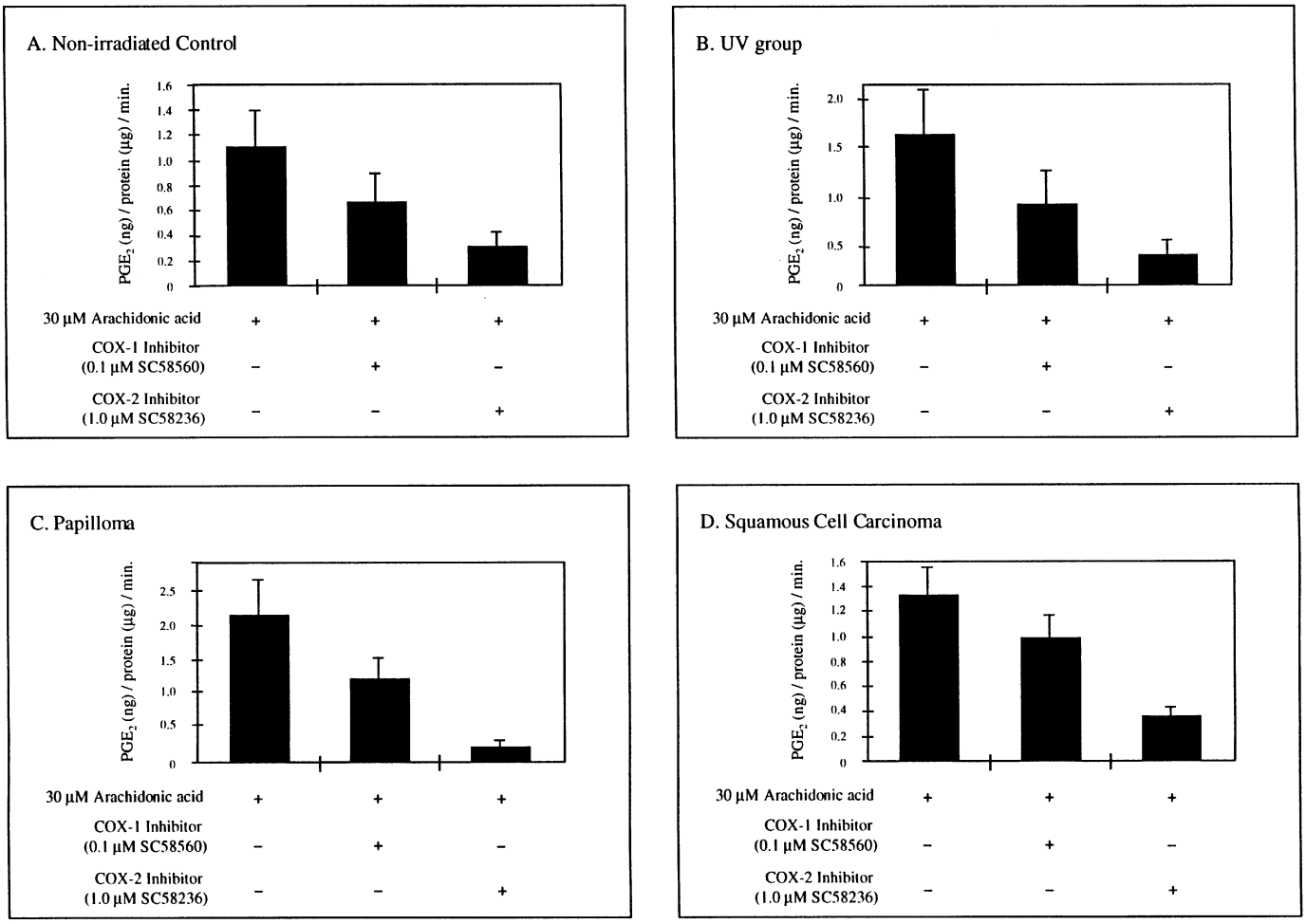

Fig. 4. Microsomal COX activity in mouse skin homogenates. Microsomes were prepared from skin homogenates as described in Materials and methods. Selected aliquots were preincubated with selective cyclooxygenase inhibitors then stimulated with 30 µM arachidonic acid and product formation determined by ELISA of PGE_2. (**A**) Control animal (no UV exposure); (**B**) UV, last irradiated 16 weeks prior to study; (**C**) papilloma from irradiated mouse; (**D**) squamous cell carcinoma from irradiated mouse.

UV irradiation) and papillomas and frank tumors to determine COX-2 expression and its cellular localization. Faint COX-2 immunoreactivity was observed in the epidermis of control animals, which was distributed throughout all layers of the epidermis (Figure 3A). There were also occasional cells in the dermis that stained strongly, which appeared to be dermal dendrocytes. In skin that had been irradiated 16 weeks previously (Figure 3B) there was mild acanthosis of the epidermis with increased thickness of the stratum granulosum. Slightly increased COX-2 staining was present in the epidermis in a patchy pattern, but the most striking change was the greatly increased density of intensely stained cells in the dermis, which appeared to be lymphocytes and histiocytes. In papillomas (Figure 3C), intense COX-2 staining in the basal layer was observed. Localization of COX-2 staining in the papillomas corresponded to the cellular compartment that showed cytological features of dysplasia, including nuclear hyperchromasia and increased nuclear to cytoplasm ratio. Squamous cell carcinomas displayed a combination of increased staining in the tumor cells themselves with an infiltrate of intensely stained inflammatory cells (Figure 3D).

COX-2 activity is increased in papillomas and tumors

To verify that the staining observed in the epithelium and inflammatory tissue was related to increases in the synthesis of prostaglandins, COX activity in tissue extracts was determined. Normal skin, previously irradiated skin, papillomas and tumors from mice not receiving drug were snap frozen, pulverized and microsomes were prepared. Because PGE_2 is the predominant product formed in skin, the PGE_2 formed by the microsomes was then determined by ELISA as a marker for COX activity. COX-1 and COX-2 selective inhibitors (SC58560 and SC58236, respectively) were pre-incubated with the microsomes to determine the preponderant isoform of COX present. These studies revealed that the activity in non-irradiated control skin reflects the action of both isoforms present in approximately equal amounts (Figure 4). In previously irradiated skin the total capacity to synthesize PGE_2 was increased, and the isoform responsible for the activity was predominantly COX-2. Further increases in the PGE_2 synthetic capacity of papillomas and tumors were found, also related to increased activity inhibitable by the selective COX-2 inhibitor SC58236.

Discussion

It often occurs in clinical practice that a patient diagnosed with squamous cell carcinoma of the skin seeks to prevent the occurrence of a second tumor. Even more frequently individuals who have developed actinic keratosis, a precursor of squamous

cell carcinoma of the skin, request measures to stop the development of new lesions. In this study, we demonstrate the capacity of celecoxib to inhibit the development of new skin cancers in previously irradiated mice. The results of this study suggest that such an approach may be quite useful clinically in at-risk individuals.

The participation of COX-2 in skin carcinogenesis demonstrated in these experiments most likely occurred via inhibition of tumor promotion. Because the drug was not started during the irradiation portion of the protocol, it cannot have acted via the well-described tumor initiation events associated with UV exposure, such as thymine dimer formation and UV-mediated production of free radicals. Nonetheless, there may still be some protective effect against tumor initiation by celecoxib if COX activity itself contributes to tumor initiation in this process (19–21). During oxygenation of arachidonic acid as a substrate, COX generates highly reactive alkoxy radicals and peroxyl radicals. Malondialdehyde generated subsequent to the activity of COX is also highly reactive. The generation of these free radicals and the potential formation of DNA adducts may continue the initiation process begun by irradiation simply because of the chronic inflammatory infiltrate produced by chronic irradiation (19–21). In this series of experiments, inflammation was clearly still present 16 weeks after the last exposure to light had occurred. Celecoxib would inhibit this process by its action in inhibiting the COX enzyme.

Tumor promotion is the process of selective clonal expansion of initiated cells that have defects in terminal differentiation, programmed cell death, growth control or resistance to cytotoxicity. The actions of prostaglandins in promoting growth must also be key in the process observed in these studies. The role of prostaglandin synthesis in this capacity is well documented in mouse skin (22,23), although their role is strain and stimulus dependent (24). In the work presented here, COX-2 overexpression was intense in papillomas in the areas of dysplastic epidermis. The results suggest that COX-2 induction in dysplastic epidermis, combined with PGE_2 formation by nearby inflammatory cells, is a potent proliferative stimulus which is able to overcome the normal cellular commitment to differentiation resulting in tumor promotion. The fact that none of the existing tumors decreased in size or changed their rate of growth suggests that the PGE_2 effect is most important in the very early stages of the process of tumor promotion. This observation also indicates that it will be necessary to be consistent over a long period of time when treating with a COX-2 inhibitor, since brief periods in which PGE_2 is present in normal amounts may be sufficient to permit the progression of initiated cells to form tumors.

Data suggesting a modest effect of COX products in UV-induced tumor promotion have been previously collected. Indomethacin treatment of UV-irradiated hairless mice significantly delayed tumor onset and decreased tumor number, however, the effect was much less pronounced than in the current study (25). The effect was likely attenuated in that study because the dose of indomethacin used had to be reduced so that gastric ulceration did not occur. Bleeding and gastric distress are also important problems in human subjects treated with drugs that inhibit COX (26). Commonly used drugs such as aspirin and indomethacin are associated with gastrointestinal ulceration and renal toxicity under prolonged administration and have little selectivity for COX-1 or COX-2. Studies of celecoxib have shown that this selective COX-2 inhibitor significantly reduces these side-effects (26).

Selective Cox-2 inhibitors may also work by modulating immune function. Fisher *et al.* have shown that UVB-induced skin tumors in mice are highly antigenic and that their growth is controlled by the immune system of the UV-irradiated host (27). In addition, it is known that UV-induced up-regulation of PGE_2 contributes to systemic immune suppression (28). Recently, selective COX-2 inhibition has been shown to block the induction of IL-4 and IL-10, cytokines critical for inducing systemic immune suppression (29). This decreased immune suppression may potentially allow for the immunological destruction of UV-induced tumors.

In summary, this study suggests that COX-2 is important in UV-induced tumorigenesis of skin in mice. Oral administration of the selective COX-2 inhibitor celecoxib potently reduces UV-induced tumor multiplicity in hairless mice. The key mechanism suggested is an effect on local proliferation of epidermis. Whether this same effect may be present in human populations needs to be examined. The fact that COX inhibitors are ubiquitously available may mask the utility of this class of drugs for the extremely common problems of actinic keratosis and squamous cell carcinoma in humans. This work suggests that they may be very useful in preventing UV-induced skin tumors in humans, but further studies will be needed to document this.

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