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

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Introduction

UV light has been well documented as a complete carcinogen responsible for initiation and promotion of both basal and squamous cell skin cancers (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 A₂ 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.
Mice were sacrificed by CO₂ 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 IC₅₀ 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 E₂ (PGE₂) content of the samples was analyzed by ELISA (Caymen Chemical, Ann Arbor, MI). PGE₂ detected (428 ± 267 pg) in unstimulated samples is subtracted from values shown in Figure 4.

**ELISA**
The quantity of PGE₂ and thromboxane B₂ (TXB₂) 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/ml for PGE₂. The antibody recognizes PGE₂ well, but cross-reacts with PGE₁ 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/cm² 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.
NSAID prevention of skin cancer

Fig. 2. Tumor growth in UV- versus UV
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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.

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 $2.12 \pm 0.73 \mu g/ml$, 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 ($8.0 \pm 3.0$ ng versus $11.0 \pm 3.0$ 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 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

<table>
<thead>
<tr>
<th>Group</th>
<th>UV</th>
<th>UV + celecoxib</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of tumors</td>
<td>19.2</td>
<td>8.54</td>
</tr>
<tr>
<td>Tumor size (mm$^3$)</td>
<td>5.09 $\pm$ 0.67</td>
<td>1.45 $\pm$ 1.99</td>
</tr>
<tr>
<td>Tumor weight (g)</td>
<td>3.52 $\pm$ 0.36</td>
<td>1.25 $\pm$ 0.13</td>
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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 PGE2 is the predominant product formed in skin, the PGE2 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 PGE2 was increased, and the isoform responsible for the activity was predominantly COX-2. Further increases in the PGE2 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
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 PGE₂ 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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References


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