Treatment with 5-Fluorouracil and Celecoxib Displays Synergistic Regression of Ultraviolet Light B-Induced Skin Tumors

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Standard chemotherapeutic agents used for the treatment of pre-cancerous skin lesions and non-melanoma skin cancers are not completely effective. Several studies have suggested that repeated inflammatory sunburn reactions, which include the induction of cyclooxygenase-2 (COX-2) and the subsequent production of prostaglandins, play a role in skin cancer development. COX-2 inhibition has been demonstrated to be a potent means of preventing skin cancer development in mice; however, COX-2 inhibitors alone are not effective as chemotherapeutic agents. Data in a variety of cancer types suggest greater efficacy in treating tumors with combination chemotherapies. Therefore, we hypothesized that a combination of the chemotherapeutic agent 5-fluorouracil (5-FU) and the COX-2 inhibitor and anti-inflammatory drug celecoxib would act synergistically to regress tumors in a murine model of ultraviolet light B- (UVB-) induced carcinogenesis. We found that topical treatment with 5-FU and celecoxib together was up to 70% more effective in reducing the number of UVB-induced skin tumors than 5-FU treatment alone. Our data suggest that more effective chemotherapy regimens can be developed to treat the millions of pre-cancerous and cancerous skin lesions that arise every year, which could ultimately lead to a significant reduction in costs and cosmetic defects (scarring) associated with surgical interventions.

Key words: chemotherapy/cyclooxygenase-2/murine/non-melanoma skin cancer/prostaglandin E_2
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Numerous studies have now demonstrated a role for the cyclooxygenase-2 (COX-2) enzyme in the development of many different types of tumors (Koki *et al*, 2002). These include studies in the skin, which have revealed the overexpression of COX-2 in skin and tumors after ultraviolet (UV) light exposure (Buckman *et al*, 1998). In addition, blocking COX-2 activity through the use of specific COX-2 inhibitors, such as celecoxib, has been demonstrated to effectively inhibit the development of murine UVB-induced skin tumors (Fischer *et al*, 1999; Orengo *et al*, 2002; Wilgus *et al*, 2003a). Whereas COX-2 inhibitors have been successfully used to combat the formation of tumors, when used alone in chemotherapy protocols they have not been effective in inducing the regression of established skin tumors (Pentland *et al*, 1999; Fischer *et al*, 2003; Wilgus *et al*, 2003b). While disappointing, the results presented here and those of Fischer *et al* (2003) show that COX-2 inhibitors do have the potential to increase the effectiveness of other drugs, thereby still being useful in a chemotherapeutic regimen. Furthermore, the fact that COX-2 inhibitors are anti-inflammatory (Wilgus *et al*, 2000) and have the ability to reduce damage in the skin after exposure to physical agents such as UV light (Wilgus *et al*, 2003a) or ionizing radiation (Liang *et al*, 2003), suggests they could potentially be used to combat the damage and negative effects associated with the use of common topical chemotherapy drugs.

5-Fluorouracil (5-FU) is a chemotherapy drug commonly used for the treatment of solid tumors of the GI tract, breast, pancreas, ovaries, and colon (Chu *et al*, 2003). This antimetabolite drug mimics uracil and is incorporated into RNA and DNA, where it inhibits the thymidylate synthetase enzyme necessary for DNA synthesis (Heidelberger *et al*, 1957; Chu *et al*, 2003). 5-FU is preferentially incorporated into the DNA of rapidly proliferating tumor cells, subsequently leading to the destruction of the tumor. The observation that the systemic use of this drug could resolve pre-cancerous actinic keratoses (AK) lesions in the skin (Falkson and Schulz, 1962) led to the development of topical preparations (Dillaha *et al*, 1963). The topical formulation Efudex (ICN Pharmaceuticals, Inc., Costa Mesa, California), is now commonly prescribed to treat pre-cancerous AK lesions, and sometimes non-melanoma skin cancer (NMSC) lesions themselves. However, it displays variable efficacy (Ashton *et al*, 1970; Belisario, 1973). Topical 5-FU's utility and effectiveness are hampered by the fact that there are dramatic side-effects associated with its use, including irritation, pain, inflammation, scaling, erosion, and even scarring (Tsuji *et al*, 1984; Gupta *et al*, 2001). These side-effects and the resulting cosmetic problems often lead

Abbreviations: AK, actinic keratoses; COX-2, cyclooxygenase-2; 5-FU, 5-fluorouracil; NMSC, non-melanoma skin cancer; PCNA, proliferating cell nuclear antigen; PGE_2, prostaglandin E_2; UV, ultraviolet light

to sporadic use by patients, which can be a detriment to the potency of this drug.

The value of the inflammatory phase induced by topical 5-FU to the regression of skin lesions has been debated for many years. Several studies suggest that it is not necessary for the resolution of the lesion (Breza *et al*, 1976; Pearlman *et al*, 1986). Therefore, there could be value in combining 5-FU with an anti-inflammatory drug such as celecoxib. The effectiveness of 5-FU is less than ideal, resulting in incomplete lesion removal and common recurrences of the treated skin lesions (Sander *et al*, 1997; Salim *et al*, 2003). Therefore, enhancing the tumor fighting capabilities of 5-FU by targeting additional pathways involved in tumor growth may prove to be beneficial in inducing tumor regression. Since celecoxib and 5-FU target different pathways to inhibit tumor growth, and because COX-2 inhibitors have been suggested to enhance the effectiveness of other chemotherapeutic drugs, we studied the effects of the addition of the COX-2 inhibitor celecoxib (Celebrex G. D. Searle LLC, St. Louis, MO) to the standard chemotherapeutic agent 5-FU (Efudex) on the regression of UVB-induced murine skin tumors.

Results

Induction of PGE_2 in unirradiated skin by 5-FU treatment The effects of combination treatment with 5-FU and celecoxib on PGE_2 production in unirradiated skin were assessed using an enzyme immunoassay (Table I). Three weeks of topical 5-FU treatments caused a significant increase in the levels of PGE_2 in the skin compared to the vehicle control ($^*p = 0.0218$), suggesting upregulation of PGE_2 may contribute to the observed inflammatory side-effects of topical 5-FU treatment. Adding the COX-2 inhibitor celecoxib to the 5-FU treatments either directly (5-FU/celecoxib) or indirectly (5-FU + celecoxib) caused PGE_2 levels to return to those found in vehicle treated skin. Celecoxib was able to effectively control 5-FU-induced PGE_2 production, implying anti-inflammatory agents have the potential to limit the damaging effects of topical 5-FU treatment.

Effects of combination treatment on tumor regression In order to formally estimate differences in tumor multiplicity between the treatment groups (Fig 1*A*), a repeated measures model was fitted to the data and an autoregression correlation structure was selected. Model results found that there was a significant group × time interaction, implying that the change in the total number of tumors over time depends on the treatment group ($p < 0.0001$). The number of tumors in vehicle-treated mice increased by an average of 1.27 tumors per week ($p = 0.0017$), whereas mice in the most effective treatment group (5-FU/celecoxib) lost an average of 2.34 tumors per week ($^\#p < 0.0001$). The other groups did not significantly change over time.

In addition to group × time interactions, group differences in tumor multiplicity (mean number of tumors per mouse) were estimated from the model at the different weeks of treatment. At the start of treatments (week 0), there were no significant differences in tumor multiplicity between the groups. Groups were declared statistically

Table I. PGE_2 levels in unirradiated skin after 3 wk of treatment[a]

Treatment	PGE_2 concentration (pg)
Vehicle	115 ± 6.35
Celecoxib	120 ± 4.91
5-FU	148 ± 8.03*
5-FU/celecoxib	113 ± 6.10
5-FU + celecoxib	122 ± 22.16

[a]Values represent mean ± standard error.
*$p = 0.0218$ compared to vehicle treatments.
PGE_2, prostaglandin E_2; FU, fluorouracil.

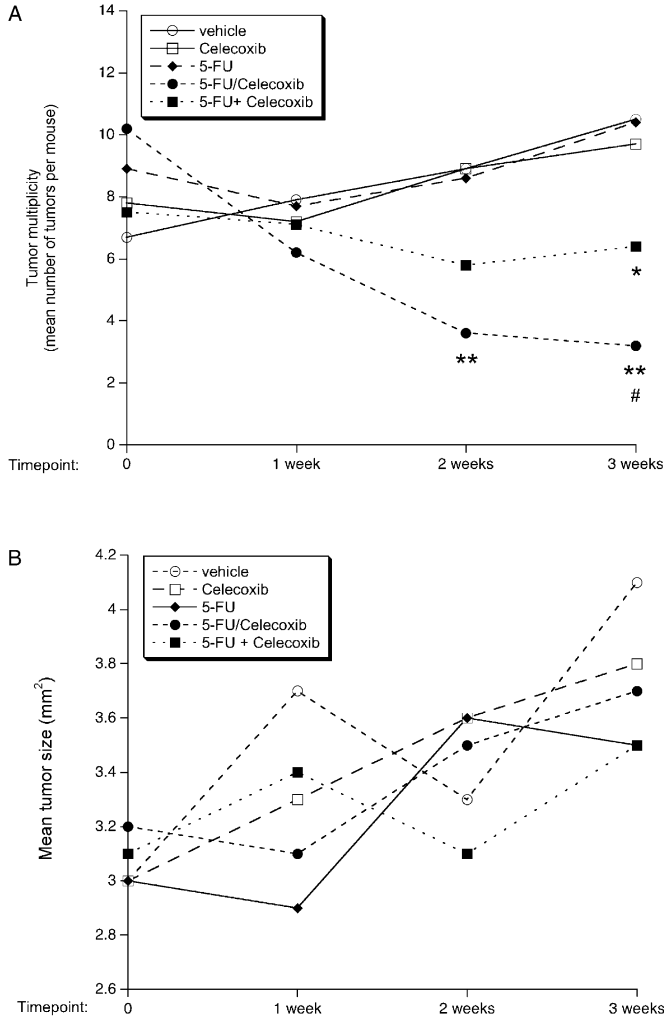

Figure 1
Chemotherapeutic efficacy of 5-fluorouracil (5-FU) and celecoxib. The effect of 3 wk of topical treatments on the regression of ultraviolet B- (UVB-) induced skin tumors was assessed by determining tumor multiplicity, or the mean number of tumors per mouse (*A*) and mean tumor size (*B*). Tumor counts and size measurements were performed weekly over the treatment time course, with $n = 10$ mice per treatment group. A * denotes fewer tumors with borderline significance ($p < 0.05$) and ** represents significantly fewer tumors ($p < 0.01$) compared to 5-FU. A # indicates a significant negative time × group interaction over the treatment period ($p < 0.0001$). Tumor size is presented in mm^2 (length × width).

significant if the p-value was less than 0.01, whereas p-values less than 0.05 but greater than 0.01 were considered borderline significant. Pairwise analysis revealed that after 3 wk of treatment, 5-FU had no significant effect on tumor multiplicity compared to vehicle controls. 5-FU/celecoxib treated mice achieved a difference of 4.4 fewer tumors per mouse compared to 5-FU treated mice at week 2 ($^{**}p = 0.0146$) and 7.2 less tumors by week 3 ($^{**}p = 0.0003$). Therefore, 5-FU/celecoxib treatments were almost 70% more effective in reducing tumor number compared to 5-FU alone. Although not as effective as the direct addition of celecoxib to 5-FU (5-FU/celecoxib), treating with the two drugs separately (5-FU + celecoxib) was also able to affect tumor multiplicity. Mice in the 5-FU + celecoxib treatment group had an average of 3.9 less tumors compared to 5-FU treated mice by week 3 ($^{*}p = 0.0457$), which corresponds to nearly a 40% enhancement in tumor regression with the 5-FU + celecoxib treatments. Although significant differences in tumor multiplicity were revealed between groups over the treatment period, no statistically significant differences in the size of remaining tumors were found (Fig 1*B*).

Combination treatment effects on tumor cell proliferation Besides causing a significant reduction in the average number of tumors per mouse (Fig 1), combination treatment with 5-FU and celecoxib resulted in a synergistic reduction in the percentage of PCNA-positive cells within the remaining tumors compared to 5-FU treatments alone (Fig 2). Tumors from both 5-FU/celecoxib (Fig 2*A* and *C*) and 5-FU + celecoxib (Fig 2*A* and *D*) treatment groups contained a significantly lower percentage of proliferating tumor cells ($^{*}p < 0.001$) compared to tumors receiving only 5-FU treatments (Fig 2*A* and *B*).

Effects of topical treatments on tumor PGE_2 content Although the addition of celecoxib to 5-FU had a profound effect on tumor multiplicity (Fig 1) and could reduce 5-FU-induced PGE_2 levels in unirradiated skin (Table I), celecoxib did not have an effect on PGE_2 levels in skin tumors (Table II). As in unirradiated skin, 5-FU treatment increased the amount of PGE_2 in skin tumors compared to vehicle-treated tumors ($^{*}p < 0.001$), but the addition of celecoxib was not able to significantly alter these levels. Similar results were seen in uninvolved irradiated skin (data not shown). These data suggest that celecoxib may have been acting synergistically with 5-FU to reduce tumor numbers in a PGE_2-independent method.

Effects of combination treatment on tumor recurrence Following the 3-wk treatment period, a random subset of mice were chosen to remain in the study with no additional treatments to determine the effect a combination treatment strategy would have on the rate of tumor regrowth (Fig 3).

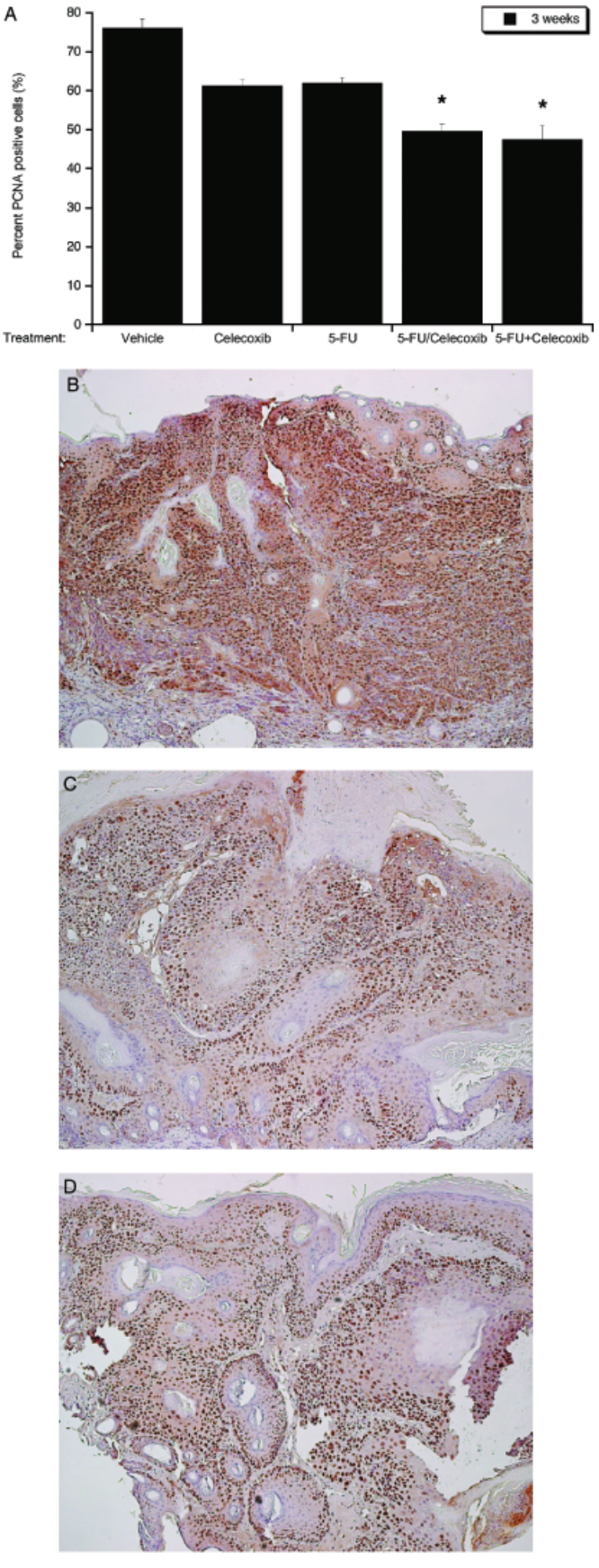

Figure 2
Effects of 5-fluorouracil (5-FU) and celecoxib on proliferation. Immunohistochemical staining for proliferating cell nuclear antigen (PCNA) was used to assess the levels of cellular proliferation within remaining skin tumors after 3 wk of topical treatments. The percentages of PCNA-positive cells per field were determined and the mean percentage ± standard error are depicted graphically in A. Four × 60 fields for each tumor section were analyzed, n = 6 tumors per treatment group. Representative photomicrographs are shown (× 10 magnification): B-5-FU, C-5-FU/celecoxib, and D-5-FU + celecoxib. A * represents statistical significance compared to 5-FU treatments, with a p value < 0.001 according to a Student's *t* test.

Table II. PGE_2 levels in skin tumors after 3 wk of treatment[a]

Treatment	PGE_2 concentration (pg)
Vehicle	183 ± 6.79
Celecoxib	189 ± 3.35
5-FU	218 ± 3.26*
5-FU/celecoxib	213 ± 3.20
5-FU + celecoxib	220 ± 4.08*

[a]Values represent mean ± standard error.
*$p < 0.003$ compared to vehicle treatments.
PGE_2, prostaglandin E_2; FU, fluorouracil.

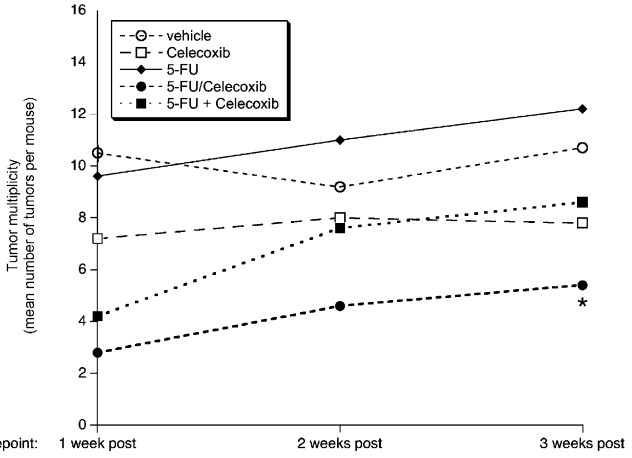

Figure 3
Effect of 5-fluorouracil (5-FU) and celecoxib on tumor recurrence. The lasting effects of 3 wk of topical treatments on the recurrence of skin tumors was assessed by monitoring tumor multiplicity over a 3-wk period after topical treatments were discontinued ($n = 5$ mice per treatment group, *$p = 0.017$ compared to 5-FU).

The assessments for tumor multiplicity and tumor size for the recurrence phase used the week 3 treatment values as the baseline values. Using the same statistical modeling described for Fig 1, a significant weeks variable was found during the recurrence phase (weeks 1–3 post-treatment), which denotes significant changes in tumor multiplicity over time ($p = 0.0001$). But there were no significant time × group interactions, so each group displayed increases in tumor multiplicity at the same rate. Although the combination of 5-FU and celecoxib did not affect the rate of tumor regrowth, pairwise analyses indicated that 5-FU/celecoxib mice still harbored significantly fewer tumors during the 3-wk period following the discontinuation of treatment, with an average of 6.7 fewer tumors than 5-FU (*$p = 0.017$). As in the treatment period, no significant differences in the size of the tumors were found between treatment groups (data not shown).

Discussion

The current treatment strategies for non-melanoma skin cancers and pre-malignant lesions such as AK are less than optimal. Treatment options most commonly include topical chemotherapies, such as 5-FU (Efudex and similar drugs) or surgical excision. Although topical therapies have an advantage over surgical interventions with regard to cost, trauma, and cosmetic outcome, they too have disadvantages. Current topical chemotherapy regimens have severe side-effects, including inflammation, pain, erosion, crusting, and scarring (Tsuji *et al*, 1984; Gupta *et al*, 2001), and there is a significant risk of recurrence after treatment (Sander *et al*, 1997; Salim *et al*, 2003). Whether this is a result of compliance issues and erratic use of the drugs by the patients due to the side-effects or a low drug efficacy remains to be seen. In any case, patients with skin cancers and pre-cancerous lesions need more effective treatments and a larger variety of treatment options to choose from.

The pre-clinical studies presented here demonstrate the potential value of combining a 5-FU, in a commonly used formulation (Efudex), and celecoxib, a COX-2 inhibitor and anti-inflammatory drug, as a new treatment option. Oral or topical treatment with celecoxib alone has previously been shown to have no effect on established skin tumors. But its addition to 5-FU significantly enhanced this drug's chemotherapeutic power. The dose of 5-FU that was used for these studies (0.5%) was a fraction of the dose typically used in humans (5%). In fact, this dose, which was chosen based on our preliminary toxicity studies, did not have a therapeutic effect on UVB-induced tumors in the present studies. Although this low dose of 5-FU when used alone did not result in the expected regression of skin tumors, this result does help illustrate the powerful synergism between 5-FU and celecoxib.

Although the combination treatments were able to dramatically reduce the number of skin tumors in this study, no significant effects on tumor size were found. This is likely due to the fact that there were very few tumors left to measure in the mice treated with the combination of the two drugs. Therefore, we were likely measuring a subset of tumors which were somewhat resistant to the treatments. This inherent bias brought forth by the overwhelming response of the majority of the tumors to the combination treatments may also explain why there were no effects on PGE_2 levels in the tumors or a more pronounced reduction in PCNA with the combination treatments.

Previously published studies have shown that COX inhibitors can synergize with chemotherapeutic drugs as well as enhance the anti-tumor effects of ionizing radiation (Ogino, 1996; Kishi *et al*, 2000; Fischer *et al*, 2003; Milas *et al*, 2003; Yao *et al*, 2003). The exact mechanism of how the combination of treatments act in an additive manner is not fully understood. In this study, the significant decrease in PCNA-positive epidermal cells within remaining tumors in mice treated with the combination of 5-FU and celecoxib compared to 5-FU alone suggests that these drugs may inhibit separate pathways of tumor cell proliferation. Treatment with 5-FU inhibits proliferation via the inhibition of DNA synthesis while celecoxib inhibits proliferation through the blockade of the COX pathway. Another possible explanation is that celecoxib was facilitating an enhanced uptake of 5-FU into the tumor cells, leading to increased regression of the tumors. An increase in toxicity and uptake of 5-FU into cancer cells *in vitro* has been demonstrated

using other non-steroidal anti-inflammatory drugs (Ogino and Hanazono, 1999; Ogino and Minoura, 2001; Mizutani *et al*, 2002). This may account for some of the increased tumor regression seen with the combination treatments, since the direct addition of celecoxib to 5-FU (5-FU/celecoxib) was more effective in destroying tumors than when the treatments were given separately (5-FU + celecoxib). Further studies will be performed to determine the *in vivo* mechanism of synergy between the two drugs used here.

The recurrence phase of this study demonstrates the need for continued treatment, since all treatment groups displayed a similar rate of tumor regrowth once the topical treatments were discontinued. These results are similar to those described in a recently published report examining the effects of treatment with a combination of oral celecoxib and difluoromethylornithine on murine UVB-induced skin tumors. As seen in our study, when the treatments were discontinued, tumor regrowth occurred at the same rate in the combination group as in all other treatment groups (Fischer *et al*, 2003). It is plausible, however, based on the fact that celecoxib can inhibit the development of new tumors (Wilgus *et al*, 2003a, b), that continued treatments with celecoxib alone during the regrowth phase would have inhibited tumor recurrence in the combination 5-FU and celecoxib treatment groups. Regardless, these studies and those of Fischer *et al* (2003) suggest that some form of treatment should be continued after tumors have undergone regression following a combination chemotherapy regimen.

Whereas the addition of the anti-inflammatory drug celecoxib to 5-FU was able to reduce PGE_2 levels in unirradiated skin and did result in the enhanced destruction of UVB-induced skin tumors, we did not find evidence of reduced PGE_2 levels in irradiated and treated skin (data not shown) or tumor tissue. The reduction in tumor number without an associated decrease in PGE_2 levels is not entirely unexpected, as reports of celecoxib acting on tumor cells in a PGE_2-independent mechanism have been reported (Hsu *et al*, 2000).

We initially expected the addition of celecoxib to the treatment regimen to decrease the 5-FU mediated inflammation in the skin. But the dose of celecoxib used in this study did not reduce the inflammatory effects of topical 5-FU treatment. At the dose and combination used in this study, celecoxib may have been acting solely to enhance the effects of 5-FU as a chemotherapeutic agent in the irradiated skin and tumors rather than acting as an anti-inflammatory agent. These results were disappointing since the side-effects associated with 5-FU treatment are likely to severely hamper its proper use by patients and in turn its efficacy. But because celecoxib combined with 5-FU reduced PGE_2 levels induced by 5-FU treatment in unirradiated skin, we expect that a formulation containing altered ratios of 5-FU and celecoxib can be developed which would retain the ability to more effectively treat skin tumors while alleviating the inflammation-related side-effects associated with the use of 5-FU. Reducing inflammation associated with topical chemotherapy treatments could be an important goal, not only to increase patient compliance but also because inflammation has been linked with the progression of pre-malignant skin tumors to squamous cell carcinomas (Berhane *et al*, 2002). Although this study examined pre-cancerous skin tumors since these are the type of lesions most commonly treated with topical 5-FU, it is plausible, based on the enormous synergism seen with these two drugs, that they may be effective for the treatment of NMSC lesions as well. In any case, the current data suggest that the combination of 5-FU and celecoxib in a topical formulation has the potential to be used in a clinical setting as an improved chemotherapy regimen. This treatment strategy is yet another step towards the ultimate goal of enhancing the effectiveness of skin cancer treatments and reducing the number of surgical excisions performed on the millions of people with NMSC and pre-cancerous skin lesions.

Materials and Methods

Animal treatments Female Skh/hr hairless mice (6–8 wk, Charles River, Wilmington, Massachusetts) were used for the studies. The mice were housed in the vivarium at the Ohio State University according to the requirements established by the American Association for Accreditation of Laboratory Animal Care. All procedures were approved prior to beginning the study by the appropriate Institutional Animal Care Utilization Committee. Mice were exposed dorsally to 2240 J per m^2 of UVB light (one minimal erythemic dose) from Phillips FS40UVB lamps (American Ultraviolet Company, Murray Hill, New Jersey), fitted with Kodacel filters (Eastman Kodak, Rochester, New York) three times weekly for 16 wk. Four weeks following the end of UVB exposure (week 20), at which point 100% of the animals had at least one pre-malignant skin lesion, or papilloma, mice were randomly assigned to one of five treatment groups ($n = 10$ mice per group). The mice received one of the following daily topical treatments in a volume of 200 μL: (1) vehicle (KY Jelly; Ortho Pharmaceutical Corp., Raritan, New Jersey); (2) vehicle containing 2 mg celecoxib (Celebrex, SC-58635, 4-(5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-bezenesulfonamide, G.D. Searle & Co., St Louis, Missouri), (3) 0.5% 5-FU (5% 5-FU cream diluted to 0.5% with vehicle) three times weekly with vehicle treatments on remaining days; (4) 0.5% 5-FU containing 2 mg celecoxib (5-FU/celecoxib) three times weekly with celecoxib (2 mg) treatments on remaining days; or (5) 0.5% 5-FU in the morning and 2 mg celecoxib 6 h later (5-FU + celecoxib) three times weekly with celecoxib (2 mg) treatments on remaining days. Due to toxicity problems seen in preliminary dose trials, 5% 5-FU (5% Efudex cream) was diluted with KY to obtain the 0.5% concentration used in the above treatments. Incidentally, this 0.5% dose (Carac, Aventis Pharmaceuticals, Inc., Bridgewater, New Jersey), although in a different delivery vehicle, is now being used clinically (Gupta *et al*, 2001). Celecoxib was dissolved in acetone (0.25 mg per μL of acetone) and mixed with vehicle or 0.5% 5-FU to obtain an even mixture at a concentration of 2mg per 200μL for celecoxib and 5-FU/celecoxib treatments, respectively. The mice were subjected to the topical treatments described above for a total of 3 wk. Age matched control mice ($n = 3$ mice per group), that had not been exposed to UVB, received the same treatments to assess the effect of the treatments on unirradiated skin. Five mice per group were randomly chosen and euthanized after 3 wk of topical treatments, 24 h after the last treatment. The remaining five mice in each group were monitored for an additional 3 wk after the cessation of treatments, without additional drug administration to determine tumor recurrence rates. The number of tumors per mouse and the size of each tumor (width by length) were determined weekly starting at week 19, the week prior to the beginning of the topical treatments, until the time of euthanization.

Immunohistochemical detection of proliferating cell nuclear antigen (PCNA) Immediately following euthanization, skin sections and tumors were placed in 10% neutral-buffered formalin for 2 h, washed with PBS, processed, and embedded in paraffin blocks. Tissue sections (5 μm) were cut and mounted onto Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, Pennsylvania). Immunohistochemical staining using a primary anti-PCNA antibody (1:100; Signet Pathology Systems, Dedham, Massachusetts) was used to detect PCNA-positive tumor cells as previously described (Wilgus *et al*, 2003a). Image analysis was used to quantitate the number of PCNA-positive tumor cells as well as the total number of cells in each high power field (60 ×). The percentage of PCNA-positive cells per field was calculated. Four high power fields were analyzed per tumor, and six tumors per treatment group were analyzed.

Prostaglandin E_2 (PGE_2) enzyme immunoassay Biotrak enzyme immunoassays (Amersham-Pharmacia, Piscataway, New Jersey) were used according to the manufacturer's instructions to determine the concentration of PGE_2 (pg) per 5 μg of total protein isolated from whole tissue as described previously (Wilgus *et al*, 2003a). Tissues from 3 mice per group for unirradiated skin and 5 mice per group for irradiated skin were analyzed. For the analysis of PGE_2 content in tumors, at least 3 tumors per mouse were pooled and 5 pooled samples per treatment group were used for the analysis.

Statistical methods The Biostatistics Core at the Ohio State University Comprehensive Cancer Center was consulted throughout this project regarding the statistical analysis of tumor multiplicity and size. Basic descriptive statistics and graphs for tumor number and size were generated to compare the five treatment groups (vehicle, celecoxib, 5-FU, 5-FU/celecoxib and 5-FU + celecoxib) comparing the average number of tumors as well as tumor size (length × width) for both the treatment and recurrence phases of the study. Two separate repeated measures models were fit in order to separately model data from the treatment and post-treatment periods. The models were of the general form $Y_{ijk} = \mu + \alpha_j + \beta_k + \gamma_{jk} + \varepsilon_{ijk}$, where i is the index for the mice, μ is the overall mean, α_j is the mean for treatment group j, β_k is the mean for week k, and γ_{jk} is the interaction between treatment j and week k. The error terms are assumed to be correlated within mice. The first set of models compare the total number of tumors in all mice from treatment weeks 0–3 (10 mice per group), and the second set of models consider the mice (5 mice per group) used for the follow-up period (weeks 1–3 post-treatment), using measurements at week 3 as a covariate in the model to control for differences at the beginning of the follow-up period. Additionally, difference in weeks 0–3 using only the mice that survived to the follow-up period were estimated. The statistical significance of factors, treatment group, time (measured in weeks), and the interaction between group and time in the model were tested. Pairwise group estimates at each week were also performed. All of the analyses were conducted using SAS version 8.02 (SAS Institute Inc., Cary, North Carolina). Statistical differences in PCNA and PGE_2 levels between the treatment groups were determined using a Student's *t* test generated using StatView software (Abacus Concepts, Berkeley, California), where $p < 0.05$ was considered statistically significant.

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