The Inhibition of colon-26 Adenocarcinoma Development and Angiogenesis by Topical Diclofenac in 2.5% Hyaluronan

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Abstract

Topical diclofenac in 2.5% hyaluronan inhibits basal cell carcinoma, actinic keratosis, and murine colon-26 growth in vivo. Colon-26 tumor growth was preceded by angiogenesis and reduced apoptotic and mitotic indices. Diclofenac reduced proliferation and viability in vitro, and stimulated apoptosis. Hyaluronan inhibited proliferation and viability at 1 mg/ml but was inactive below this level. Topical application of diclofenac inhibited tumor prostaglandin synthesis and retarded angiogenesis and tumor growth (ratio of treatment:control, 0.174). The mitotic index remained unaltered in vivo, whereas the apoptotic index and necrosis were increased. Topical vehicle exhibited slight antitumor and antiangiogenesis activity. The substantial quantities of diclofenac delivered locally in hyaluronan may exhibit antitumor activity in similar fashion to those seen in vivo and explain its clinical efficacy.

Introduction

The dual cyclo-oxygenase-1 and 2 inhibitor diclofenac reduces granulomatous tissue angiogenesis (1) and induces remission in basal cell carcinoma (2) and actinic keratosis (3) when formulated in 2.5% hyaluronan and applied topically. Possible mechanisms by which the antitumor effects may occur include the release of PGE2-mediated inhibition of immunity (4), angiogenesis inhibition (5, 6), and the induction of apoptosis (7), all of which could be dependent on or independent of cyclo-oxygenase 1 and 2 inhibition. The topical application of diclofenac formulated in hyaluronan results in negligible serum diclofenac in humans, unlike other formulations (8, 9). Hyaluronan retains and controls the release of diclofenac from the epidermal layer of human skin (10), which may be the result of binding to the hyaluronan receptors CD44 and the receptor for hyaluronan-mediated motility (RHAMM). We have reported previously that the topical application of [14C]-diclofenac in 2.5% hyaluronan results in the significant accumulation of drug within murine skin to pharmacologically significant levels (11), radioactivity also being found within both s.c. granulomatous tissue and colon-26 tumors. To provide insights into the mechanisms by which this formulation may act, we have investigated the effectiveness of topical diclofenac formulated in hyaluronan in inhibiting tumor growth and angiogenesis as well as its effects on tumor proliferation, apoptosis, necrosis, and PGE2 synthesis in a model shown previously to be sensitive to inhibition by diclofenac in vivo (12).

Materials and Methods

Experimental colon-26 Tumors. Colon-26 adenocarcinomas were established as described previously (13). Sponges (8 × 4 × 4 mm) were implanted s.c. into female anesthetized (halothane) BALB/c mice (20 ± 0.5 g, Bantin and Kingman, Hull, United Kingdom) and seeded with 106 colon-26 cells 3 days later. Briefly, colon-26 cells (a gift from the Imperial Cancer Research Fund, London, United Kingdom) were cultured in DMEM supplemented with 10% FCS, penicillin-streptomycin (100 units/ml and 100 µg/ml, respectively), and gentamicin (100 µg/ml) at 37°C with 5% CO2 (all cell culture reagents from Sigma Chemical Co., Poole, United Kingdom). Confluent monolayers were trypsinized (0.025% trypsin with 0.02% EDTA), and after centrifugation for 10 min, the cells were adjusted to a final concentration of 1 × 105/0.2 ml saline for injection. Tumors were dissected and dried at 56°C for 24 h for the assessment of dry mass. The T:C ratio was calculated from the mean dry masses of tumors from drug-treated and control mice.

Assessment of Vascular Density. The carmine/vascular cast method was used to assess vascular index as described previously (1, 14). Mice were anesthetized with Hypnorm/Hypnovel (1:1 v/v), with peripheral vasodilatation induced by warming on a heated platform and followed by i.v. injection of 1.0 ml of 5% carmine in 10% gelatin at 40°C. Carcasses were chilled overnight, and tumors were dissected, dried (24 h at 56°C), and weighed. Dried tumors were digested with papain in 4.5 ml of buffer [12 units/ml in 50 mM phosphate buffer and 2 mM N-acetylcysteine (pH 7.0) overnight], and the carmine was solubilized by the addition of 0.5 ml of 5% sodium hydroxide.

After centrifugation at 2000 × g for 10 min, the supernatants were filtered (0.22 µm, nitrocellulose) and 200 µl samples assayed spectrophotometrically at 490 nm. Results were expressed as vascularity index calculated from µg of carmine/mg of dry mass tissue (mean ± SE) and analyzed by ANOVA and Bonferroni t test (Graphpad Instat, Amsterdam, the Netherlands).

To visualize tumor capillaries, capillary endothelial cells were stained for the constitutive marker platelet/endothelial cell adhesion molecule 1 (CD31). Tumors were excised and snap-frozen in n-hexane in a bath of liquid nitrogen. For the detection of endothelial cells, 12-µm acetone-fixed cryostat sections were incubated with rat antimouse CD31 (PharMingen, San Diego, CA), and positive binding was detected by sequentially incubating with biotinylated rabbit antirat antibody and developed with avidin-biotin peroxidase complex (Vectorstain avidin-biotin peroxidase complex, Vector Laboratories, Peterborough, UK). Sections were then counterstained with hematoxylin.

Cell Proliferation, Viability, and Apoptosis in Vitro. Confluent colon-26 monolayers were trypsinized as above and transferred into 96-well plates and incubated for 24 h. The medium was replaced with 0.5% FCS in DMEM (200 µl/well) and incubated for an additional 24 h to ensure a homogeneous growth phase. Cells were then cultured for another 24 h with either diclofenac (0.03–300 µM) or hyaluronan (10 ng/ml to 1 mg/ml) in DMEM with 5% FCS. Fifty µl of [3H]thymidine (4 µCi/ml) were added to the culture medium for 16 h. Cells were then washed three times with Dulbecco’s PBS A followed by an additional three washes with ice-cold 10% trichloroacetic acid to remove free [3H]thymidine. The cells were then disrupted with 0.1 M sodium hydroxide (200 µl) and left overnight at 4°C followed by liquid scintillation counting.

Cell viability was assessed as dehydrogenase activity, measured as the
dehydrogenation of MTT to form the formazan product. Fifty μl of MTT were added to make 0.5 mg/ml in each well and incubated until the control lanes were developed (20 min). The cells were then washed twice in Dulbecco’s PBS A, the formazan product was dissolved by adding 100 μl DMSO to each well, and the plates were read at 570 nm. All treatments were measured in duplicate, expressed as a percentage of untreated control lane A50. The results from six to eight experiments were expressed as mean ± SE, and the absolute data were compared by linear ANOVA and Bonferroni t test (Graphpad Instat).

The identification of apoptotic and necrotic cells in vitro was carried out by using acridine orange and the vital stain ethidium bromide. Colon-26 cells were grown to 80% confluence on coverslips. Diclofenac or hyaluronan was added for 24 h, and the coverslips were washed in HBSS (Sigma). Coverslips were incubated with 100 μg/ml acridine orange and 100 μg/ml ethidium bromide in HBSS for 2 min at room temperature and washed once more in HBSS. The coverslips were mounted with Aquamount, and the cells were viewed by fluorescence microscopy. Early apoptotic cells were identified as those with nuclei staining green with acridine orange, or exhibiting apoptotic bodies within the nuclei (these cells excluded ethidium bromide). Late apoptotic cells were identified with the orange ethidium bromide-stained dense nuclei, often lacking cytoplasm. Necrotic cells are revealed with diffuse orange staining with ethidium bromide.

Colon-26 Proliferation, Apoptosis, and Necrosis in Vivo. Proliferating cells within the colon-26 tumors were visualized as mitotic figures, whereas apoptosis and zones of necrosis were assessed using the Apoptag DNA fragmentation detection kit (Oncor).

Tumors were snap frozen in n-hexane at −70°C, and 5-μm cryostat sections were taken and fixed in 10% neutral buffered formalin. To detect apoptosis, sections were stained with anti-digoxigenin-peroxidase antibody using the terminal deoxynucleotidyl transferase-mediated nick end labeling method (Apoptag). For the detection of cell division, nuclei were stained with hematoxylin.

Assay of Medium and Tissue PGE2. Tumors and overlying skin were dissected quickly and placed immediately on Cardice. The tissues were then homogenized in 1 ml protease-inhibitory buffer [Tris (pH 7.5), 21 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin, 1 μM pepstatin A, and 100 μM aspirin] on ice, and 50 μl were removed for the assay of protein (Bradford assay). The proteins were precipitated, and prostaglandins were extracted by adding to the samples 1:4 ethanol and 20 μl of glacial acetic acid. The samples were centrifuged at 4°C for 10 min, and the supernatant was removed and loaded onto 10% ethanol-primed Waters Sep Pak C18 cartridges washed with 1 ml of distilled water and 1 ml of hexane, and eluted with 1.5 ml of ethyl acetate and freeze dried. Samples were reconstituted in iced Tris buffer (0.05% Na3P, and 0.1% gelatin). PGE2 content was measured by RIA and expressed as mean ± SE ng/mg protein and compared by ANOVA with Bonferroni t test (Instat).

Drug Administration. Diclofenac sodium (Sigma) was administered once daily from 1 h prior to tumor seeding at 6 mg/kg topically as 0.1 ml of aqueous cream BP, and topical 6 mg/kg diclofenac in 2.5% hyaluronan (c and d) compared to 0.1 ml of aqueous cream BP (a—d). Results are means ± SE; n = 8—10 per group. * P < 0.05; ** P < 0.01; *** P < 0.001 versus control.

Results

Tumor Growth and Angiogenesis. BALB/c mice received implantations of s.c. sponges that were seeded with syngeneic colon-26 tumor cells, which induce blood vessel growth and develop into discrete, dissesectible tumors (13). The tumors developed over 12 days after an initial lag period (Fig. 1), peaking at day 4 and acquiring maximal growth rate from day 6.

The vascular content of the tumors preceded the rise in tumor mass by 2 days, resulting in a substantial rise in vascular density between days 4 and 6 postseeding (Fig. 1). This demonstrates that the development of the vasculature within the sponge matrix reaches a critical density required for maximal tumor growth. This is evidenced by the vascular density remaining relatively constant, whereas the tumor undergoes rapid development from days 8—12.

Compared with the topical aqueous cream BP, the topical application of 0.1 ml 2.5% hyaluronan vehicle alone resulted in a reduced tumor mass at day 14 (T:C ratio, 0.534; Fig. 2a) with a transient inhibition of vascular development that returned to control levels between days 6 and 10 (Fig. 2b).

Fig. 1. Tumor development (■) and vascular index (□) of colon-26 murine adenocarcinomas in vivo. The vascular index was calculated from gelatin vascular casts containing carmine and expressed as μg carmine/mg dry tumor mass. Results are mean ± SE; n = 8 per group.

Fig. 2. The inhibition of tumor growth and angiogenesis by topical hyaluronan vehicle (a and b, 0.1 ml/day 2.5% hyaluronan), and topical 6 mg/kg diclofenac in 2.5% hyaluronan (c and d, □) compared to 0.1 ml of aqueous cream BP (a—d, O). Results are means ± SE; n = 8—10 per group. * P < 0.05; ** P < 0.01; *** P < 0.001 versus control.
Fig. 3. The reduction in colon-26 tumor capillary vasculature, visualized by CD-31 immunohistology, by topical 6 mg/kg diclofenac in 2.5% hyaluronan [aqueous cream day 8 control (a), topical diclofenac (b) ×300], as well as induction of tumor apoptosis as visualized by DNA end labeling by Apoptag [aqueous cream day 14 control (c); diclofenac in hyaluronan day 14 (d) ×300]. Necrosis was seen as diffuse staining with Apoptag. In three of four hyaluronan-treated tumors, this feature was absent (e; ×70), but it was present in four of four tumors treated with diclofenac in hyaluronan (f; ×70). Apoptosis was also induced in vitro by diclofenac as visualized by acridine orange and ethidium bromide incorporation (g, control; h, 300 μM diclofenac; ×350).
However, daily treatment with topical diclofenac in hyaluronan (6 mg/kg) resulted in a dramatic and complete cessation of tumor growth, significant from day 10 (Fig. 2c), with a T:C ratio of 0.174. This was accompanied by a retardation of vascular development, with the vascularity gradually returning to control levels by day 14 (Fig. 2d).

Immunostaining for CD34 revealed a dense network of capillaries that were spread evenly throughout the tumors (Fig. 3a). The effectiveness of topical diclofenac in reducing blood vessel density is shown in Fig. 3b, confirming the reduction in vascular density calculated from the carmine/gelatin vascular casts.

**Tissue PGE2 Levels.** The level of PGE2 in skin overlying colon-26 tumors was 236 ± 81 (n = 14) ng/mg protein (n = 8). The oral administration of diclofenac for 5 days at 6 mg/kg resulted in little change in this level (218 ± 87 ng/mg protein; n = 7). Topical diclofenac in 2.5% hyaluronan dramatically reduced prostaglandin synthesis to 46 ± 13 ng/mg tissue (n = 6, P < 0.01) in the same experiment, an inhibition of 80.5%. The application of 2.5% hyaluronan alone induced no change in resident PGE2 levels (2.7% ± 0.13%; n = 4 experiments) over 5 days of application when compared to water-rubbed controls.

The tumors from animals treated with water p.o. for 5 days contained 67% more PGE2 than the overlying skin [395 ± 94 (n = 9) compared to 236 ± 81 pg PGE2/mg protein]. The oral administration of 6 mg/kg diclofenac daily for 5 days resulted in a 48% reduction in tumor PGE2 synthesis to 207 ± 103 pg/mg tissue (n = 9; P < 0.01). When applied topically in 2.5% hyaluronan, this inhibition was increased by an additional 40% to 125 ± 24 pg/mg (n = 5; P < 0.01).

**Tumor Proliferation and Viability in Vitro.** colon-26 murine adenocarcinoma cell proliferation was inhibited in a concentration-dependent fashion by diclofenac (Fig. 4a). The effect of diclofenac was in association with a dramatic reduction in cell viability (Fig. 4b). Hyaluronan alone exhibited little effect on colon-26 proliferation and viability in vitro at concentrations between 10 ng/ml and 0.1 mg/ml, whereas at 1 mg/ml, hyaluronan inhibited both parameters (Fig. 4b).

The incubation of colon-26 cells with diclofenac (30–300 μM) resulted in a dramatic induction of apoptosis (Fig. 3, g and h) compared to vehicle control as assessed by morphology under fluorescence after acridine orange and ethidium bromide staining; apoptotic cells were not evident at concentrations below 30 μM. A reduction in the number of cells in each field of view was evident at 100 μM and 300 μM. Cells with diffuse orange staining with ethidium bromide, indicative of cytotoxicity, were essentially absent in all groups.

**Tumor Proliferation, Apoptosis, and Necrosis in Vivo.** The tumor proliferation indices were not significantly altered over time nor modified by any treatment (see Table 1). However, the apoptotic index of untreated tumors was reduced over 10-fold between days 7 and 14; these days corresponded to the initiation of tumor growth and its peak, respectively, in treatment-matched animals in the same experiment (Fig. 2, c and d). Topical diclofenac in hyaluronan significantly raised the apoptotic index on day 14 when compared to day 14 control, and at 20 days (see also Fig. 3, c and d). The index at 20 days remained equivalent to those at the control preangiogenic phase at day 7. Hyaluronan alone significantly increased the apoptotic index on day 7 when compared with day 7 control, but no significant differences were noted at day 14.

Areas of necrosis identified by diffuse DNA end labeling within the tumors were noted in all of four analyzed animals being treated with the combination of diclofenac and hyaluronan after 20 days of development (Fig. 3, e and f). No areas of necrosis were noted in aqueous cream-treated controls up to day 14; tumors were not taken to 20 days because of animal welfare considerations. However, of the hyaluronan-treated animals, only one of four day 20 tumors showed histological evidence of necrosis. Tumor necrosis was not noted at any other time point.

**Discussion**

Tumor development, angiogenesis, and apoptosis have been investigated in murine colon-26 tumors in vivo. An inverse relationship exists between angiogenesis and apoptosis in the control of tumor development. Lewis lung microtumors have a high apoptotic index, which is reduced substantially on development of the microvasculature (15) followed by unrestricted growth of the tumors. This appears also to be true for the colon-26 adenocarcinoma reported here. Angiogenesis develops from between days 4 and 6 to reach a peak by day 10. However, tumor growth accelerates from between 6 and 10 days depending on the experiment. The apoptosis index reduces substantially over the period of active angiogenesis and is matched to tumor growth in matched controls (Fig. 2).

Diclofenac is a dual cyclo-oxygenase 1 and 2 inhibitor (16), and reports of antitumor activity of this NSAID in sensitive tumors are scarce (2, 3), despite its widespread use as an antiarthritic agent in the

**Table 1. The apoptotic and mitotic indices in s.c. colon-26 tumors from mice treated topically daily with 0.1 ml of aqueous cream BP, 2.5% hyaluronan vehicle, or diclofenac (6 mg/kg) in hyaluronan**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apoptotic index (%)</th>
<th>Mitotic index (%)</th>
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<tbody>
<tr>
<td>Aqueous cream</td>
<td>Day 7 (4)</td>
<td>0.32 ± 0.05</td>
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<tr>
<td></td>
<td>Day 14 (3)</td>
<td>0.04 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.5% hyaluronan</td>
<td>Day 7 (3)</td>
<td>0.56 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Day 14 (4)</td>
<td>0.05 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Day 20 (4)</td>
<td>0.05 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diclofenac in 2.5% hyaluronan</td>
<td>Day 7 (4)</td>
<td>0.33 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>Day 14 (3)</td>
<td>0.15 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Day 20 (4)</td>
<td>0.32 ± 0.04&lt;sup&gt;d&lt;/sup&gt;</td>
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<sup>a</sup> Mean ± SE.  
<sup>b</sup> P < 0.001 versus day 7.  
<sup>c</sup> P < 0.01 versus day 7.  
<sup>d</sup> P < 0.001 versus hyaluronan day 14.  
<sup>e</sup> P < 0.01 versus day 7.  
<sup>f</sup> P < 0.01 versus hyaluronan day 20.
elderly. We have found that oral diclofenac retards both colon-26 tumor growth and angiogenesis (12). The cyclo-oxygenase product PGE₂ may mediate angiogenesis, including the angiogenic activity of fibroblast growth factor 2, and its effect on plasma exudation could accelerate this process in a fashion similar to vascular endothelial cell growth factor (reviewed in Refs. 5 and 6).

*In vitro*, the inhibition of colon-26 cell proliferation and viability was observed with diclofenac, albeit at high concentrations. In addition to these effects, apoptosis was observed in those samples with the lowest viability. NSAIDs, especially diclofenac, have been reported to stimulate apoptosis at similar concentrations in chick embryonic fibroblasts (7), and the mechanisms by which NSAIDs may induce apoptosis have been reviewed (7). New protein synthesis is required for NIA. One mechanism may be via the inhibition of the inducible enzyme cyclo-oxygenase 2, which is linked to apoptosis inhibition and overexpression of the p53 down-regulator and apoptosis inhibitor bel-2 as found in intestinal epithelium (17). Transfection of v-src induces PGE₂ synthesis, which is itself apoptotic, but these cells are also NIA sensitive. PGE₂ replacement does not reverse NIA in these transformed cells. NIA is probably thus prostaglandin independent, but NIA potency is proportional to Cox-2 selectivity. Certainly, the IC₅₀ for PGE₂ synthesis inhibition by diclofenac is substantially lower (16) than that found to inhibit tumor cell growth and viability in this study.

The high concentrations of diclofenac required to induce apoptosis in tumor cells *in vivo* may be achieved by delivering the drug locally. Diclofenac formulated in hyaluronan has already been shown to be effective against basal cell carcinoma (2) and actinic keratosis (3) when applied topically. This formulation results in substantial quantities of diclofenac being retained within murine skin (23 µg/area applied, 24 h after one application) and increasing with every application (1.11 mg/area applied after five once-daily applications (10)). This formulation we have shown to reduce PGE₂ synthesis to negligible quantities within both skin and the underlying tumor relative to when diclofenac is administered p.o. Angiogenesis was retarded and tumor apoptosis was raised. The tumors did not show reduced proliferation indices in contradiction to the *in vitro* results but appeared to exhibit striking areas of necrosis after 20 days of therapy. This may be a result of the inhibition of angiogenesis in addition to other cytotoxic events.

Hyaluronan alone appeared to have a small effect on both tumor angiogenesis and growth. High molecular weight hyaluronan and its fragments have opposing effects on angiogenesis (reviewed in Ref. 5), the former being angiostatic. Mᵦ 500,000 hyaluronic acid had no effect on colon-26 cell proliferation or viability except at high concentrations at which physicochemical properties may be relevant. The topical application of hyaluronan to mice results in its accumulation within the s.c. layer and secretion by the lymphatic system (18). However, human skin is less permeable to hyaluronan than murine skin, with hyaluronan permeating the stratum corneum and accumulating at the epidermal layer (11). In addition to delivering diclofenac to the tumor on topical application, exogenous hyaluronan in such quantities may interfere with cell-matrix interactions via hyaluronan receptors such as RHAMM and CD44 in a fashion similar to soluble CD44 (19). These high-affinity receptors may concentrate hyaluronan at the site of tumor pathology, and we have certainly found that hyaluronan is targeted to colon-26 tumors *in vivo* (10).

Diclofenac applied topically in hyaluronan inhibits s.c. tumor growth, which occurs concomitantly with inhibition of angiogenesis, increased apoptosis, and tumor necrosis. These events may be due to the high concentrations of drug delivered to the tumors and may in part explain the clinical efficacy of this formulation.

**References**

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