Amfenac increases the radiosensitivity of uveal melanoma cell lines

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Abstract

Purpose To evaluate the proliferation rates of five human uveal melanoma (UM) cell lines after treatment with amfenac, a cyclooxygenase (COX)-2 inhibitor, and subsequent radiation exposure.

Methods Five human UM cell lines (92.1, SP6.5, MKT-BR, OCM-1, and UW-1) and one human fibroblast cell line (BJ) were incubated with amfenac. Treated and non-treated cell lines were then exposed to various doses of γ radiation: 0, 2, 4, 6, and 8 Gy. Sulphorhodamine-B assay was used to assess proliferation rates 48 h post-radiation.

Results Treatment of UM cell lines with amfenac prior to radiation led to a marked reduction in proliferation rates. This difference was statistically significant in all cell lines at every radiation dose ($P < 0.005$), with the exception of 92.1 at 2 Gy ($P = 0.157$). Fibroblasts treated with amfenac showed significantly higher proliferation rates after 2 and 8 Gy, with no significant differences at 0, 4, and 6 Gy.

Conclusions The radiosensitivity of UM cell lines was increased by the administration of amfenac, the active metabolite of nepafenac. There appears to be a radioprotective effect of amfenac on human fibroblasts. The topical administration of nepafenac may decrease tumour recurrence and radiation-induced complications while broadening the indications for radiotherapy by treating larger tumours.

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Introduction

Uveal melanoma (UM) is the most common primary intraocular malignant tumour in adults with an incidence of seven cases per million.[1] Enucleation of the affected eye was the only available treatment for UM for most of the last century.[2] However, with the advent of more conservative alternatives to treat the primary tumour, enucleation rates have substantially declined in recent years. The procedure is now reserved for large tumours or those cases where there is no hope of regaining vision.[3]

Radiation is the preferred form of treatment for most cases of UM.[4] UMs are typically treated with a calculated apex dose of 70–85 Gy.[5] Iodine-125 is the most commonly used isotope for plaque radiotherapy of choroidal melanomas,[6] although cobalt-60, ruthenium-106, iridium-192, strontium-90, and palladium-103 have also been used.[3] Modern techniques for brachytherapy involve suturing a shielded plaque containing seeds of the radioactive isotope to the sclera. This remains in place for a specified number of days to deliver the proper dose of radiation. Although the rates of tumour control are high, visual acuity is compromised in 25–35% of cases.[6] Some of the complications associated with the treatment are neovascular glaucoma, cataract, radiation retinopathy, and optic nerve neuropathy.[7] Thus, a substantial number of eyes are still enucleated due to failure of tumour control or radiation-related complications.

There are two forms of the cyclooxygenase (COX) enzyme, COX-1 and COX-2. While COX-1 is expressed constitutively in normal tissues,[8] COX-2 is an inducible enzyme expressed in response to a variety of inflammatory and mitogenic stimuli.[9] COX-2 expression has been reported in a wide variety of malignant tumours,[10–12] including uveal melanoma.[13] The expression of COX-2 has been linked to various processes, including tumour proliferation,[14] immunosuppression,[15] and metastasis.[16,17] Specific COX-2 inhibitors are currently in use for patients diagnosed with familial

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adenomatous polyposis, a genetic disorder, which predisposes patients to colon cancer.[18] The effectiveness of these selective inhibitors has been investigated in a variety of tumours and shows promise for use as an adjuvant therapy.[19]

COX-2 inhibitors have also previously been shown to increase the radiosensitivity of lung[20] and breast[21] cancer cell lines. Moreover, it has been demonstrated that a COX-2 inhibitor is capable of minimizing radiation damage to non-neoplastic tissues.[22] Nepafenac is a COX-2 inhibitor formulated for topical administration to the eye.[23,24] The purpose of our study was to determine the effects of amfenac, the active metabolite of nepafenac, on the radiosensitivity of five human UM cell lines and one human fibroblast cell line.

Methods

Cell culture

Four previously characterized human UM cell lines (92.1, SP6.5, MKT-BR, OCM-1), one transformed human uveal melanocyte cell line (UW-1) and one human fibroblast cell line (BJ, American Type Culture Collection, USA) were incubated at 37°C in a humidified 5% CO_2-enriched atmosphere.[25] The cell lines used have previously been graded in terms of proliferative and invasive abilities and metastatic potential.[25] The UM cell lines were cultured in RPMI-1640 medium (Invitrogen, Burlington, ON, Canada), supplemented with 5% heat inactivated fetal bovine serum (FBS), 1% fungizone, and 1% penicillin–streptomycin purchased from Invitrogen (Burlington, ON, Canada). The fibroblast cell line was cultured in DMEM medium (Invitrogen, Burlington, ON, Canada), supplemented with 10% heat-inactivated FBS, 1% fungizone, and 1% penicillin-streptomycin purchased from Invitrogen (Burlington, ON, Canada). Cells were cultured as a monolayer in 25 cm² flasks (Fisher, Whitby, ON, Canada) and observed two times weekly, at every media change, for normal growth by phase contrast microscopy. The cultures were grown to confluence and passaged by treatment with 0.05% trypsin in EDTA (Fisher) at 37°C and washed in 7 ml RPMI-1640 media before being centrifuged at 120 g for 10 min to form a pellet. Cells were then suspended in 1 ml of medium and counted using the Trypan Blue dye exclusion test for use in all subsequent assays.

The UM cell lines 92.1, SP6.5, and MKT-BR were established by Dr Jager (University Hospital Leiden, The Netherlands), Dr Pelletier (Laval University, Quebec, Canada) and Dr Belkhou (CJF INSERM, France), respectively. Dr Albert (University of Wisconsin-Madison, USA) established the OCM-1 and UW-1 cell lines.[26,27]

Irradiation

Prior to radiation, each cell line was seeded at a concentration of 500 000 cells per ml in micro petri dishes and incubated overnight with a 150 nM concentration of amfenac (Alcon Laboratories Inc., Fort Worth, TX, USA), the active metabolite of nepafenac. The concentration of 150 nm was the recommended 50% inhibitory concentration (IC_{50}) of COX-2 activity.[28] On the following day, the cells were exposed to graded doses of γ irradiation: 2, 4, 6, and 8 Gy (^{137}Cs source, γ Cell 1000). Controls consisted of cell lines exposed to radiation without prior incubation with amfenac.

Post radiation, all micro petri dishes containing 500 000 cells/plate were trypsinized using 0.05% Trypsin in EDTA, then washed in 5% FBS RPMI solution, and centrifuged for 5 min at 3000 g. The cells were then diluted to a concentration of 50 000 cells/ml in 5% FBS RPMI solution. These dilutions were seeded in a 96-well plate format at a concentration of 5000 cells per well and left to incubate at 37°C for 48 h to adhere to the bottom of the wells. After 48 h, we could already see colonies formed. Thus, to guarantee exponential growth, we chose to run the proliferation assay at this time point. The proliferation assay was done in triplicate per exposure condition.

Sulphorhodamine-B assay

The Sulphorhodamine-B assay kit (TOX-6, Sigma-Aldrich Co., St Louis, Missouri, USA) was used to measure total cell material as described previously.[29] Each of the treated and non-treated cell lines were seeded into wells at a concentration of 5×10^3 cells per well, with a minimum of three wells per cell line/radiation dose. The cells were then allowed to incubate for 48 h at 37°C. Following the incubation period, the cells were fixed to the bottom of the wells using a solution of 50% Trichloroacetic acid for 1 h at 4°C. The plates were then rinsed with distilled water, to remove trichloroacetic acid and medium, and later air dried. The Sulphorhodamine-B dye solution was next added to each well and allowed to stain for 25 min. The Sulphorhodamine-B solution was subsequently removed by washing with a 10% acetic acid solution and once more allowed to air dry. The dye that had become incorporated into the fixed cells at the bottom of the wells was solubilized in a 10 mM solution of Tris. The absorbance of the solute was measured using a microplate reader at a wavelength of 510 nm.

Data analysis

The Student's *t*-test was used to compare proliferation results between treated and non-treated cell lines after

various doses of radiation. A value of $P < 0.05$ was considered statistically significant.

Results

The results of the Sulphorhodamine-B proliferation assay for each cell line are presented in Figure 1. The actual values, SD, and *P*-values are shown in Table 1. The effect of radiation was significantly more pronounced in the UM cell lines treated with amfenac. The difference in the proliferation rates between treated and non-treated cell lines was statistically significant in all cell lines at all radiation doses ($P < 0.05$), except for the 92.1 cell line exposed to 2 Gy ($P = 0.157$).

The effect of amfenac on different doses of radiation is seen in Figure 2. The proliferation rates of the treated cell lines are represented as a percentage of the untreated controls. A positive value means that the proliferation rate was higher after the addition of the drug, thus suggesting a radioprotective effect. In contrast, a negative value means that the cell line proliferated less when the compound was added, indicating that the effects of radiation were enhanced. The effect of amfenac on the radiosensitivity of all UM cell lines was more pronounced with increasing doses of radiation. The synergism between amfenac and radiation was more pronounced in the MKT-BR, OCM-1, SP6.5, and UW-1 cell lines than in the 92.1 cell line.

A human fibroblast cell line was used to test the effect of amfenac on the radiosensitivity of a non-neoplastic cell type. The results of the Sulphorhodamine-B proliferation assay did not show a statistically significant difference for radiation doses of 4 and 6 Gy. Amfenac demonstrated a radioprotective effect, in terms of statistically significant higher proliferation rates, in the treated fibroblasts after 2 and 8 Gy of radiation.

Discussion

There is increasing evidence that COX-2 inhibitors may work as radiosensitizing agents.[30] Systemic COX-2 inhibitors are being used in clinical trials for lung cancer and the results are promising.[20,31] To the best of our knowledge, this is the first time that a drug has been shown to increase the radiosensitivity of UM cell lines. Furthermore, we demonstrated that no such synergistic effect was seen in a non-neoplastic human fibroblast cell line, that was, on the contrary, protected by the addition of amfenac. The finding that the fibroblasts were actually protected from the radiation damage provides *in vitro* support to the findings of Liang *et al.*[22] The authors observed that mice treated with a COX-2 inhibitor showed less damage to the surrounding tissues than the control animals, after receiving 50 Gy of radiation. Although it was proposed that the protective mechanism would involve the reduction of cytokines and chemokines, our *in vitro* results show that there is an intrinsic effect not related to tissue inflammation or other host-related factors.
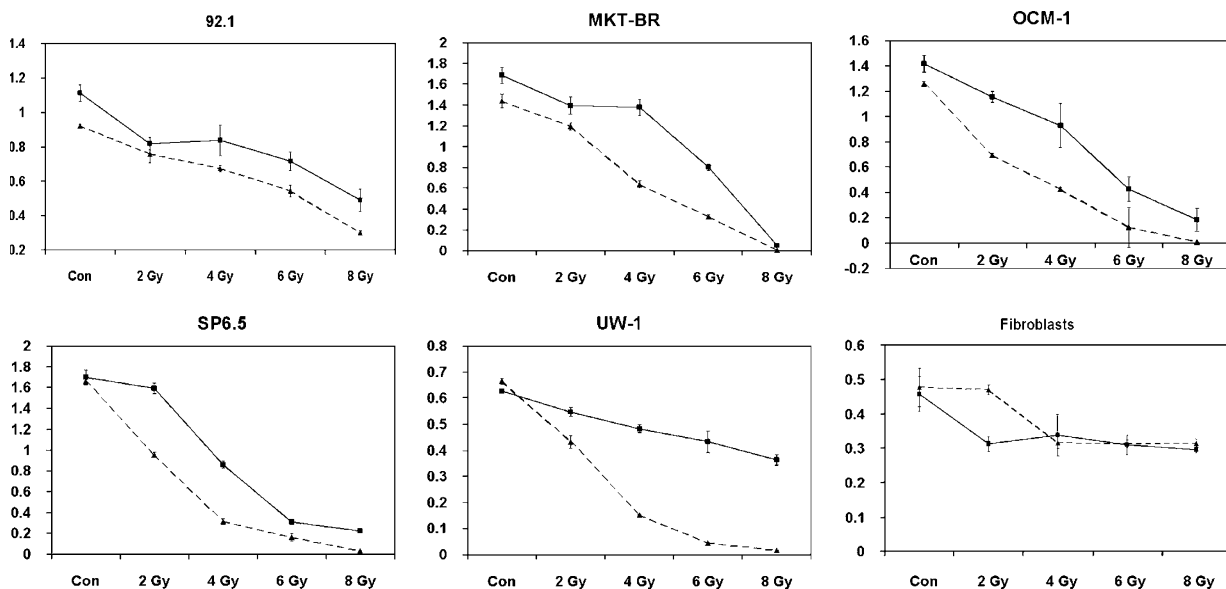

Figure 1 Graphical representation of the effect of amfenac and radiation on cell lines. The absorbance level detected by the Sulphorhodamine-B proliferation assay is seen on the *y* axis, while the doses of radiation are shown on the *x* axis. The solid and dashed lines correspond to the non-treated and treated cell lines, respectively. A significant reduction on the proliferation rates was seen only in the uveal melanoma cell lines.

Table 1 Proliferation rates of five uveal melanoma and fibroblast cell lines after radiation measured by the Sulphorhodamine-B assay kit

Radiation dose (Gy)	Without Amfenac (absorbance + SD)	With Amfenac (absorbance + SD)	P-value
92.1			
0	1.11 ± 0.08	0.92 ± 0.01	0.002
2	0.82 ± 0.04	0.76 ± 0.05	0.157
4	0.84 ± 0.09	0.67 ± 0.02	0.036
6	0.72 ± 0.05	0.54 ± 0.03	0.009
8	0.49 ± 0.07	0.3 ± 0.01	0.007
MKT-BR			
0	1.69	1.44	0.013
2	1.39	1.19	0.017
4	1.38	0.63	<0.001
6	0.8	0.33	<0.001
8	0.05	0.005	<0.001
OCM-1			
0	1.42	1.26	0.017
2	1.15	0.69	<0.001
4	0.93	0.43	0.007
6	0.42	0.12	0.047
8	0.18	0.009	0.029
SP6.5			
0	1.7	1.66	0.42
2	1.59	0.95	<0.001
4	0.86	0.31	<0.001
6	0.3	0.16	0.003
8	0.22	0.03	<0.001
UW-1			
0	0.63	0.66	0.01
2	0.55	0.43	0.003
4	0.48	0.15	<0.001
6	0.43	0.04	<0.001
8	0.36	0.02	<0.001
Fibroblast (BJ)			
0	0.46	0.48	0.555
2	0.31	0.47	<0.001
4	0.34	0.32	0.411
6	0.31	0.31	0.825
8	0.29	0.31	0.014

UM is known to be a radioresistant tumour. *In vitro* studies have shown that downstream defects in the p53 pathway may be involved in resistance to apoptosis, even though the radioresistance of UM is unlikely to be attributed to a single genetic defect.[32] UM cell lines show a wide range of radiosensitivity, which may be explained by varied induction of apoptosis, and cell cycle disruption.[33] The mechanism underlying the synergistic effect of COX-2 inhibitors and radiation remains elusive. One of the proposed mechanisms is the accumulation of cells in the G_2S/M phase of the cell cycle, which is most sensitive to ionizing radiation.[34] Aside from the intrinsic effect of COX-2 inhibition in cancer cells, the anti-angiogenic properties of COX-2 inhibitors may further facilitate induction of apoptosis *in vivo* and hinder tumour growth.[21]

Nepafenac has a unique prodrug structure and exhibits superior ocular bioavailability properties compared to diclofenac, another NSAID available for topical administration. Nepafenac is an inactive form that requires intraocular bioactivation to become the effective prostaglandin H synthase inhibitor, amfenac.[35] Little hydrolytic conversion of nepafenac is seen during transit. Following penetration, nepafenac diffuses through the anterior and posterior chambers of the eye and is accumulated and bioactivated in vascularized tissues: iris, ciliary body, retina, and choroid.[36] The end result is a prolonged suppression of COX activity, which is a distinct advantage over an NSAID with a free carboxylic acid function. Nepafenac was the only topical anti-inflammatory medication shown to inhibit the blood–retinal barrier breakdown in an animal model of ocular inflammation.[24] Thus, it is believed that nepafenac is the only topical NSAID to reach therapeutic levels in the posterior segment of the eye. Concentration of the drug achieves its highest peaks in ocular tissues 30 min after topical administration.[24]

Complications secondary to treatment by plaque radiotherapy occur in up to 70% of patients, especially when treating larger tumours.[7] Radiotherapy does not affect only the neoplastic tissue in the eye. Histopathological studies show increased necrosis, inflammation, fibrosis, and tumour blood vessel damage in irradiated eyes.[37] The combination of COX-2 inhibitors and radiation is a promising emerging therapy since COX-2 inhibition can selectively enhance the radiosensitivity of a tumour while allowing non-neoplastic tissues to be spared.[30] The concept that an eyedrop, without significant side effects,[38] can deliver the drug to an intraocular UM is even more appealing since the systemic complications related to COX-2 inhibition can be circumvented.[20]

In light of our preliminary results, we would encourage the use of nepafenac as an adjunct to radiotherapy in the treatment of UM. However, further studies are needed to characterize the exact mechanism behind the observed synergism. Animal studies would also be valuable before the institution of clinical trials in humans.

In summary, we showed that the radiosensitivity of UM cell lines could be increased by the administration of amfenac, the active metabolite of a commercially available anti-inflammatory topical eye medication. The administration of such a drug to uveal melanoma patients, before radiotherapy, may increase success rates regarding local tumour treatment and control.
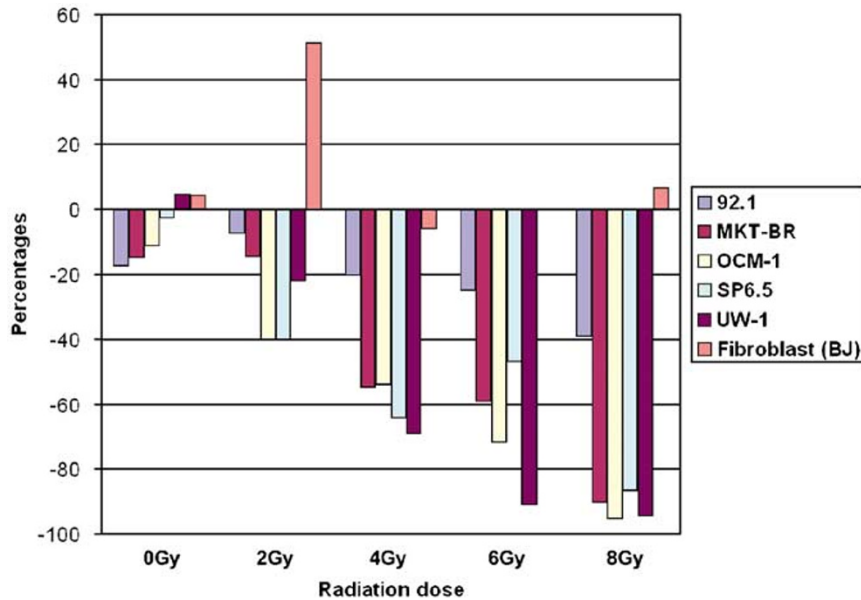
Figure 2 The proliferation rates of the treated cell lines are represented as a percentage of the untreated controls. A positive value reflects a radioprotective effect of amfenac, whereas negative values were a sign of increased radiosensitivity.

Acknowledgements

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