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LAB. INVESTIGATION-HUMAN/ANIMAL TISSUE

Radiosensitizing potential of the selective cyclooygenase-2 (COX-2) inhibitor meloxicam on human glioma cells

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Abstract The COX-2 protein is frequently overexpressed in human malignant gliomas. This expression has been associated with their aggressive growth characteristics and poor prognosis for patients. Targeting the COX-2 pathway might improve glioma therapy. In this study, the effects of the selective COX-2 inhibitor meloxicam alone and in combination with irradiation were investigated on human glioma cells *in vitro*. A panel of three glioma cell lines (D384, U87 and U251) was used in the experiments from which U87 cells expressed constitutive COX-2. The response to meloxicam and irradiation (dose-range of 0–6 Gy) was determined by the clonogenic assay, cell proliferation was evaluated by growth analysis and cell cycle distribution by FACS. 24–72 h exposure to 250–750 μM meloxicam resulted in a time and dose dependent growth inhibition with an almost complete inhibition after 24 h for all cell lines. Exposure to 750 μM meloxicam for 24 h increased the fraction of cells in the radiosensitive G_2/M cell cycle phase in D384 (18–27%) and U251 (17–41%) cells. 750 μM meloxicam resulted in radiosensitization of D384 (DMF:2.19) and U87 (DMF:1.25) cells, but not U251 cells (DMF:1.08). The selective COX-2 inhibitor meloxicam exerted COX-2 independent growth inhibition and radiosensitization of human glioma cells.

Keywords COX-2 inhibitor · Meloxicam · Growth inhibition · Radiosensitization · Human Glioma cells

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Introduction

Malignant gliomas account for approximately 30% of all intracranial tumors, with glioblastoma multiforme to be the most frequent and aggressive type. For many years, surgical resection followed by radiation therapy has been the standard treatment for gliomas, resulting in a median survival of less than 1 year after initial diagnosis [1–3]. Since the study of Stupp et al. [4], showing a significant prolongation of survival by additional administration of temozolomide, the new standard of care for glioblastoma multiforme now consists of surgery, radiotherapy and temozolomide [4]. However, regardless of treatment, almost all of these patients ultimately succumb to their disease. Further optimization of therapy is required.

The cyclooxygenase (COX) enzyme exists in two main isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most tissue types and plays a role in housekeeping functions. COX-2 expression is low in most normal tissues and can be upregulated under various pathological conditions and also by irradiation. Overexpression of COX-2 has been found to be important in the development of several human tumor types (e.g. colon, gastric, pancreatic) [5], including gliomas [6] and has been associated with high tumor aggressiveness and poor patients' prognosis [7, 8]. The COX-2 protein is overexpressed in the majority of gliomas, therefore it is considered to be an attractive therapeutic target [6, 8, 9].

In recent years, drugs with high affinity to COX-2 and low affinity to COX-1, the so-called selective COX-2 inhibitors, have become available. An advantage of this selective affinity is that COX-1 mediated processes are not affected. Therewith, these compounds show a low probability of the occurrence of side effects like gastroulceritis, dyspepsia, or even acute renal failure. Selective inhibitors of COX-2 are

prescribed for various inflammatory diseases (e.g. arthritis). The anti-tumor effect of selective COX-2 inhibitors, reported both *in vitro* and *in vivo* experimental studies [10–12], supported the introduction of these compounds in the clinic. However, the mechanism of action of these inhibitors is not well understood. Angiogenesis regulation, inhibition of cell proliferation as well as apoptosis induction have been reported to be involved in their anti-tumor effect [5, 13, 14]. Interestingly, selective COX-2 inhibitors also demonstrated to act as a radiosensitizer [5, 15–21]. The selective COX-2 inhibitor meloxicam has been shown to inhibit cell proliferation of different cancer cell lines and animal tumors [10, 12, 22–25]. However, no data are available yet on glioma cells. Differences in the profiles between meloxicam and other selective COX-2 inhibitors are explained by its different chemical structure and its unique pharmacological action on COX-2. The underlying molecular mechanism of the anti-inflammatory action of many COX-2 inhibitors is quite well understood, but it is not clear how meloxicam exerts its anticancer effect. Since gliomas represent a group of heterogeneous tumors, and often overexpress COX-2 [6, 9], a better understanding of the basic biology of gliomas and on the response to COX-2 inhibition might contribute to the improvement of glioma therapy.

The objective of the present study was to determine the effectiveness of the COX-2 inhibitor meloxicam alone and in combination with irradiation on cell proliferation, cell survival and radiosensitization on human glioma cell lines, with different COX-2 protein expression levels.

Materials and methods

Cell culture

The established human glioma cell line U87 was obtained from the American Type Culture Collection. U251 cells were isolated from a glioblastoma multiforme specimen [26] and D384 cells were cloned from a cell line derived from an astrocytoma [27]. Both U251 and D384 are established cell lines and were kindly provided by Dr. C. H. Langeveld (Dept. Pharmacology, Vrije Universiteit, Amsterdam, The Netherlands) [27]. All cell lines were confirmed to be mycoplasma free and cultured in DMEM supplemented with 10% fetal calf serum, 2% glutamine, 100 IU/ml penicillin, and 100 IU/ml streptomycin. Cells were grown as monolayers in 25 cm^2 culture flasks and were maintained in a humidified 7% CO_2 atmosphere at 37°C.

Growth assay

1.10^5 (D384 and U251) or 1.10^6 (U87) cells/flask were plated, a density that enables linear growth. Cells were given 24 h to attach to the bottom of the culture flask, after which cells were exposed to 250, 500 and 750 μM of meloxicam (Boehringer Ingelheim, Germany) or vehicle (0.38% DMSO). Duplicate culture flasks of each day were trypsinized and counted each day for a maximum of 3 days using coulter counter (Coulter® Z™ series).

Clonogenic assay

Subconfluently growing D384, U251 and U87 cells were exposed to 750 μM meloxicam or vehicle (0.38% DMSO) for 24 h and irradiated with single doses of γ-radiation (0–6 Gy) using a ^{60}Co source (Gammacell 200, Atomic Energy of Canada Ltd). 150–5000 cells/flask were plated immediately after irradiation for colony-forming ability in medium without meloxicam. After 10 days, colonies were fixed with 100% ethanol and stained with 10 % Giemsa solution. Colonies containing more than 50 cells were counted. Cell survival curves were estimated after normalization for cytotoxicity induced by meloxicam alone. Data from three independent experiments were combined and the average survival levels were fitted by least squares regression using the linear quadratic model. The Dose Modifying Factor (DMF) (the ratio of the radiation dose plus vehicle and the radiation dose plus meloxicam) was estimated at a surviving fraction (SF) of 0.5. Survival curves were analysed using Statistical Package for Social Sciences (SPSS; Chicago, IL, USA) statistical software as previously described by van Bree et al. [28].

Fluorescence-activated cell sorter (FACS) analysis of cell cycle distribution

After treatment of 5.10^5 cells/flask with vehicle or 250, 500 and 750 μM meloxicam, cells were trypsinized and resuspended in fresh medium. Cells were washed twice in PBS, centrifuged for 4 min at 1000 rpm, and fixed with 70% ethanol. Subsequently, cells were incubated with RNAseA (0.25 mg/ml) for 20 min at room temperature and stained with propidium iodide (0.1 mg/ml) for 30 min in dark on ice. DNA content of the cells was analyzed by FACS (Becton Dickinson) with an acquisition of 50,000 events.

Western blotting

After 24 h treatment with vehicle or 750 μM meloxicam, cells were scraped in ice-cold PBS and collected for treatment with 25 μl lysis buffer (50 mM Tris/HCL, 1% NP40, 0.5% Na-deoxycholate, 150 mM NaCl, 0.05% SDS, and 1:1000 pefablock) to obtain whole cell lysates. Cell lysates were kept on ice for 1 h and centrifuged at 14,000*g* at 4°C for 10 min. The protein concentration of the supernatant was measured using the Bio-Rad Assay

(BioRad Laboratories, Germany) according to the manufacturer's instructions. A total of 100 µg protein was subjected to 7.5% SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Immobilon-P, Millipore). Membrane was blocked in 5% non-fat milk in TBST (Tris buffered saline, 0.1% Tween 20) for 1 h at room temperature. Subsequently, the membrane was incubated with the primary antibody (1:1000 mouse-α-COX-2 monoclonal antibody, Cayman Chemical, USA) in TBST (with 0.5 % non-fat milk) overnight at 4°C. The blot was washed 3 times in TBST (with 0.5% non-fat milk) and incubated with the secondary antibody (1:1000 goat-α-mouse-HRP, DAKO Cytomation, Denmark) in TBST (with 0.5% non-fat milk) for 1 h at room temperature. After incubation, the membrane was washed in TBST (with 0.5% non-fat milk) and developed using an ECL system (Amersham Pharmacia, England) on a hyperfilm (Amersham Bioscience, England).

Results

Effect of meloxicam on cell growth and cell survival

Meloxicam exposure to D384, U87 and U251 cells resulted in a time and dose-dependent growth inhibitory response (Fig. 1). An almost complete growth arrest was induced after exposure to 750 µM meloxicam, which remained arrested for up to 3 days. After 24 h of treatment of D384, U87 and U251 cells with 750 µM meloxicam the surviving fraction was 0.57, 0.81 and 0.74, respectively.

Combination of meloxicam with irradiation

To determine the radiosensitizing potential of meloxicam, cells were incubated for 24 h with 750 µM meloxicam and then irradiated. Cell survival curves (Fig. 2) show that meloxicam treatment enhanced the radiation response of D384 cells (DMF of 2.19, $P < 0.01$) and of U87 cells (DMF of 1.25, $P < 0.01$), but not that of U251 cells (DMF of 1.08, n.s.).

COX-2-protein expression

COX-2 protein expression levels were assessed to determine whether or not the growth inhibition and radioenhancement after meloxicam treatment were related to COX-2. Subconfluently growing D384 and U251 cells did not show COX-2 expression. U87 cells expressed COX-2 constitutively at a level exceeding the 10 ng reference standard (Fig. 3).

Cell cycle distribution

The effect of meloxicam on cell cycle distribution was analyzed by flow cytometry. As illustrated in the DNA histograms in Fig. 4, both D384 and U251 cells accumulated in the G_2/M phase after 24 h of treatment with

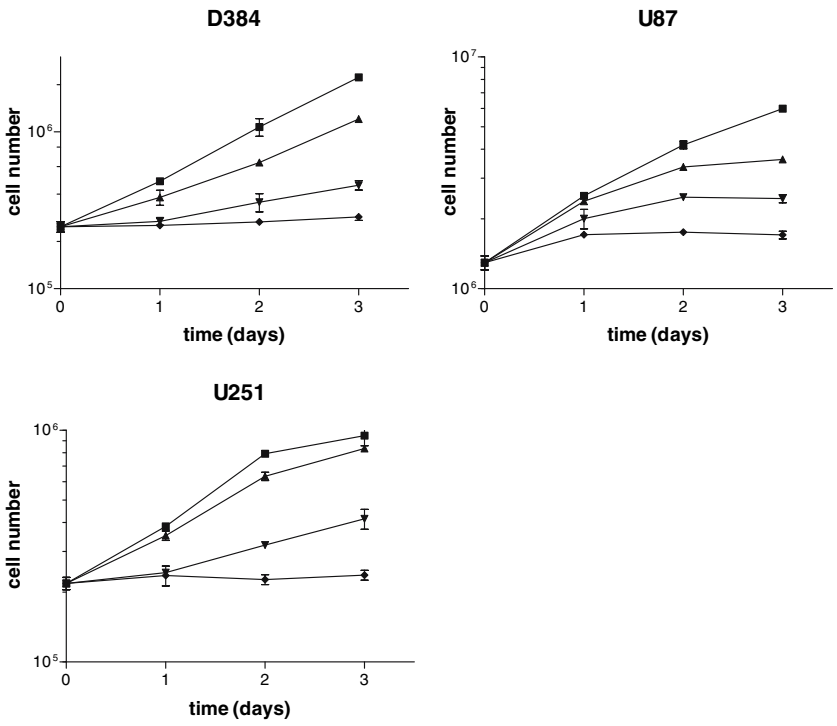

Fig. 1 Dose-dependent growth inhibition after meloxicam exposure. Subconfluently growing D384, U87 and U251 cells were exposed to vehicle (0.38% DMSO) (■), or to 250 µM (▲), 500 µM (▼), and 750 µM (◆) meloxicam for depicted exposure times. Error bars represent SD of three independent experiments and are indicated for each data point

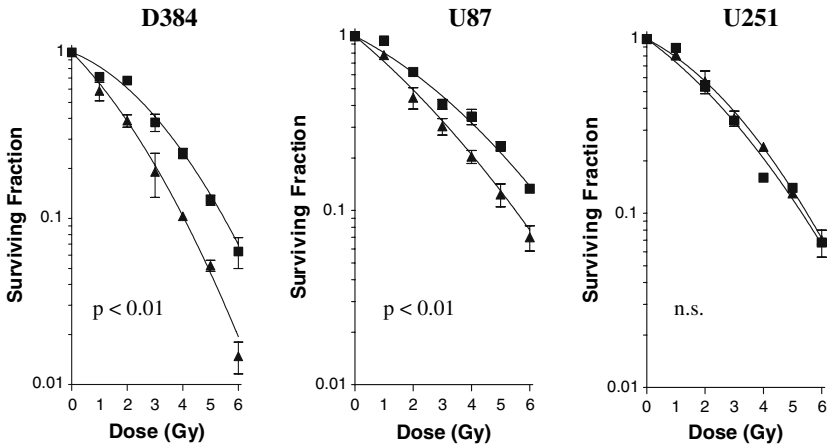

Fig. 2 Radiation cell survival curves for D384 cells, U87 cells and U251 cells. Cells were pre-treated for 24 h either with vehicle (0.38% DMSO) (■) or 750 μM meloxicam (▲). Error bars represent SD of three independent experiments. (n.s. is not significant)

750 μM meloxicam. Following exposure to meloxicam, the fraction of U87 cells in the G_0/G_1 phase increased from 53 to 75% (Fig. 4).

Discussion

Previous studies have demonstrated tumor growth inhibition and radioenhancement by selective inhibition of the COX-2 protein [11, 16–19, 15]. The COX-2 inhibitor meloxicam was reported to inhibit growth of various tumor cell types both *in vitro* and *in vivo* [10, 12, 22, 23, 29, 30]. Until now, neither the growth inhibitory capacity nor the radiosensitizing potential of meloxicam was tested on human glioma cells.

The present study shows that meloxicam inhibits cell growth of three glioma cell lines and significantly radiosensitizes two of them. Exposure to 750 μM meloxicam almost completely suppressed cell growth, but only slightly reduced cell survival. Our observations agree with other reports, showing inhibition of cell proliferation at comparable doses of meloxicam [24, 25, 31]. Since the glioma cells we tested had different levels of constitutive COX-2 protein expression, the growth inhibitory response was probably not related to the COX-2 protein level. COX-2 independent growth inhibition has also been reported by Patel et al. [32] after treatment of human prostate cancer cells with celecoxib or rofecoxib both *in vitro* and *in vivo*.

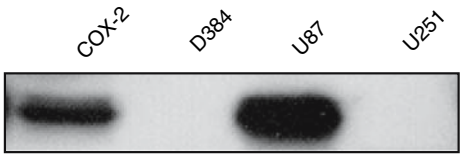

Fig. 3 Representative western blot showing COX-2 expression in D384, U87 and U251 cells relative to the reference standard

Furthermore, celecoxib was reported to inhibit growth of 9L rat gliosarcoma cells that were orthotopically transplanted in rat brains, leading to a decreased expression of Bcl-2, Bcl-XL, phosphorlyated Akt and EGFR [11]. However Hsu et al. [33] reported celecoxib to induce apoptosis by blocking the activation of anti-apoptotic Akt in prostate cancer cells via an action that was independent of Bcl-2.

Little information is available about the molecular mechanisms involved in COX-2 mediated growth inhibition. Both cell cycle blockade and induction of apoptosis are reported after in vitro exposure of cells to the inhibitors meloxicam, celecoxib and rofecoxib. The meloxicam concentration and exposure time we used in our experiments did not result in the induction of apoptosis, but caused a cell cycle arrest. Because cell cycle arrest was induced at a concentration of meloxicam higher than needed for inhibition of COX-2 function, this effect was probably independent of the COX-2 protein. COX-2 independent anti-tumor effects of selective COX-2 inhibitors have recently been discussed in detail by Grosch et al. [34].

Petersen et al [17], using the COX-2 inhibitor SC-236, reported radioenhancement after treatment of U251 cells (DMF of 1.4 at SF 0.1). The absence of a radioenhancement on U251 cells in the present data (Fig. 2), might be ascribed to a different mechanism of action of the inhibitor meloxicam. Meloxicam enhanced the radiation response of D384 and U87 (Fig. 2). Because only U87 cells constitutively expressed the COX-2 protein, this observation suggests that the radiosensitizing effect was not related to the COX-2 protein level at the time of irradiation. However, using the selective COX-2 inhibitor NS-398, Pyo et al. [18] found a preference for radiosensitization of cells that express COX-2.

Cells in the G_2/M phase of cell cycle are known to be sensitive to radiation [35]. Previous studies reported

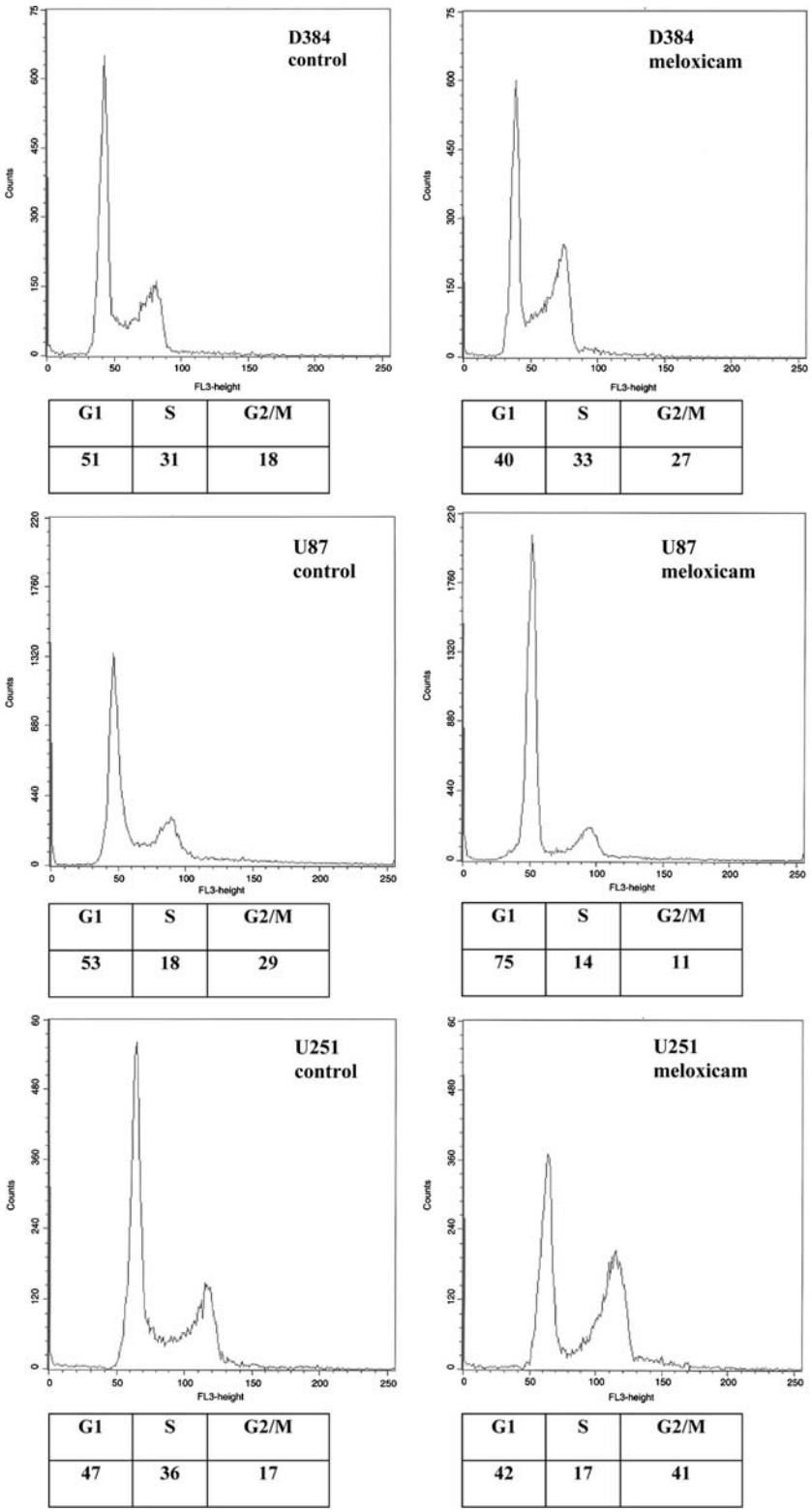

Fig. 4 FACS analysis (n=2) of D384, U87 and U251 cell cycle distribution 24 h following exposure to 750 μM meloxicam or vehicle (0.38% DMSO). Proportions of G_1, S- and G_2/M phase are given in percent

accumulation of cells into the G_2/M phase by inhibitors of the COX-2 protein [19, 36]. The radiosensitizing effect of meloxicam we observed in our experiments was probably not caused by cell cycle redistribution (cf. Figs. 2 and 4).

Growth inhibition and radiosensitization can be mediated by the prostaglandins. The COX-enzymes are the rate-limiting enzymes in the prostaglandin pathway, i.e. the conversion of arachidonic acid to prostaglandins. The

COX-2 enzyme is responsible for the synthesis of prostaglandin subtypes involved in pathological conditions. The major metabolite of COX-2 is PGE_2, which is reported to inhibit apoptosis [37] and to act as radioprotector [38, 39]. Hence, the radiosensitizing action of meloxicam might be ascribed to inhibition of the PGE_2 production, as previously reported [23]. Kang et al [40] reported radiosensitization of U87 cells by celecoxib after high dose irradiation in vitro by PGE_2 inhibition. This phenomenon might also explain our data, although the meloxicam concentration in our experiments was higher than reported to be required for inhibition of PGE_2 production [19, 41]. Experimental studies on the growth inhibitory and radiosensitizing effects of meloxicam should focus on PGE_2 synthesis and on apoptosis induction.

A recent report showed that meloxicam may exert its anticancer effect by binding of Cu(II)-complexes of meloxicam with the DNA backbone, resulting in DNA distortion [42]. Further studies should attempt to determine a possible interaction of DNA-intercalated Cu(II)-meloxicam complexes with irradiation and the effect on the induction and repair of DNA damage.

The present findings on three human glioma cell lines demonstrate that the COX-2 inhibitor meloxicam inhibits cell proliferation and may enhance the radiation response, independent of COX-2 protein expression. Because of the radiosensitizing potential of meloxicam and in view of the recently reported interaction between irradiation and celecoxib [40] as well as between temozolomide and celecoxib [43] in experimental gliomas, selective COX-2 inhibitors yield promising perspective to further improve the therapy of glioma patients.

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