

Effects of Ibuprofen on Orthotopic Glioma Model in Rats

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ABSTRACT

Objective: To determine the effects of ibuprofen (Ibp) on the vessel proliferation and necrosis in a rat glioma model.

Study Design: Experimental, randomized interventional trial.

Place and Duration of Study: 1st Neurosurgery Clinic in Bakirkoy Mental Diseases Hospital, Bakirkoy, Istanbul, Turkey, in the year 2010.

Methodology: After stereotactic injection of C6/LacZ rat glioma cells into the Wistar rats brain, the rats were randomly assigned to two treatment groups (group 1, control; group 2, Ibp treatment). Rats were sacrificed 18 days after treatment, and number of intra-/peri-tumoural vessels, microendothelial proliferations, immunohistochemistry and necrotic area were evaluated.

Results: Ibp treatment significantly decreased tumour tissue, intratumoral vessel number and total tumour area level. The level of Ki67 was significantly decreased in the tumour tissue of group 2. Additionally, the total necrotic area / total tumour volume (%) was significantly less in the tumour tissue of the ibuprofen-treated rats compared to the controls.

Conclusion: The data show that the Ibp produced an important reduction in glioma tumour cell proliferation in the rat model.

Key words: Rat. Orthotopic glioma. Non-steroidal anti-inflammatory drugs. Ibuprofen. Stereotactic injection. C-6 glioma cell line.

INTRODUCTION

Glioblastoma is a common and aggressive type of brain tumour.¹ The typical symptoms of glioblastoma multiforme are progressive headaches, nausea, confusion or seizures and progressive loss of brain function. The current therapy for patients suffering from malignant glioma is surgery followed by radiation therapy, with a more limited role for adjuvant chemotherapy.² A combination of surgery, radiotherapy, and treatment with the alkylating agent has recently been shown to increase survival for patients with glioblastoma. Despite these developments, there is much demand for new therapies.^{3,4}

In previous studies, aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) have largely supported a reduced risk against colorectal cancer and adenoma.^{5,6} Additionally, prostaglandins (PG) which are the known mediators of inflammation, have a role in carcinogenesis by direct mutagenesis, tumour cell proliferation, tissue

invasiveness, metastasis, immune suppression, and hormone responsiveness.⁷⁻⁹

NSAIDs are potent cyclo-oxygenase (COX) inhibitors. The COX isozymes COX-1 and COX-2 are presented in the control of inflammatory reactions and catalyze the conversion of arachidonic acid to PGH-2, the pre-cursor of PGs.^{10,11} Ibuprofen (Ibp) is a non-selective COX inhibitors, which inhibits both COX-1 and COX-2.^{12,13}

In the present study, the aim was to evaluate the effects of treatment with ibuprofen on angiogenesis and necrosis in a rat brain tumour model using C6 glioma cell line.

METHODOLOGY

This interventional study was conducted at the 1st Neurosurgery Clinic in Bakirkoy Mental Diseases Hospital, Bakirkoy, Istanbul, Turkey, in the year 2010.

The experiment was approved by Istanbul University Animal Experimentation Committee. Male Wistar rats, weighing between 240 and 280 grams, were anaesthetized by intramuscular injection of a solution containing 42.8 mg/ml of 10% ketamine and 8.6 mg/ml of 2% xylazine (this dosage did not exceed 0.7 mg/kg of body weight in total). The rats were secured into a stereotaxis apparatus (Trent Welles Inc., South Gate, Ca, USA). After antiseptic preparation of the scalp with betadine, the skin was incised in the mid-sagittal line for 1 cm. A burr hole (1.5 mm wide) was made on the right side 3 mm lateral to midline, and 2 mm proximal to the bregma using a dental drill. Injection of the C6 glioma cell suspension was made using a Hamilton syringe with a 27 gauge needle, which was fixed to the manipulation arm of the stereotaxis apparatus and advanced to the

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center of the right caudate nucleus which was 5 mm deep to the surface of the brain. A total of 5 ml solution was injected over a 5 minute of time in 0.5 μ l aliquo. The needle was kept in same place for an additional 3 minutes and then withdrawn very gently over 2 minutes. The operation area was irrigated with saline and the burr hole was covered with bone-wax. The scalp was sutured using 5-0 vicryl.

All animals underwent implantation of guide screws and after 1 week they were divided into two groups. For all animals (14 rats), 5×10^5 C6/LacZ glioma cells were injected and the animals were sacrificed 18 days later following specific treatment. Animals in group 1 (control, 7 rats) were treated by saline (23 ml/kg/day) p.o. daily from 11th to 15th day. Animals in group 2 (Ibp treated, n = 7) were treated by ibuprofen, which was administered at a dose of 30 mg/kg by gastric catheter (6[°]F) doses of ibuprofen (Brufen® syrup, Eczacibasi) on days 11 – 15 after tumour implantation.

The rat glioblastoma cell line C6 was obtained from ATCC (Rockville, USA) and serial passages were made at the Histology and Embryology Department of Istanbul Medical School in modified eagle medium of dulbecco, which contained 15% heat inactivated fetal calf serum, 0.2 mM glutamine, 50 mg/ml neomycin, and 100 mg/ml streptomycin. Culture flasks were kept in electronic incubator (Sanyo) under humidified atmosphere with 5% CO₂ at 37°C. Cells were harvested via 2 ml trypsin-EDTA solution C (Biological Industries, Israel) and centrifuged after the addition of 1.5 cc of F12 medium and fetal calf serum mixture at 1 x 10³ rpm for three minutes. After removing the supernatant, pellet was re-suspended with 2 cc of medium. Cell suspension was concentrated so that 5 microliters of any injection volume would contain 5×10^5 cells, and then placed in a micro-centrifuge tube, and kept in a water-ice mixture environment during the whole implantation procedure, which always lasted less than 2 hours.

Brains fixed in 10% buffered formalin were dissected in 10 mm coronal slices via taking inoculation hole as origin. All samples were photographed. Glass slides of 4 microns thickness were prepared. Histology sections from these were stained with conventional haematoxylin-eosin technique for routine analysis. Number of intra-/peri-tumoural vessels and glomeruloid microendothelial proliferations were counted in five high power field (HPF) area (0,238 mm²). Two indexes were calculated, i1 and i2 respectively where i1 was number of intratumoural vessels / total tumour area and i2 was number of peritumoural vessels / intratumoural vessel.

For immunohistochemistry, primary antibody Ki-67 (clone SP6, Lab Vision, Fremont, CA, USA) was used. Immunoreactivity was visualized by a biotin-streptavidin-peroxidase kit (Nichirei, Tokyo, Japan) and 3,3'-diaminobenzidine solution. To assess Ki-67 labelling index, more than 1000 neoplastic cells from each histologic type of brain tumour were counted.

Haematoxylin and eosin (H&E) sections (4 micron thick, paraffin embedded) of each tumour were analyzed by a pathologist. For tumour *necrotic area* quantification (in μ m²), images were captured using a digital camera (Olympus Camedia 7070) and analyzed using SPOT version 3.2.4 software. (Diagnostic Instruments, Inc.). Total necrotic area / total tumour volume was given as a percentage (%).

Data are expressed as mean \pm S.D. and evaluated statistically by Statistical Package for Social Sciences (SPSS) version 11.5 with independent student's t-test. P-values; p < 0.05 was recognized as statistically significant.

RESULTS

The i1, i2, Ki67 and necrotic area values for the different groups are shown in Table I. The i1 levels were increased in the group 1 (Figure 1a). However, the i1 levels in tumour tissue were significantly decreased by ibuprofen treatment (p < 0.05) (Figure 1b). The i2 levels in group 2 were found not to be different than that of the control group.

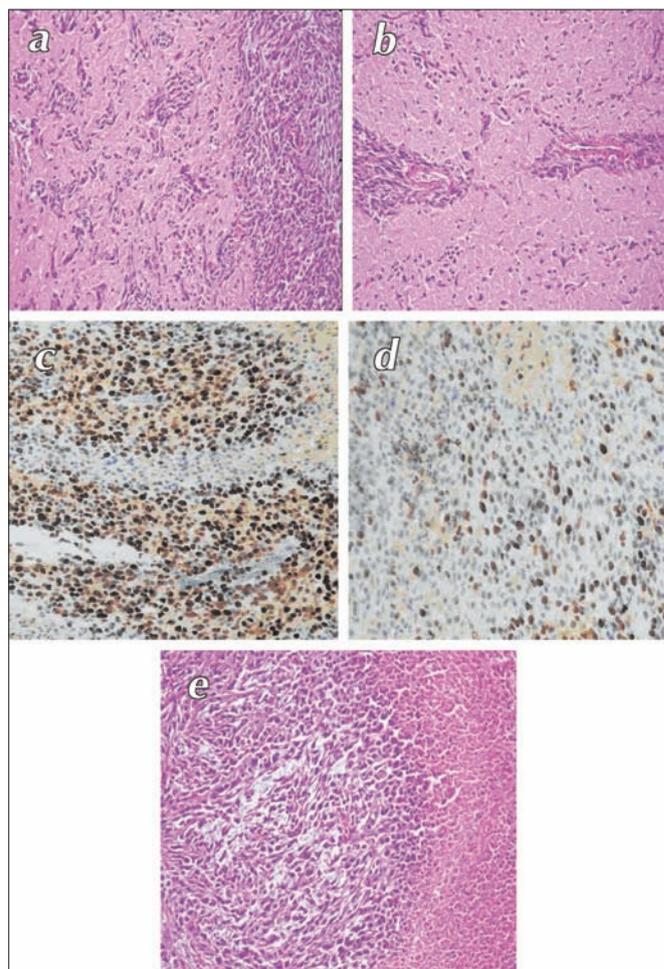


Figure 1: (a) Intense vascular endothelial proliferation in control group (H&E, x 200). (b) Mild vascular endothelial proliferation in Ibp treated group (H&E, x 200). (c) Glioma cell proliferation in control group was assessed by immunostaining for Ki67 intense positive glioma cell nuclei (x 200). (d) In Ibp group was stained Ki67 mild positive (x 200). (e) Mild necrosis with pseudopalisading in group 2 (H&E, x 200).

Table I: Number of intra-/peri-tumoural vessels, glomeruloid micro-endothelial proliferations and total necrotic area.

Groups	i1	i2	Ki67	Total necrotic area/ total tumour volume (%)
Group 1	5.07 ± 0.6	3.48 ± 0.2	73.4 ± 7.2	0.23 ± 0.9
Group 2	4.03 ± 0.5*	3.52 ± 0.6	55.6 ± 9.3*	0.15 ± 0.12*

Values are as mean ± SD. Abbreviations are: i1 = Intratumoral vessel number / total tumour area, i2 = Peritumoral vessel number / intratumoral vessel number. Group 1: Control, Group 2: Ibuprofen treated. *p < 0.05 vs. group 1.

The level of Ki67 was significantly decreased in the tumour tissue of the group 2 (p < 0.001) compared to the group 1 (Figure 1c and d).

The total necrotic area / total tumour volume (%) was decreased significantly in tumour tissue of the group 2 rats (Figure 1e) compared to group1 (p < 0.05).

DISCUSSION

Although the mainstay of malignant glioma management is surgery, radiotherapy, and chemotherapy, treatment remains challenging. With the help of advances in molecular biology, tissue microarray and signal transduction pathways are better defined for tumours such as malignant glioma. Targeting specific steps in the transduction cascade has become the focus of many treatment trials. One of these transduction cascade steps that have gained interest is cyclo-oxygenase. In the present study, lbp treatment significantly decreased the tumour tissue and number of intratumoural vessels.

Some recent studies have demonstrated that gastric, colonic and other carcinogenic processes could be hindered by NSAIDs.^{14,15} Although the exact mechanisms by which NSAIDs contribute to anti-tumoural activity remain controversial, these agents cause the induction of apoptosis, in the control of cell proliferation and invasion and/or in the inhibition of angiogenesis.^{14,16} The cyclo-oxygenase enzyme is the rate-limiting enzyme that catalyzes the first step in the synthesis of prostanoids, such as PG-D2 (in CNS), PG-E2 (in vascular beds), PG-F2 α (in smooth muscles), PG-I2 (in the vascular endothelium) and thromboxane (in the platelets). These metabolites influence cellular functions such as mitogenesis, cellular adhesion, invasion, and apoptosis.¹⁷ Bernardi *et al.* reported that indomethacin, a powerful NSAIDs derived from indolacetic acid, caused anti-proliferative effects on glioma cell lines due to an arrest of cell cycle progression.¹⁸ In another study, Naumann *et al.* reported that anti-inflammatory steroids such as dexamethasone inhibit drug cytotoxicity of human malignant glioma cells.¹⁹ Sivak-Sears *et al.* showed that between NSAIDs use and glioblastoma multiforme presence of an inverse association.²⁰ Their work compared NSAID use among glioblastoma multiforme cases and controls. The principal findings were that cases with self-reported glioblastoma multiforme were less likely than controls who had used 600 or more NSAIDs pills within the 10 years prior to

diagnosis; the associations being similar among men and women across the various categories of NSAIDs. Altinoz *et al.* studied two liposoluble drugs, medroxy-progesterone acetate (MPA) and the analgesic ibuprofen, on glioma vascularization *in vivo*.²¹ MPA had a slight though insignificant activity to reduce the fatality of C6 glioma.²¹ But ibuprofen both alone or with MPA had no effect on survival.²¹ On histological analysis, progesterone seemed to increase peritumoural vessels. Co-administration of ibuprofen acted to suppress the peritumoural vessels increase, and to enhance lymphomonocytic infiltration around tumour vessels. Kang *et al.* found that in celecoxib and temozolomide combination group, tumour cell apoptosis was increased as well as decreased microvessel density and tumour cell proliferation relative to the control and single agent therapy.²²

Results obtained from the rat glioma model of the present study indicate that lbp inhibits cell proliferation, angiogenesis and induce the stimulation of apoptosis. The Ki-67 protein is a cellular marker for proliferation.²³ Ki-67 immunostaining of brain tumour is associated with a high proliferation rate.

In this study, the level of Ki67 was decreased in the tumour tissue of the group 2 compared to the group 1. Additionally, the total necrotic area / total tumour volume (%) was decreased in tumour tissue of the group 2 rats compared to group 1. Clinically, concomitant radiation and chemotherapy shows a survival gain in glioblastoma patients. Combination treatment of the lbp with other active chemotherapy agents used for the treatment of brain tumours may offer a greater inhibitory effect on tumour and may also prevent the development of undesirable side effects.

CONCLUSION

According to our data, lbp treatment demonstrated an inhibition to rat glioma tumours, a significant decrease in cell proliferation, and an increase in apoptosis. These results imply that this drug can be considered as a potential candidate with other agents for glioma treatment. Additionally, the results of the present study may provide an experimental basis for further clinical studies.

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