Protective Effect of Autophagy in Laser-Induced Glioma Cell Death In Vitro

Aleksandar J. Krmpot, PhD,[1] Kristina D. Janjetovic, MSc,[2,3] Maja S. Misirkic, MSc,[2,3] Ljubica M. Vucicevic, MSc,[2,3] Dejan V. Pantelic, PhD,[1] Darko M. Vasiljevic, PhD,[1] Dusan M. Popadic, MD, PhD,[2] Branislav M. Jelenkovic, PhD,[1] and Vladimir S. Trajkovic, MD, PhD[2]***

[1]*Institute of Physics, University of Belgrade, 11080 Belgrade, Serbia*

[2]*Institute of Microbiology and Immunology, School of Medicine, University of Belgrade, 11000 Belgrade, Serbia*

[3]*Institute for Biological Research, 11000 Belgrade, Serbia*

Background and Objective: Laser phototherapy could be potentially used for cancer treatment, but the mechanisms of laser-induced cell death are not completely understood. Autophagy is the process in which the damaged cellular proteins and organelles are engulfed by and destroyed in acidified multiple-membrane vesicles. The aim of the present study was to investigate the role of autophagy in laser-induced tumor cell death in vitro.

Study Design/Materials and Methods: The monolayers of U251 human glioma tumor cells were exposed to 532 nm laser light from a single mode frequency-doubled Nd-YVO4 laser. A flattened Gaussian radial profile of laser beam (0.5–4 W) was used to uniformly illuminate entire colony of cells for various amounts of time (15–120 seconds) in the absence of cell culture medium. The cells were grown for 24 hours and the cell viability was determined by crystal violet or MTT assay. The presence of autophagy was assessed after 16 hours by fluorescence microscopy/flow cytometric analysis of acridine orange-stained autophagolysosomes and Western blot analysis of the autophagosome-associated LC3-II protein. The concentration of the principal pro-autophagic protein beclin-1 was determined after 6 hours by cell-based ELISA.

Results: The intracytoplasmic accumulation of autophagic vesicles, increase in LC3-II and up-regulation of beclin-1 expression were clearly observed under irradiation conditions that caused approximately 50% cytotoxicity. Post-irradiation addition of three different autophagy inhibitors (bafilomycin A1, chloroquine, or wortmannin) further increased the laser-induced cytotoxicity, without affecting non-irradiated cells.

Conclusions: These data indicate that beclin-1-dependent induction of autophagy can protect glioma cells from laser-mediated cytotoxicity. Lasers Surg. Med. 42:338–347, 2010. © 2010 Wiley-Liss, Inc.

Key words: apoptosis; cancer; necrosis; phototherapy

INTRODUCTION

Laser irradiation is known for its ability to cause cell death, a feature that has been used over past decades for therapeutical killing of tumor cells [1–6]. However, the value of laser-based anticancer therapy is limited due to many side-effects and insufficient efficacy [7], the main limitation being the failure to achieve complete tumor destruction, particularly at the margins of larger tumors [8,9]. In order to increase efficiency and/or decrease the side-effects of laser anticancer therapy, a more thorough knowledge is required on the mechanisms underlying laser-induced cytotoxicity.

In addition to tissue vaporization at very high temperatures ($>100^{\circ}C$) [10], laser irradiation can also cause necrotic cell death at high power [11–13] and apoptotic cell death at low power [14–16]. Necrosis is characterized by vacuolation of the cytoplasm, breakdown of the plasma membrane and release of cellular contents, resulting in the induction of inflammation around the dying cell [17]. Apoptosis or type I programmed cell death, on the other hand, is typified by fragmentation of DNA without plasma membrane breakdown, followed by packaging of the deceased cell into apoptotic bodies that are recognized and removed by phagocytic cells in the absence of inflammation [17]. Importantly, apoptosis was observed in a fraction of tumor cells surrounding the necrotic lesion induced by high-power laser therapy [18]. Since the secondary outgrowth of these marginal zone cells may form the basis of a reformation of the tumor mass [18], the complete elucidation of the mechanisms underlying their survival and death is of paramount importance for improving the efficiency of laser-based anticancer treatment.

Macroautophagy (referred to hereafter as autophagy) is a self-cannibalization process that involves sequestration of cell structures in double-membraned organelles, called autophagosomes [19]. This is followed by fusion of

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*Correspondence to: Vladimir S. Trajkovic, MD, PhD, Institute of Microbiology and Immunology, School of Medicine, University of Belgrade, Dr. Subotica 1, 11000 Belgrade, Serbia.
E-mail: vtrajkovic@eunet.rs

**Correspondence to: Aleksandar J. Krmpot, PhD, Institute of Physics, University of Belgrade, Pregrevica 118, 11080 Belgrade, Serbia. E-mail: krmpot@phy.bg.ac.rs

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autophagosomes with lysosomes and formation of autophagolysosomes in which the cellular content is degraded by acidic lysosomal hydrolases [20]. The physiological role of autophagy is to remove long-lived proteins and damaged organelles, but when it is extensive, activated inappropriately or in cells which are unable to die by apoptosis, autophagy acts as an alternative cell-death pathway called autophagic or type II programmed cell death [21]. In apoptosis-competent cells, on the other hand, autophagy mainly acts as a survival mechanism that can rescue cells from apoptotic death induced by nutrient deprivation or pharmacological agents [22]. Although autophagy can either promote or impair cell death in a context-dependent manner, no study so far, to the best of our knowledge, has addressed the ability of laser light to induce autophagy in mammalian cells.

In this study we present evidence that laser irradiation can cause autophagy in human U251 glioma cell line. Moreover, our data demonstrate that autophagy is not involved in cell death, but actually protects glioma cells from laser-induced cytotoxicity.

MATERIALS AND METHODS

Cells and Cell Culture

The rat glioma cell line U251 was kindly donated by Dr. Pedro Tranque (Universidad de Castilla-La Mancha, Albacete, Spain). The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2, in a HEPES (20 mM)-buffered RPMI 1640 cell culture medium (Sigma–Aldrich, St. Louis, MO) supplemented with 5% fetal calf serum (FCS), 2 mM L-glutamine, 10 mM sodium pyruvate and penicillin/streptomycin (all from Sigma–Aldrich). The cells were prepared for experiments using the conventional trypsinization procedure with trypsin/EDTA and incubated as a monolayer in 96-well flat-bottom plates (1×10^4 cells per well). Cells were rested for 24 hours, washed with phosphate buffered saline (PBS; Sigma–Aldrich) and exposed to laser light in the absence of cell culture medium. Following irradiation, fresh cell culture medium was added and the cells were incubated for an additional 6, 16, or 24 hours for the measurement of beclin-1 expression, autophagy or cell number, respectively. These end-points were determined in preliminary experiments (data not shown) as the time points in which the particular cellular responses were most pronounced (6 and 16 hours) or clearly observable (24 hours). In some experiments, after irradiation cells were treated with autophagy inhibitors bafilomycin A1 (50 nm), chloroquine (20 μM) or wortmannin (100 nm) (all from Sigma–Aldrich) and the cell viability was determined after 16 hours.

Laser Treatment and Temperature Measurement

For irradiation of cells we used a continuous wave single mode frequency-doubled diode-pumped Nd-YVO4 laser (Verdi V10, Coherent, Inc., Santa Clara, CA), which delivers green light (532 nm) up to 10 W power. The beam from the laser was directed to the cells passing through a lens and a diffuser to get flattened profile of ~4 mm diameter (Fig. 1), as measured by the beam profilometer (BCi4, by C-Cam Technologies, Leuven, Belgium). The cells were irradiated with different laser

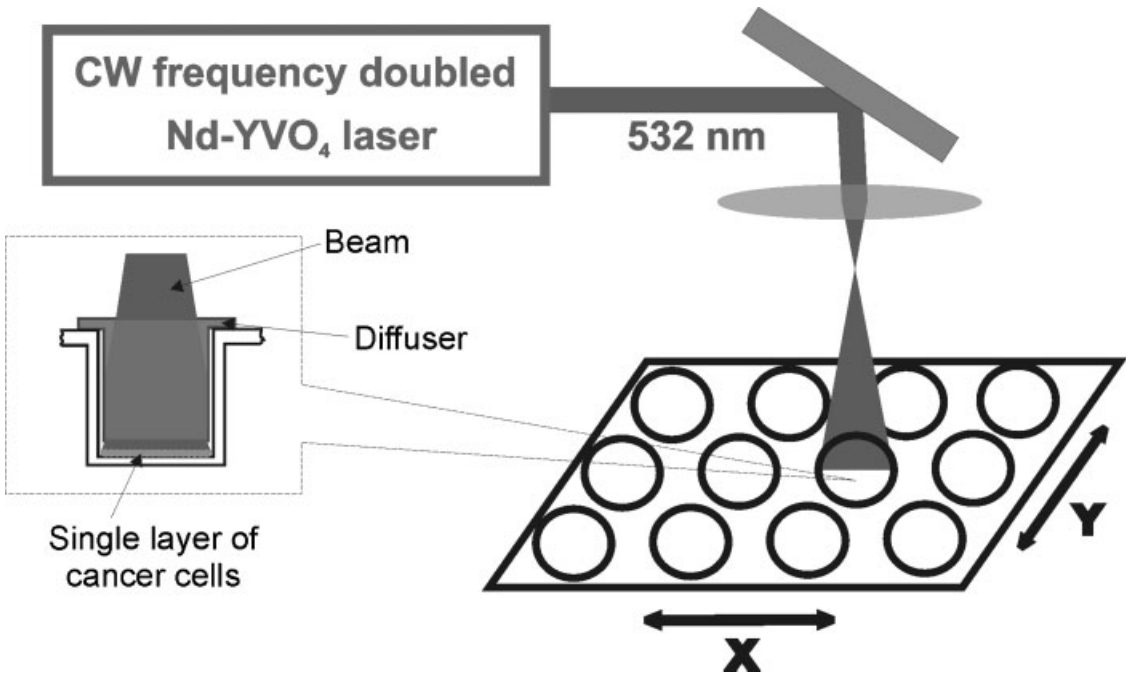


Fig. 1. The setting for laser irradiation of tumor cells. The beam from the laser (532 nm) was directed to the cell monolayer (without cell culture medium) passing through a lens and a diffuser to get flattened profile of ~4 mm diameter.

powers (0.5–4 W) for various amounts of time (15–120 seconds) at room temperature and in the absence of cell culture medium. To prevent irradiation of adjacent wells, the convex side surface of the diffuser was coated with a light-reflecting silver dye. The effectiveness of this approach was confirmed in preliminary experiments, in which no reduction of cell number, autophagy, or significant increase in temperature ($>37^{\circ}C$) was observed in the wells adjacent to those irradiated with laser (data not shown). The temperature of the cell culture well surface with or without cells was measured during 60 seconds irradiation with 2 W laser beam using an infrared termovision camera with 640×480 pixel resolution and a spectral range of 7.5–13 μm (FLIR ThermaCAM SC640, FLIR Systems, Boston, MA) and the appropriate software.

Cytotoxicity Assays

The cytotoxic effect of laser irradiation was assessed by measuring the number of viable cells using crystal violet or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Crystal violet cytotoxicity test is based on the inability of dead cells to remain adherent to cell culture plastic [23]. Cells were washed with PBS to remove dead, non-adherent cells. The remaining adherent, viable cells were fixed with methanol and stained with 1% crystal violet solution for 10 minutes. The plates were thoroughly washed with water, and crystal violet was dissolved in 33% acetic acid. The absorbance of the dissolved dye, corresponding to the number of viable cells, was measured in an automated microplate reader at 570 nm. Mitochondrial dehydrogenase activity, as another indicator of cell number, was assessed by the mitochondrial-dependent reduction of MTT to formazan [24]. At the end of the incubation, MTT solution (0.5 mg/ml) was added to cell cultures and the cells were incubated for an additional 1 hours. Thereafter, medium was removed and the cells were lysed in dimethylsulfoxide. The conversion of MTT to formazan by metabolically viable cells was monitored by an automated microplate reader at 570 nm. The results of MTT and crystal violet tests were presented as % of the control value obtained in untreated cells.

Autophagy Assessment

The acidic autophagic vesicles were visualized by supravital staining with a pH-sensitive dye acridine orange, as previously described [25]. Briefly, cells were washed with PBS and stained with acridine orange (1 μM; Sigma–Aldrich) for 15 minutes at 37°C. Subsequently, cells were washed and analyzed under the inverted fluorescence microscope. Depending on their acidity, autophagic lysosomes appeared as yellow-orange to bright-red fluorescent cytoplasmic vesicles, while nuclei were stained green. Alternatively, acridine orange-stained cells were trypsinized, washed, and analyzed on FACSCalibur flow cytometer using Cell Quest Pro software. The intensity of autophagy was quantified as a red/green fluorescence ratio (FL3/FL1), which increases during autophagy. The induction of autophagy was confirmed by Western blot analysis of LC3, which exists in two forms, the 18-kDa cytosolic protein (LC3-I) and the processed 16-kDa form (LC3-II), the latter being associated with autophagosome membranes [19]. Approximately 2×10^6 cells were lysed on ice in Tris-saline containing 1% NP-40, 1 mM phenylmethylsulfonylfluoride and protease inhibitor cocktail (all from Sigma–Aldrich), centrifuged at 18,000*g* (15 minutes, 4°C), and the supernatants were collected as the cell lysates. Equal amounts of protein from each sample was separated by SDS–PAGE on polyacrylamide gels and transferred to nitrocellulose membranes. Following incubation with rabbit anti-human LC3 (Cell Signaling Technology, Danvers, MA) as a primary antibody (1:1,000) and peroxidase-conjugated goat anti-rabbit IgG (Jackson IP Laboratories, West Grove, PA) as a secondary antibody (1:3,000), specific bands corresponding to LC3-I and LC3-II were visualized using enhanced chemiluminescence reagents for Western blot analysis (Amersham Pharmacia Biotech, Piscataway, NJ).

Beclin-1 Expression Analysis

The cell-based Enzyme-Linked Immunosorbent Assay (ELISA) was performed as previously described [26]. After irradiation, cells were fixed in 4% paraformaldehyde, endogenous peroxidase was quenched with 1% H_2O_2 in PBS containing 0.1% Triton X-100 (PBST), and unspecific binding of antibodies blocked with PBST solution containing 10% FCS. Primary rabbit polyclonal antibodies specific for human beclin-1 (1:250; Abcam, Cambridge, UK) were applied in PBST supplemented with 2% bovine serum albumin (PBSTB), followed by secondary peroxidase-conjugated goat anti-rabbit IgG (1:3,000 in PBSTB; Jackson IP Laboratories). Both incubations were undertaken at 37°C for 1 hours. After incubation with the peroxidase substrate tetramethylbenzidine (Sigma–Aldrich), the reaction was stopped with 0.1 M HCl and the absorbance was measured in an automated microplate reader at 450 nm. The obtained absorbances were corrected for the cell number determined by crystal violet staining, as described in the original protocol [26]. The results are presented as relative expression in comparison with the control value, which was arbitrarily set to 1.

Induction of Hyperthermia

Cell suspension (10^5 or 10^6 cells in 100 μl for the flow cytometry or Western blotting, respectively) was transferred to a sterile thin wall 0.2 ml tube (Eppendorf, Hamburg, Germany) and placed in a preheated (30°C) thermoblock of the thermocycler (Reaplex^2, Eppendorf) programmed to gradually increase temperature to 47°C within 30 seconds. This was achieved by setting control heating parameters to a ramping rate of 10% in block (simulated) tube mode with switched-off lead heating and input of the correct volume of the cell suspension (100 μl) in the sample volume box. Correct heating of the block was confirmed using an external Pt100 thermal probe (Omega Engineering, Manchester, UK) placed in the well juxtaposed to the cell-containing well. Immediately after reaching 47°C cells were transferred to 24-well plates (10^5 cells per well) for the flow cytometry analysis or 6-well plates (10^6 cells/well) for Western blot and incubated for an

additional 18 hours. Cells were then collected by trypsinization and autophagy was examined by flow cytometry following acridine orange staining, while LC3 conversion was assessed by Western blotting. We also included the positive control for hyperthermia-induced autophagy by heating the cells continuously for 30 minutes at 43°C.

Statistical Analysis

The data are presented as mean ± SD values of triplicate observations from at least three experiments, or as mean ± SD values from three independent experiments. The statistical significance of the differences between treatments was assessed using *t*-test and the value of $P < 0.05$ was considered significant.

RESULTS

Cytotoxic Effect of Laser Irradiation

In order to establish the appropriate conditions for the assessment of autophagy, we first exposed the cells to laser irradiation of increasing power (0.5–4 W) for various amounts of time (15–120 seconds) and measured cell numbers 24 hours after laser exposure. Both crystal violet and MTT assay demonstrated that the cytotoxicity of laser irradiation was energy-dependent, with 50% cytotoxicity achieved with ~60 J = 2 W × 30 seconds (Fig. 2A,B). The light microscopic analysis of cell morphology has shown that, in conditions causing complete cell death (2 W, 120 seconds), the cells were totally destroyed, probably

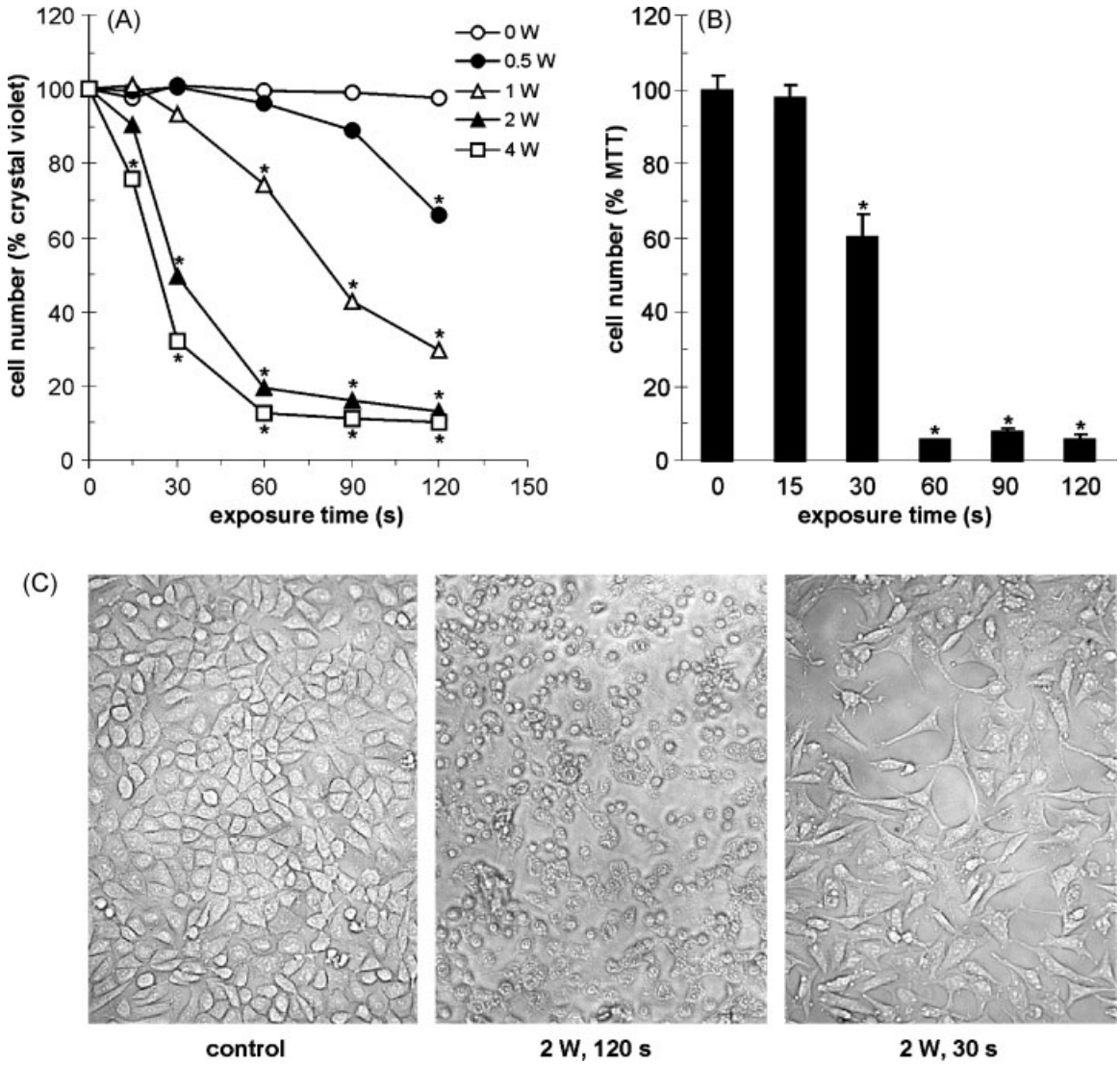


Fig. 2. The dose- and time-dependent effects of laser light on viability of glioma cells. U251 cells were exposed to laser light of different power (**A**) or 2 W power (**B**,**C**) for the indicated periods of time. The cell viability was investigated by crystal violet (A) or MTT assay (B), while the cell morphology was examined by inverted light microscopy (C). The SD values in A were less then 10% of the mean and were omitted for clarity (*$P < 0.05$, *t*-test, $n = 4$).

due to induction of necrosis (Fig. 2C). On the other hand, in conditions causing 50% reduction in cell numbers (2 W, 30 seconds) the cell morphology was mainly preserved, while some round cells detached from the surface could be observed (Fig. 2C). Therefore, the conditions causing ~50% cytotoxicity (2 W, 30 seconds) were chosen for further experiments.

Laser-Mediated Cytotoxicity Is Accompanied by the Induction of Autophagy and Up-Regulation of Beclin-1

To assess the presence of autophagy in laser-treated cells, we used a pH-sensitive dye acridine orange, which stains the acidic autophagosomes orange-red. As demonstrated by fluorescence microscopy, acridine orange-stained cells that were previously exposed to laser, but not control, untreated cells, clearly displayed the presence of orange-red autophagic vesicles within their cytoplasm (Fig. 3A,B). To further investigate the induction of autophagy in laser-treated cells, we used flow cytometry to measure the intensity of red fluorescence in acridine orange-stained cells. Accordingly, a significant increase in red/green (FL3/FL1) fluorescence ratio was observed in laser-exposed, compared to control, untreated cells (Fig. 3B,C). These results were confirmed by Western blot analysis of LC3 conversion, showing the increase of autophagosome-associated LC3-II isoform in laser-irradiated cells (insert in Fig. 3C). One of the mechanisms for the induction of autophagy is the up-regulation of pro-autophagic protein beclin-1, which is required for the formation of autophagocytic vesicles. Accordingly, we have performed cell-based ELISA with beclin-1-specific antibodies to determine whether the intracellular concentration of beclin-1 increases following exposure to laser light. Indeed, the data presented in Figure 3D clearly show that beclin-1 level in cells exposed to laser is significantly higher than in untreated cells. These results indicate that laser-mediated cytotoxicity is associated with the induction of autophagy, probably as a consequence of the increase in beclin-1 expression.

Hyperthermia Is Not Involved in Laser-Mediated Autophagy Induction

The cytotoxic efficiency of laser irradiation correlated with the temperature measured at the surface of laser-exposed cell culture wells. A linear increase in temperature up to 60°C was observed in cell culture wells exposed to 2 W laser light during 60 seconds (Fig. 4A). Similar increase in temperature was observed irrespectively of the presence of cells in the cell culture wells (data not shown). While hyperthermia induced after 30 seconds of laser irradiation (up to 47°C) was clearly incapable of thermal ablation, we sought to examine whether it could be involved in the induction of autophagy. To produce laser-like temperature increase in the absence of light, we heated the cells in a PCR thermocycler programmed to gradually increase the temperature to 47°C within 30 seconds (Fig. 4B). As a positive control for hyperthermia-induced autophagy, we used cells exposed to 43°C during 30 minutes. After an additional 18 hours of incubation, the increase in red/green acridine orange fluorescence ratio as a marker of autophagy was clearly observed in the positive control, but not in cells exposed to a linear increase to 47°C during 30 seconds (Fig. 4C). Accordingly, no increase in LC3-II was observed in cells exposed to short-term (30 seconds) hyperthermia (insert in Fig. 4C). These data indicate that hyperthermia was not responsible for the laser-induced autophagic response in tumor cells.

Autophagy Protects Cells From Laser-Induced Death

Finally, we wanted to examine to role of autophagy in laser-induced cytotoxicity. To that end, we treated the cells immediately after the laser exposure with three different autophagy inhibitors: bafilomycin A1 or chloroquine, which both prevent acidification of autophagosomes [27,28], or wortmannin, a phosphatydilinositol 3-kinase blocker that prevents autophagosome formation [29]. Since the autophagy inhibitors alone were apparently able to slightly reduce glioma cell viability after 24 hours of incubation (data not shown), we have shortened the incubation time to 16 hours for this experiment. While in these conditions the autophagy inhibitors did not significantly affect the viability of cells that were not exposed to laser, they all markedly increased cell death caused by exposure to laser for various amounts of time (Fig. 5). Similar results were obtained with both crystal violet (Fig. 5A) and MTT assay (Fig. 5B). These data clearly demonstrate that autophagy exerts a protective role in laser-induced cell death.

DISCUSSION

The present study for the first time demonstrates the ability of laser light to induce autophagy associated with up-regulation of the pro-autophagic protein beclin-1 in mammalian cells. Interestingly, the induction of autophagy protected human U251 glioma cells from laser-mediated cytotoxicity. It should be noted that the observed effect was apparently not species- or cell type-specific, as similar results were obtained in L929 mouse fibrosarcoma cell line [Krmpot et al., unpublished observation].

Although laser light has been used for decades for various medical indications, the mechanisms of laser-induced cell death are still not completely elucidated. In contrast to necrotic cell death caused by high-power laser-mediated thermal destruction [11–13], low-power laser treatment can lead to type I programmed cell death, apoptosis, in which various intracellular mechanisms are activated in a highly controlled manner to eventually cause cell demise [14–16]. Although we did not closely investigate the cell features characteristic for necrosis or apoptosis, the cell morphology and temperature measurement data support the assumption that the laser exposure setting in our experiments led to a non-thermal cell proliferation arrest and apoptotic cell death. More importantly, we have shown that laser-induced cytotoxicity is associated with the autophagic digestion of intracellular proteins. The two main mechanisms for the initiation of autophagosome formation are the inhibition of mammalian target of

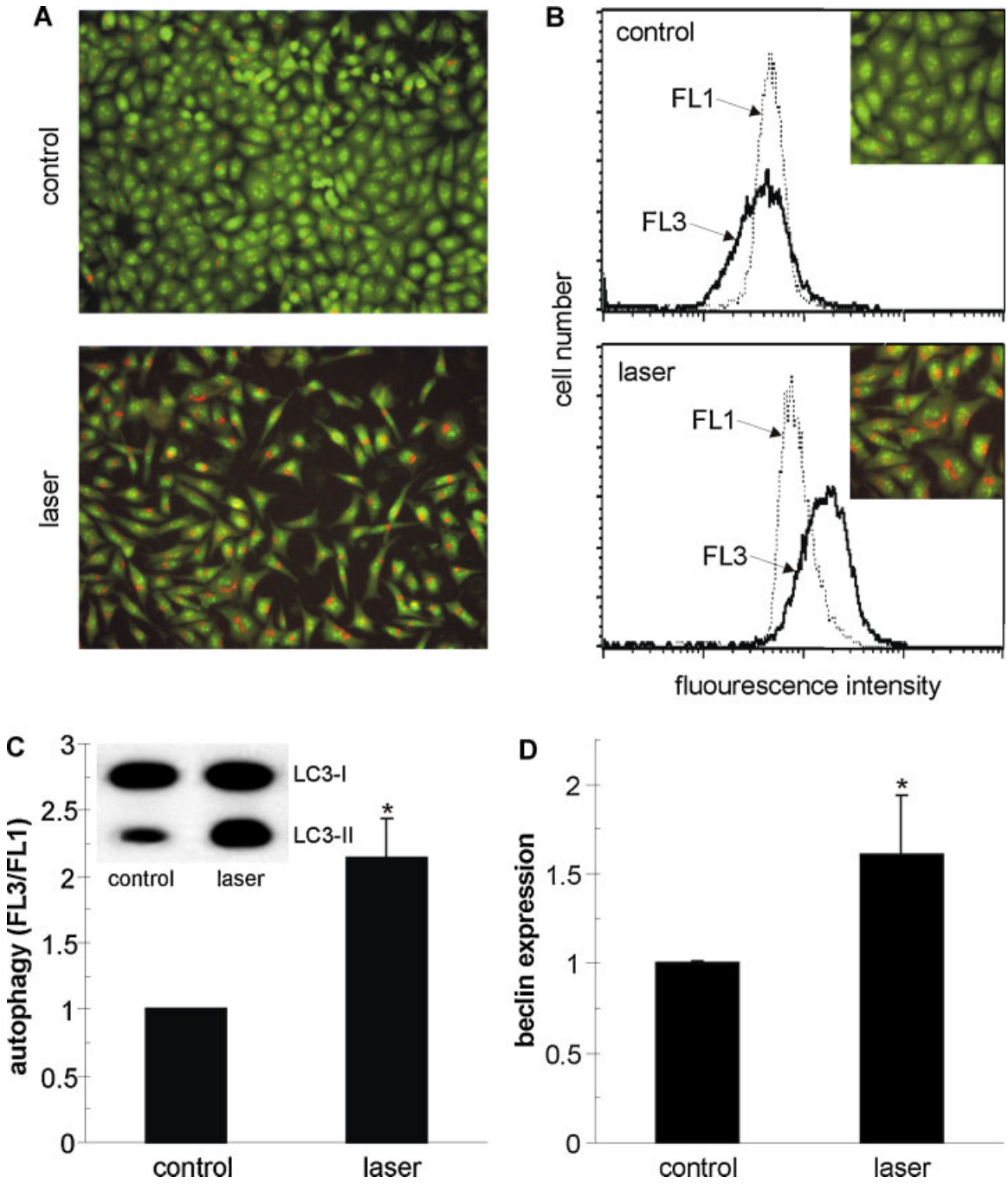
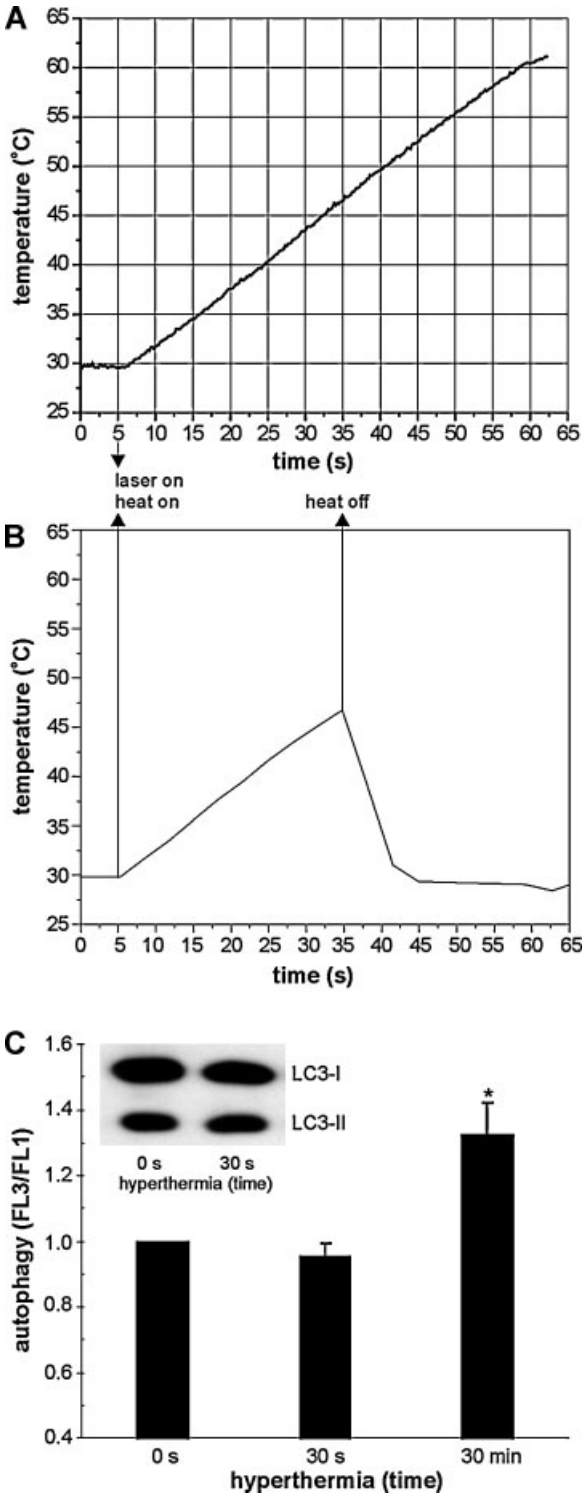


Fig. 3. The induction of autophagy and beclin-1 in laser-exposed glioma cells. **A**–**D**: U251 cells were exposed to laser light (2 W) for 30 seconds. The presence of autophagic vesicles was examined after 16 hours in acridine orange-stained cells by fluorescent microscopy (A, inserts in B) or flow cytometric analysis (B,C), while the level of autophagosome-associated LC3-II was analysed by Western blotting (insert in C). The expression of beclin-1 was assessed after 6 hours by cell-based ELISA (D) (*$P < 0.05$, t-test, $n = 3$).

rapamycin (mTOR) and up-regulation of the crucial pro-autophagic protein beclin-1 [30]. While we did not assess the mTOR activity in response to laser irradiation, our data clearly demonstrate that laser-induced autophagy is associated with the increase in beclin-1 expression. It should be also noted that the laser irradiation in our experiments caused hyperthermia, and the recent results from others and us indicate that hyperthermia could

initiate autophagy [31,32]. However, the experiments in which cells were exposed to temperature increase (up to 47°C during 30 seconds) in the absence of light indicate that short-term hyperthermia induced by laser irradiation was not responsible for the induction of autophagy in our study.



Another possible mechanism for laser-mediated induction of autophagy is the production of reactive oxygen species and the subsequent oxidative damage of intracellular components, which has recently been implicated in autophagy initiation by photodynamic therapy [33–35]. This assumption is consistent with the findings that laser irradiation alone can induce production of reactive oxygen species [36–38] and is currently being investigated in our laboratory.

Recent data indicate that authophagy, depending on conditions, can either impair or promote cell survival [21,22]. The induction of autophagy by anticancer agents such as tamoxyfen, rapamycin, arsenic trioxide, temozolomide, brevinin-2R or fullerenes is deleterious to tumor cells [25,39–44], implicating autophagy as a "magic bullet" for treatment of apoptosis-resistant cancers. On the other hand, a considerable body of evidence indicates that autophagy can also act as a survival mechanism providing constituents necessary for sustaining essential cell metabolism in stress conditions such as nutrient deprivation [45]. Furthermore, inhibition of autophagy increases the sensitivity of apoptosis-competent cancer cells to hyperthermia, ionizing irradiation, camptothecin, alkylating agents, hystone deacetylase inhibitors, sulindac sulfide, tumor necrosis factor-α, and cisplatin [31,46–52], suggesting that tumor cells in some conditions might use autophagy to evade therapy-induced apoptotic death. Accordingly, our results with three different autophagy inhibitors clearly demonstrate that autophagy protects glioma cells from laser-induced cytotoxicity. While further confirming the concept of autophagy as a cell-protective mechanism, these data could have important implications for the outcome of various laser-based therapeutic procedures. For example, one could expect that pharmacological inhibition of autophagy might enhance the ability of laser light to kill cancer cells, particularly those at the margin of laser-induced tumor necrosis. On the other hand, it has been shown that low-power laser irradiation can protect cells from apoptosis in various experimental settings [53–55], but the involvement of autophagy in the observed protective effect was not evaluated. We are currently investigating the possibility that laser-induced autophagy might protect cells from other apoptotic stimuli.

Fig. 4. The role of hyperthermia in laser-induced autophagy. **A**: The bottom of the cell culture well was exposed to laser light (2 W) using the setting described in Figure 1. The temperature increase was monitored during 60 seconds by an infrared termovision camera. **B**: To mimic laser-induced hyperthermia, cell suspensions are heated in a thermocycler set to linearly increase the temperature to 47°C during 30 seconds. **C**: After 16 hours of incubation, autophagy was asessed in cells from (B) by flow cytometric analysis following acridine orange staining (*$P<0.05$, t-test, $n=3$). The positive control included cells heated at 43°C for 30 minutes. The absence of LC3 conversion in cells exposed to short-term (30 seconds) hypertermia was confirmed by Western blot analysis (insert in Fig. 4C).

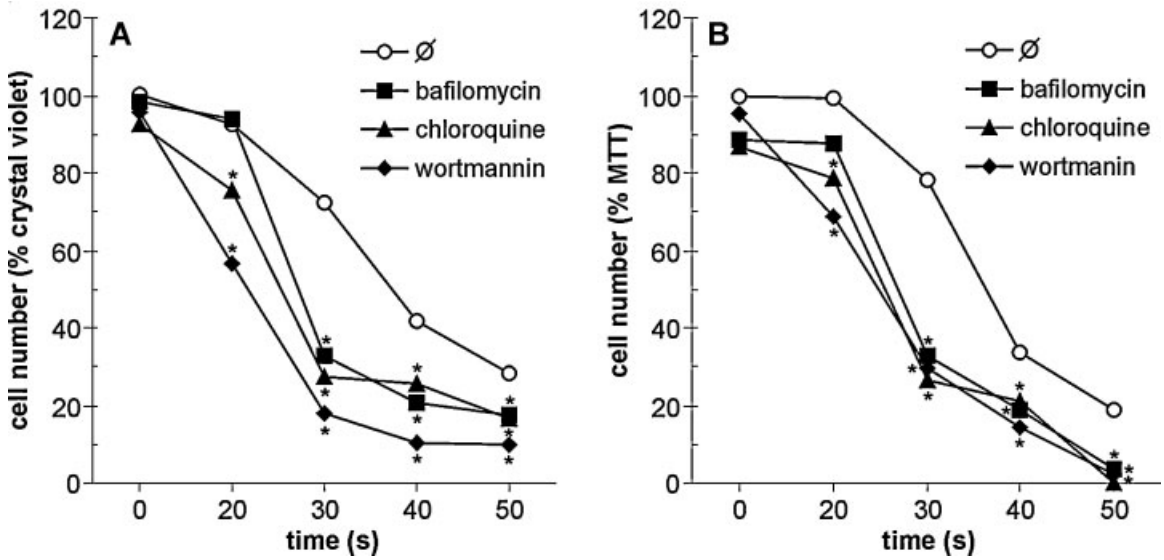


Fig. 5. The effect of autophagy inhibitors on laser-induced glioma cell death. **A**,**B**: U251 cells were exposed to laser light (2 W) for the indicated amounts of time in the absence or presence of autophagy inhibitors bafilomycin A1 (50 nm), chloroquine (20 μM) or wortmannin (100 nm). The cell viability was examined after 16 hours by crystal violet (A) or MTT assay (B). The SD values were less then 10% of the mean and were omitted for clarity (*$P<0.05$, t-test, $n=3$, refers to corresponding cell cultures without autophagy inhibitors).

In conclusion, the present study demonstrates a protective effect of beclin-1-dependent autophagy in laser-induced cytotoxicity. It should be noted that our laser setting was restricted to the use of 532 nm wavelength, which has low tissue penetration depths, but good coagulative properties, and has mainly been utilized for treatment of skin cancers, laryngeal cancers and benign prostate hyperplasia [10,56]. In the future studies we intend to include the near-infrared laser light, which has larger penetration depth and is more widely used for hyperthermic treatment of cancer [10]. In addition, due to differences in light-absorption properties of a single cell layer on plastic compared to the living tissues, our results are difficult to directly extrapolate to in vivo conditions in terms of laser power and exposure times. Nevertheless, it is conceivable to expect that autophagy will be induced by laser treatment in vivo, particularly during low-level laser therapy or at the margins of necrotic tumor lesions induced by high-power laser irradiation. Having in mind the important role of autophagy in various cellular functions [57,58], its possible impact on the outcome of various laser-based therapeutic approaches seems worthy of further investigation.

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