Effect of Light Irradiation by Light Emitting Diode on Colon Cancer Cells

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Abstract. *Background/Aim: Recent studies have demonstrated the efficacy of irradiation from light emitting diodes (LED) for wound healing, anti-inflammation and anticancer therapies. However, little is known about the effects of visible light in colon cancer cells. The purpose of this study was to evaluate the biological response (including gene expression changes) of human colon cancer cells to different wavelengths of LED irradiation. Materials and Methods: Human colon cancer cells (HT29 or HCT116) were seeded onto laboratory dishes that were then put on LED irradiation equipment with a 465 nm-, 525 nm-, or 635 nm-LED. Irradiation at 15 or 30 mW was performed 10 min/day, each day for 5 days. The cell counting kit8 was then used to measure cell viability. Apoptosis and expression of several mRNAs (caspase, MAPK and autophagy pathway) in HT29 cultures irradiated with 465 nm LED were evaluated via AnnexinV/PI and RT-PCR, respectively. Results: Viability of HT29 and HCT116 cells was lower in 465 nm-LED irradiated cultures than in control cultures, but viability of HT29 cells did not differ between control cultures and 525 nm-LED or 635 nm-LED irradiated cultures. Moreover, the expression of FAS, caspase-3, capase-8, and JUK were significantly higher in 465 nm-LED irradiated cultures than in control cultures, and expression of ERK1/2 and LC3 was lower in blue-irradiated cells. Conclusion: LED irradiation at 465 nm inhibited the proliferation of HT29 cells and of HCT116 cells. Notably, LED irradiation at 465 nm promoted apoptosis inHT29 cultures via the extrinsic apoptosis pathway and the MAPK pathway.*

Abbreviations: LED: Light-emitting diode; MAPK: mitogen-activated protein kinase; JNK: c-Jun N-terminal kinase; CRC: colorectal cancer; PDT: photodynamic therapy; ROS: reactive oxygen species; CCK8: Cell Counting Kit-8; PBS: Phosphate Buffered Saline; FACS: flow cytometry; FITC: fluorescein isothiocyanate; PI: propidium iodide; TNFα: tumor necrosis factor α; ERK1/2: extracellular signal-regulated kinases 1/2.

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Globally colorectal cancer (CRC) is the third most common cancer and the fourth most common cause of cancer-related mortality (1). Although advances in drug development and surgery have led to an increased overall survival of CRC, the prognosis for an individual with CRC is usually poor (2). Therefore, effective and novel treatment modalities are required.

Photodynamic therapy (PDT) was introduced approximately 35 years ago. PDT consists of systemic or topical administration of a photosensitizer or metabolic precursor, photoexcitation of the sensitizer by light in the visible wavelength (400-750 nm), and consequent tumor cell death induced by the release of reactive oxygen species (ROS) (3, 4). Photofrin® (Porfimer sodium) has received worldwide regulatory approval as a photosensitizer and was the basis for the growth of oncologic PDT. However, Photofrin requires approximately 6 weeks of photosensitivity precautions (4). This side-effect has limited its use as a photosensitizer for PDT.

Accumulating evidence indicates that clinical treatments involving low-intensity irradiation with a specific wavelength of visible light may promote wound healing, reduce inflammation, relieve pain, or some combination thereof;
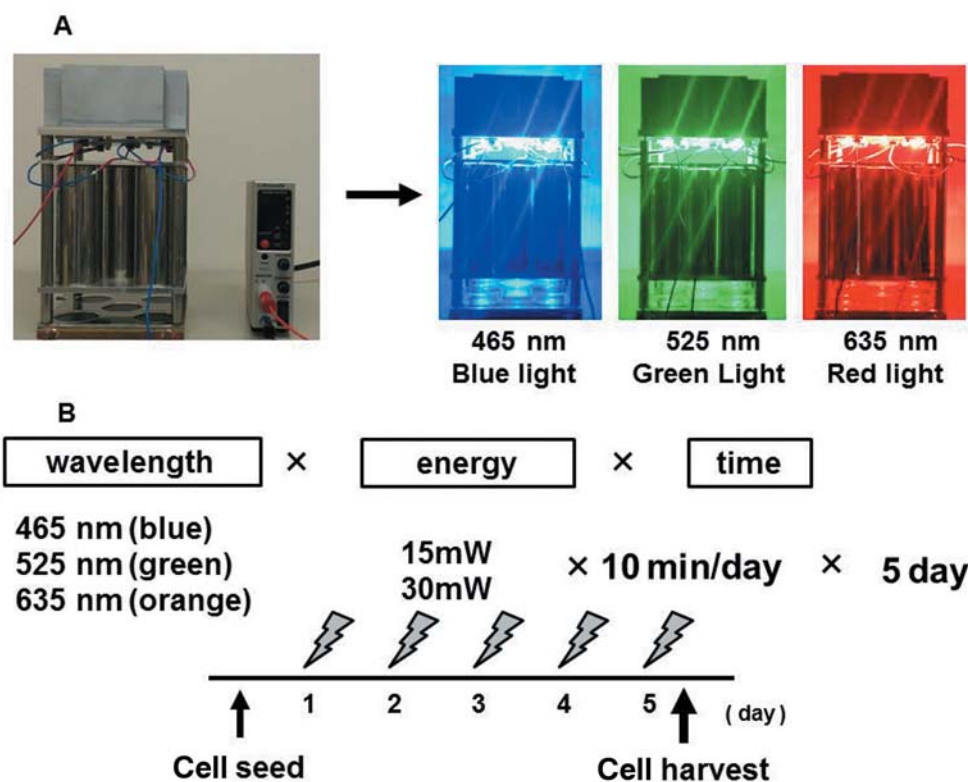
Figure 1. *A. LED irradiation device. For each of the wells, one LED was mounted above the cell culture dish. The set-up was used in the dark room. B. Irradiation protocol. Each cell line was irradiated by only one of three types LED at 30 mW or 15 mW for 10 m/day on 5 consecutive days.*

visible-light irradiation has several potential advantages over traditional X-ray irradiation (5, 6). Thus, the low-intensity irradiation may become an important therapeutic tool in inducing cell apoptosis (7, 8). Previous studies have demonstrated that light-emitting diodes (LEDs) are similar to low-intensity lasers and that LEDs have superior abilities to control light intensity, wavelength, and stability than other light sources (8, 9).

The effects of LED-based PDT on cells, disease, and wound healing have been studied. LED irradiation at 570 nm enhances fibroblast growth (10), 530 nm or 700 nm irradiation increases angiogenesis in cutaneous wounds (11), and 880 nm irradiation decreases the influx of inflammatory cells (12). However, the effects of LED irradiation on colon cancer cells have not been studied. Also, the effects of LED irradiation on apoptosis or autophagy factor synthesis in cancer cells have been the subject of very few studies. The aim of the present study was to evaluate the effects LED irradiation at different wavelengths on colon cancer cell proliferation, apoptosis, and autophagy. Here, we assessed whether various wavelengths of light emitted from an LED could regulate biologic and morphologic responses of colon cancer cells *in vitro*.

Materials and Methods

Cell cultures. HT29 and HCT116 human colon cancer cell lines were purchased from the American Type Culture Collection (ATCC). CSC-2FO human fibroblast cells were purchased from Cell Systems Corporation (CSC). All cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Sigma–Aldrich, St. Louis, MO, USA) at 37°C in an environment containing 5% CO_2.

LED irradiation. NICHIA Corporation (Tokushima, Japan) made three types of LEDs. Each LED emitted a specific wavelength; 465 nm blue light (NCSB119), 525 nm green light (NCSG119), and 635 nm red light (NCSR119) were used. A photo-radiometer (MCPD-370A Otsuka Electronics, Japan) was used to measure the light intensity.

All cells (5×10^4 cells /well) were seeded onto 35 mm plates (BD Biosciences) with liquid medium. An LED irradiation device (Department of Electrical and Electronic Engineering Faculty of Engineering, The Univ. of Tokushima) was used as a platform for the light source (Figure 1A). We used the continuous mode and a distance of 15 cm from light source to cells during each irradiation; the manufactured energy densities were set at 15 mW or 30 mW. Cells were exposed to 465 nm, 525 nm, or 635 nm wavelength light for 10 m every 24 h for 5 days in a dark room (Figure 1B). Control cells were treated in the same manner except for light exposure. None of the treatments increased the temperature of the culture medium.

Cell proliferation assay. Cell Counting Kit-8 (CCK-8) assay (DOJINDO, Japan) was used to measure cell proliferation. At the end of an experimental or control treatment period, 200 μl CCK-8 were added to each plate. After 2 h of incubation in CCK-8, the optical density (OD) was measured biochromatically, with an enzyme calibrator, at 450 nm and 630 nm.

Apoptosis detection by flow cytometry. Irradiated and unirradiated cells were labeled with both Annexin V-conjugated fluorescein isothiocyanate (FITC) and propidium iodide (PI) (eBioscience, Santiago, California, USA). Unexposed cells without Annexin V or PI were used as negative controls for the assay. Flow cytometry was used to measure the proportion of apoptotic cells in each culture; (Becton Dickinson Facscalibur, San Jose, California, USA). The quantitative analysis was performed with WinMDI 2.8 software (The Scripps Institute, San Diego, CA, USA).

Cell-cycle distribution analysis by flow cytometry. After a 5-day course of LED irradiation or control treatment, cells were harvested by brief trypsinization and centrifugation. Cell pellets were washed twice with ice-cold PBS, and 0.5×10^6 cells were suspended in 500 ml of saponin/propidium iodide solution (0.3% (w/v) saponin, 25 lg/ml propidium iodide, 0.1 mmol/L EDTA, and 10 lg/ml RNase A in PBS) and incubated at 4°C for 2 h in the dark. These cells were analyzed by flow cytometry (13).

RT-PCR analysis. The RNeasy Mini Kit (Qiagen, Valencia, CA) was used according to the manufacturer's instructions to prepare each total RNA sample. The RNA was reverse transcribed with high capacity cDNA reverse transcription kit (Applied Biosystems, Tokyo, Japan). An Applied Biosystems 7500 real-time PCR system, TaqMan gene expression assays on demand, and TaqMan universal master mix (Applied Biosystems) were used to perform quantitative real-time RT-PCR. The following TaqMan assays (assay identification number) were used: caspase3; (Hs00263337_m1), caspase8; (Hs00236278_m1), caspase9; (Hs00964603_m1), TNFα (tumor necrosis factor α); (Hs00608187_m1), Fas; Hs00236330_m1, ERK1/2 (extracellular signal-regulated kinases); (Hs00946872_m1), p38; (Hs00176247_m1), JNK (c-Jun N-terminal kinases); (Hs00177083_m1), LC3; (Hs01076567_g1), and Beclin1; (Hs01061917_g1). GAPDH (4326317E) was used as internal control for mRNA expression (Applied Biosystems). The thermal cycler conditions were as follows: 2 min at 50°C, 10 min at 95°C, then 40 cycles of 15 s at 95°C and 1 min at 60°C. Amplification data were analyzed with an Applied Biosystems Prism 7500 Sequence Detection System ver. 1.3.1 (Applied Biosystems).

Statistical analysis. Data were expressed as means±SD. The JMP 8.0.1 software (SAS Institute Inc, Cary, NC, USA) and the Mann-Whitney *U*-test were used for all statistical comparisons between irradiated and unirradiated cells. Differences were considered statistically significant at *p*<0.05.

Results

Effects of LED irradiation on cell proliferation. LEDs emitting 465 nm (blue), 525 nm (green), or 635 nm (red) light were used to assess the effects of LED irradiation on proliferation of CSC-2FO, HT29, and HCT116 cells. Cells were irradiated once a day for 10 min, for 5 days; CCK-8
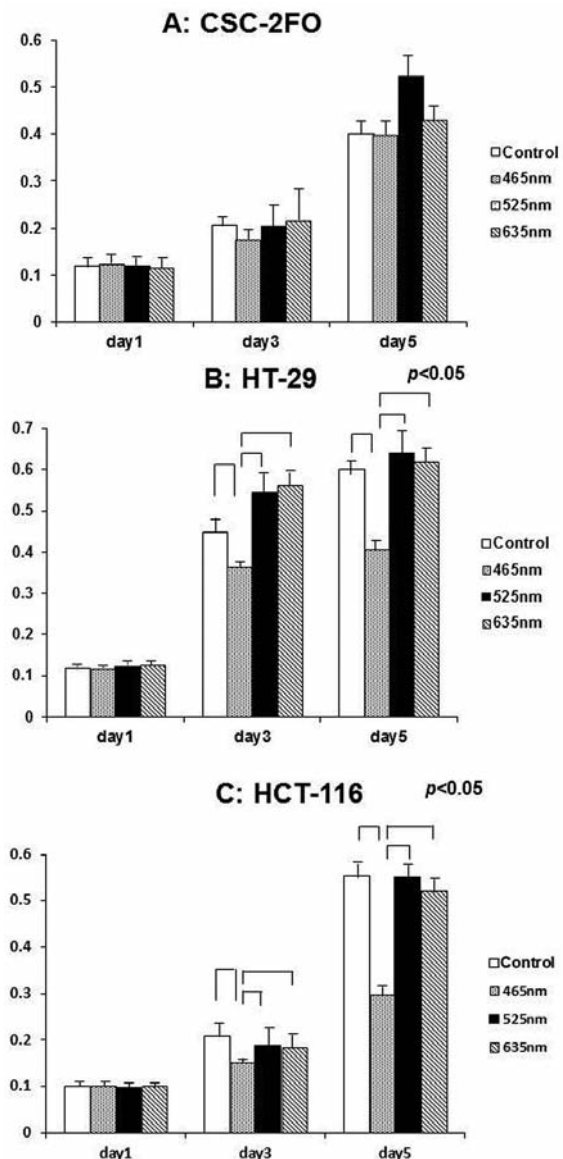
Figure 2. *Results of cell proliferation assays with 2FO-C75, HT29, or HCT116 cells on day 1, 3, or 5. Cells were irradiated with a 465 nm-, 525 nm-, or 635 nm LED or were unirradiated controls (n=10 wells, p<0.05 vs. 465 nm-LED group).*

assays were performed on days 1, 3 and 5 to measure cell number. The time-, energy- and wavelength-dependence of growth on LED irradiation were determined. For each cell type, LED irradiation did not cause any difference in cell proliferation by day 1 (Figure 2).

By day 3, LED irradiation at 465 nm tended to cause decreased proliferation of CSC-2FO cells (*p*=0.1), but there was no difference at day 5. Furthermore, 525 nm and 635 nm LED irradiation no affected CSC-2FO proliferation (Figure 2A).
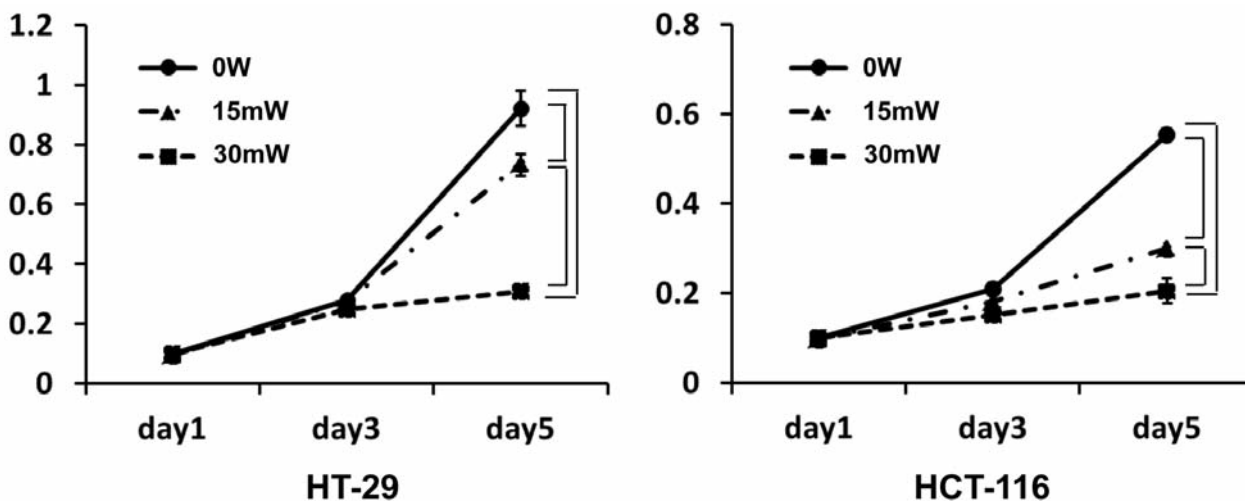
Figure 3. *Results of HT29 and HCT116 cells proliferation assay. Irradiation with a 465 nm-LED inhibits cell growth in a time- and energy-dependent manner.*

For both cancer cell lines (HT29 andHCT116), the 465-nm irradiation group exhibited a significant, time-dependent decrease in cell viability ($p<0.05$). When LED was irradiated at 465 nm and 30 mW for 5 days, HT29 and HCT116 exhibited only 24.8% or 37.1%, respectively, of the viability of control cells. But viability of HT29 and of HCT116 cells exposed to green or red was not significantly different from proliferation of control cells at any time point (Figure 2B and C). Moreover, decreasing light dose from 30 mW to 15 mW, the inhibition effect was reduced to both HT29 and HCT116 cells (Figure 3).

Blue light irradiation induces apoptosis in HT29 cells. To assess whether 465 nm LED irradiation led to increased apoptosis in HT29 cultures, annexin V flow cytometric experiments were performed. HT29 cells were stained with PI and fluorescein-conjugated annexin V; annexin V binds to phosphatidyl serine residues, and apoptosis-induced changes in phosphatidyl serine localization within the cell membrane can be detected. Co-staining with annexin V and PI permits differentiation of three classes of cells: viable cells (annexin V-negative, PI-negative), early apoptotic cells (annexin V-positive, PI-negative), and late apoptotic cells (annexin V-positive, PI-positive). The percentages of both early (bottom right quadrant) and late (top right quadrant) apoptotic cells were higher in the 465 nm-LED irradiated cells than in the control cells. The percentages of early and late apoptotic cells following 465 nm LED irradiation were 16.9% and 33.8%, respectively, but those in the control groups were 0.01% and 0.07%, respectively (Figure 4).

Blue light irradiation induces cell cycle arrest. The cell-cycle distribution within HT29 cultures experiencing LED-induced apoptosis was examined; specifically, the DNA content was measured by PI staining and subsequent flow cytometry; cell fractions were then identified as in a cell-cycle phase (G_0/G_1, G_2/M, or S DNA content) or undergoing apoptosis because of a sub-G_1 DNA content. The sub-G_1 fraction was the first peak and G_0/G_1 phase was the second peak, G_2/M phase was the smallest and third peak (Figure 5). Flow cytometric analysis revealed that the unirradiated cultures had a typical distribution of G_0/G_1, G_2/M, and S fractions. However, the cultures exposed to 465 nm-LED irradiation had reduced G_0/G_1 and G_2/M fractions relative to controls; specifically, the density of G_0/G_1 fraction was 16.4% of the whole sample for irradiated cells, but 35.4% for unirradiated cells (Figure 5). Conversely, the density of sub-G_1 fraction was 60.3% of the whole sample for irradiated cells and only 37.1% in the control group (Figure 5). These results indicated that 465 nmLED might induce HT29 apoptosis in HT29 cells and influence the G_0/G_1 phase of the HT29 cell cycle.

mRNA levels in HT29 cells irradiated with 465 nm LED. The effects of LED blue light irradiation on the expression of three apoptosis factors (caspase 9, 8, and 3) were analyzed with RT-PCR. The expression of caspase 8 and 3, two pro-apoptotic factors, was significantly higher in irradiated compared to unirradiated cells. However, the expression of caspase 9 was significantly lower in the irradiated cells (Figure 6A). We next examined whether the death receptor-initiated pathway was involved in LED-induced apoptosis. Expression of FAS was significantly higher in irradiated than in unirradiated cells, but expression of TNF did not differ significantly between the two groups. These data strongly indicate that the death receptor-initiated extrinsic apoptosis pathway was involved in this LED-induced apoptosis (Figure 6B).
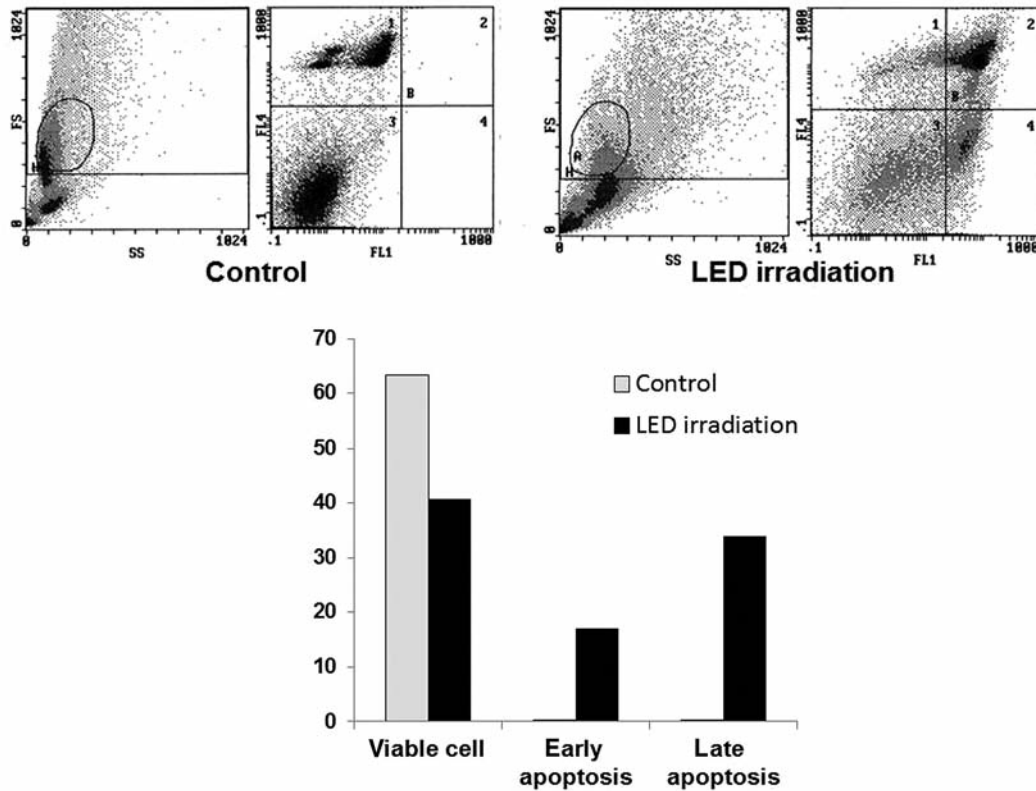
Figure 4. *HT29 apoptosis was detected via Annexin V-FITC/PI double staining and flow cytometry. Irradiation with a 465 nm-LED induced apoptosis of HT29 cells; the average percentages of apoptotic HT29 cells within irradiated and unirradiated cultures are shown.*
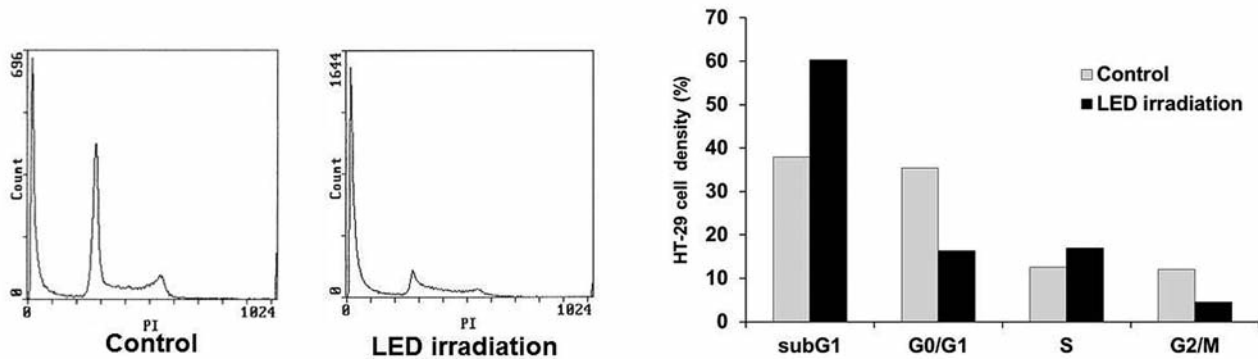

Figure 5. *Cell-cycle and apoptosis of HT29 cells were analyzed by flow cytometry after 5 days of irradiation or control treatments. Effects of irradiation with a 465 nm-LED on the distribution of cell cycle phases within HT29 cultures were assessed. The vertical axis shows the number of the cell channel, and horizontal axis shows the DNA content. G_0/G_1, G_2/M, and S indicate cell phases, and sub-G_1 DNA content indicates apoptotic cells.*
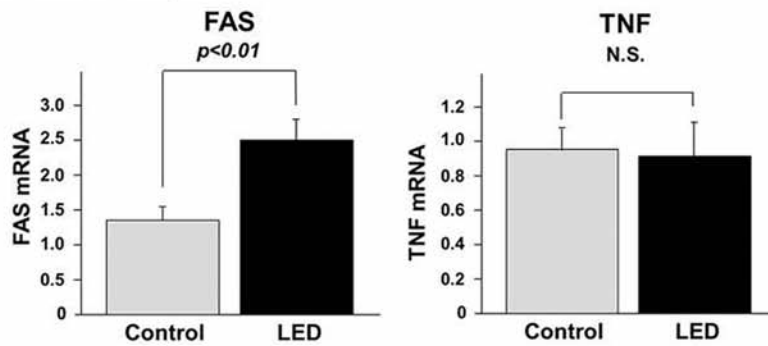
The expression of three MAPK factors (*ERK1/2, JNK,* and *p38* mRNAs) was also analyzed. *JNK* mRNA expression was significantly higher in irradiated cells than controls, but expression of *ERK1/2* and of *p38* mRNAs was significantly lower ($p<0.01$) (Figure 6C).

Expression of two autophagy factors (LC3 and Beclin 1) was also measured. *LC3* mRNA was significantly lower in irradiated cells than in control cells. Expression of Beclin 1 mRNA did not differ between the irradiation and control groups (Figure 6D).
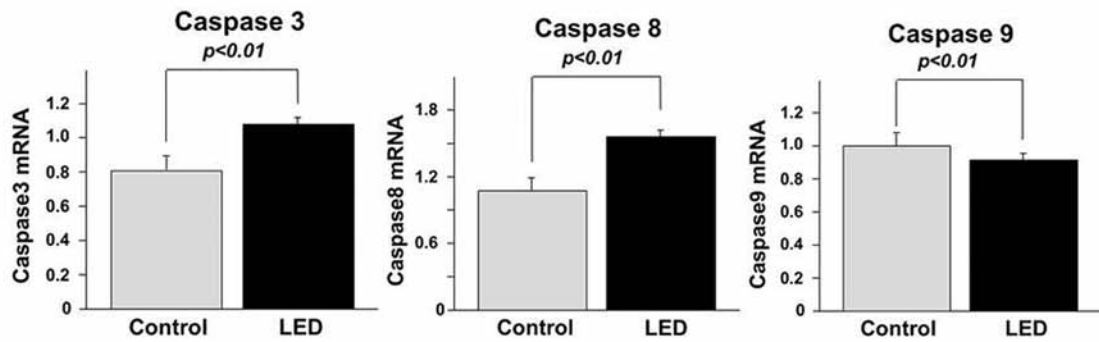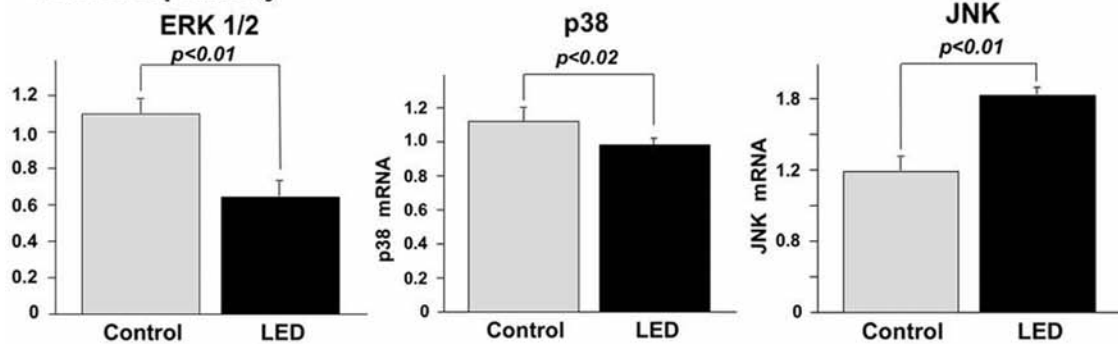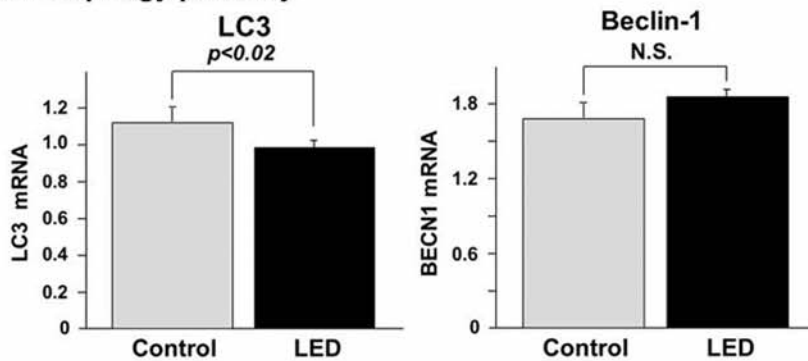
Figure 6. *Effects of irradiation with a 465 nm-LED on mRNA expression. RT-PCR was used to perform this expression analysis. A: Caspase pathway, B: death receptor, C: MAPK pathway, D: autophagy pathway.*

These patterns of expression may indicate that FAS, caspase 3, 8, and JNK were involved in the LED-induced apoptotic process and that autophagy and ERK-dependent cell proliferation were suppressed by 465 nm-LED irradiation.

Discussion

Laser-based phototherapy has been studied over the last decades, and it has many positive effects including anticancer, angiogenic and antioxidative effects (3, 14). Most research on clinical applications of phototherapy has mainly involved low-level laser studies, and the use of alternative light sources such as LEDs has received little attention. In a recent study, researchers reported that specific wavelength and energy of light from a LED source increased activity and growth of various kinds of cells. LED irradiation at 647 nm and 9.29 mW stimulates osteogenic differentiation of mesenchymal stem cells; this effect was confirmed by increased alkaline phosphatase activity, osteocalcin mRNA, and collagen type I (15). Irradiation with a 460 nm-LED is cytotoxic to anterior cruciate ligament cells, but irradiation with a 530 nm-LED or 630 nm-LED is not (16). Irradiation with a red LED at 650 nm and 4.4 mW/cm^2 promotes apoptosis of HeLa cell due to increases in intracellular calcium concentrations (13). A blue LED inhibited B16 melanoma cell growth, which was shown by the decreased number of B16 melanoma colonies, but growth and colony formation of B16 melanoma were not affected by a red or a green LED (17). These finding indicate that different wavelengths may differ in their effects on cell types, cell growth, and cell behavior. Nevertheless, the mechanisms by which LED-based phototherapy stimulates cell growth or apoptosis remain unclear. Given previous findings, we hypothesize that one specific wavelength of LED radiation would inhibit colon cancer cell proliferation, promote synthesis of pro-apoptotic factors, and induce cancer cell apoptosis; therefore, we examined the effects of three different LEDs (a red, a blue, and a green) on three cell lines. Using a 5 day irradiation of 465 nm-LED, we conducted a series of experiments on the distribution of apoptotic cells and cell cycle phases in HT29 cultures. There were fewer G$_2$/M phase cells and more S phase cells in irradiated cultures than in controls. Therefore, irradiation with a 465 nm-LED might have arrested the dividing cells at the onset of mitosis and interfered with cell-cycle progression during the S phase. These findings also indicated that HT29 cells in S phase might be more sensitive than cells at other stages of the cell cycle to irradiation with a 465 nm-LED, and that irradiation with a 465 nm-LED might be used in combination with other cell-cycle agents as a new anti-tumor treatment.

The apoptosis pathway depends on caspases and is closely connected with mitogen-activated protein kinases (MAPKs) and the autophagy pathway (18, 19). Caspase proteins exist
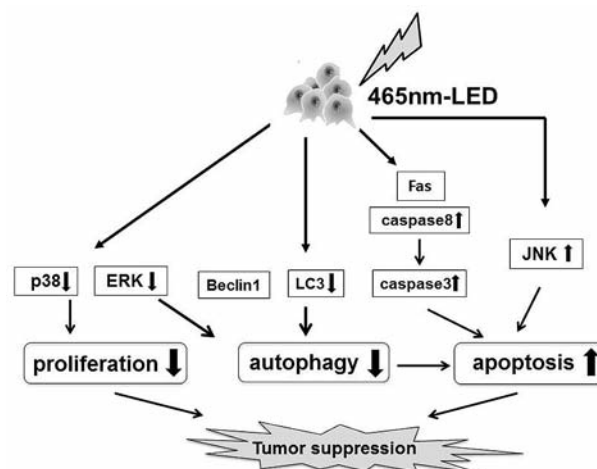

Figure 7. *Schematic representation of the major molecular mechanism by which 465 nm LED irradiation affects colon cancer cells.*

in cells as inactive pro-enzymes that can be activated by apoptosis signals (20). Thus, in our study the initiator caspases (caspase and 9) and the executioner caspase 3 in HT29 cells were measured by RT-PCR; expression of caspase 8 and 3 was higher following 465 nm-LED irradiation than in controls. Moreover, we showed that apoptosis of the irradiated HT29 cells was induced *via* activation of the extrinsic apoptosis pathway.

MAPK are proline-directed serine/threonine kinases that are activated *via* dual phosphorylation in response to extracellular stimuli. The MAPK family includes ERK, p38, and JNK. MAPKs are major signal transduction molecules involved in regulating a variety of cellular responses, including proliferation, differentiation, survival, and apoptosis. MAPKs have been shown to contribute to proliferation, migration, and invasion of many types of cancer cells (21). There is increasing evidence that the MAPK pathway has an important role in promoting cancer cell growth, survival, and invasion (22). However, MAPK also reportedly promotes apoptosis (22). We found that irradiation with a 465 nm-LED suppressed ERK1/2 and p38 in the HT29 cells and that it inhibited colon cancer cell growth and induced apoptosis *via* the JNK pathway.

Autophagy is a catabolic process in eukaryotic cells that results in the breakdown of intracellular material within lysosomes (23, 24). Under conditions of cellular stress (*e.g.*, nutrient-deprivation, DNA damage, or elevation of intracellular ROS), autophagy can be activated to provide alternative sources of energy to sustain cell survival (25, 26). The relationship between apoptosis and autophagy is rather complicated. Autophagy can either delay apoptosis or promote apoptosis (27, 28). Furthermore, activation of the

ERK1/2 signaling pathway can promote autophagy (29). The present study showed that 465 nm-LED irradiation suppresses ERK1/2 activity and autophagy in HT29 cells, while promoting apoptosis (Figure 7).

However, at present, we do not have clinical evidence of any anti-tumor effects of 465nm-LED phototherapy, and the specific photoacceptor by which 465 nm-LED induces colon cancer cell apoptosis remains unknown. In conclusion, only irradiation with the 465 nm-LED induced apoptosis of colon cancer cells; specifically, this irradiation mediated morphological changes, arrested the cell cycle, activated an extrinsic apoptosis pathway and the JNK pathway, and suppressed autophagy and the ERK pathway.

Disclosure

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