Effects of Red Light Emitting Diode on Apoptosis of HeLa Cells and Suppression of Implanted HeLa Cells Growth in Mice

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Apoptosis/Light emitting diode/HeLa cells.

Low intensity irradiation of cells by laser was an effective method of biostimulation. Here, we have extended these actions to evaluate the apoptosis effects in red light emitting diode (RLED) exposure. Through morphological observation, flow cytometric analysis, intracellular calcium measurement and RT-PCR, we found that HeLa cells in 24 h RLED irradiation in in-vitro experiments would significantly affects the induction of cellular apoptosis, and morphological changes such as the loose arrangement of cells, the noticeable development of apoptotic bodies, and the accompaniment of arrested S phase and activated caspases-3,-6,-8. Moreover, intracellular calcium concentrations markedly increased 40.3 ± 1.3% and 43.1 ± 0.8% respectively, relative to an extracellular solution containing the Ca2+ and Cu2+ free unexposed group. In in-vivo tests, RLED irradiation decreased the growth of tumors on day 50 and attenuated the elevation of vascular endothelial growth factor (VEGF) expression in HeLa cell implanted BALB/c mice. Taken together, our results suggest that RLED could induce HeLa cell apoptosis and convey potential antitumor properties.

INSTRUCTION

Tumorous development is a complex process. It occurs via the accumulation of somatic mutations of tumor-related genes that govern cell regeneration, proliferation, and apoptosis.1,2) Therefore, apoptosis may be an important pathway in mediating therapeutic antitumor strategies.3) However, with the increase of tumorous morbidity, such as acute lymphoblastic leukemia, acute myeloblastic leukemia and other tumors, current therapeutic methods still mainly depend on traditional antitumor drugs and traumatic surgeries.4–6) These methods have unknown adverse effects, such as drug-resistance and treatment-related damage.7,8) Thus, research for new antitumor therapeutic methods with no or few adverse effects will be significant.

Recently, many studies have suggested that low intensity irradiation of a specific wavelength of light could be an effective tool for wound healing, anti-inflammation and pain-killing in clinical treatments, and this irradiation has several potential advantages over traditional X-rays.9–11) Moreover, researchers presented that low intensity laser could modulate various biological processes, including synthesizing ATP, increasing mitochondrial respiration and promoting the processes of regeneration and angiogenesis.12,13) Thus, the low intensity irradiation may be an important therapeutic tool in inducing cell apoptosis. Here, we used a kind of new light irradiation named red light emitting diode (RLED), which is an improvement upon the light emitting diode (LED). Previous studies have proved that LED is similar to low intensity laser, but it is superior in light intensity, wave length, and stability, compared with other light sources.14) Thus in our study, to explore whether the low intensity light irradiation has also affected the apoptosis of tumor cells and may play an antitumor role, we have tested from in-vitro to in-vivo and investigated possible mechanisms of apoptosis.

MATERIALS AND METHODS

Cell lines

The human cervical carcinoma cell line HeLa was obtained from the American Type Culture Collection
Light sources and irradiation properties
We used a 650 nm RLED, 0.28 mW/cm² input power for in-vitro experiments and a 4.40 mW/cm² for in-vivo experiments. (Supplied by Zhengjia Li, College of Laser Engineering, president of HuaZhong University of Science & Technology, Wuhan, China).

MTT assay
The MTT assay was performed as described previously. Briefly, there were a total of 6 groups involved: 12, 24, 48, 60, and 72 h irradiated cell groups as treatment groups and one non-irradiated group as the control. All groups above were maintained in the cell incubator for 3 days after they were planted into the culture vessels with the same cell density. The MTT reagent (Sigma, St Louis, Missouri, USA) was added to 6 groups after finishing RLED irradiation. Absorbance of each group was measured with a microplate reader (Bio-Rad550, USA; A630 used for adjustment). The percentage of absorbance was used to assess cell inhibition rate.

Morphological observation
Giemsa staining (Sigma, St Louis, Missouri, USA) and in situ end labeling technique of TUNEL (Roche Boehringer Mannheim, Mannheim, Germany) were used after fixation. The stained cells were viewed, and digital photomicrographs obtained, using an inverted microscope (Olympus IX51, Tokyo, Japan) and an optical microscope (Olympus BX51, Tokyo, Japan).

Flow cytometric analysis and Annexin V/PI staining
Irradiated and unexposed cells were labeled with both Annexin V- conjugated fluorescein isothiocyanate (FITC) and propidium iodide (PI) ( eBioscience, Santiago, California, USA ). Unexposed cells without Annexin V and PI were used as controls. The irradiated groups were divided into 12 h, 24 h, 48 h and 72 h irradiation groups. The rates of apoptosis were determined by flow cytometry (Becton Dickinson Facscalibur, San Jose, California, USA), provided by Central Laboratory of Tongji Hospital, Tongji Medical College of Huazhong Science and Technology University, Wuhan, China) and the analysis was performed in the WinMDI28 software (USA). These experiments were repeated thrice. Unexposed HeLa cells and those exposed to irradiation for 24 h were harvested for extraction of low molecular weight DNA to determine the distribution of the cells among different phases of the cell cycle. The cells were irradiated when they were subcultured into the culture vessels, harvested when they were digested with 20% trypsin, and then used with the DNA Fragmentation Assay Kit (Biovision). 24 h later, the same number of cells (20,000) was counted for each treatment group. The analysis was performed with the Multicycle software (Phoenix Flow System, San Diego, CA, USA). These experiments were repeated thrice.

Expression of caspase-3, 6, 8 mRNA in RT-PCR
The RT-PCR assay was performed as described previously. Irradiated HeLa cells were collected after 0, 12, and 24 h of irradiation for analysis using the reverse transcriptase polymerase chain reaction (RT-PCR). The following primers were used: Caspase 3 (F 5'-ATTATCTTGACTGGGTGTTTA-3', R 5'-TTTGGGTTTCCAGTTAGAC-3'); Caspase 6 (F 5'-AGAGTTCTCTTTGGCActTA-3', R 5'-CCATGGA CGCGGTTCCGTTCACAGTTT-3'); Caspase 8 (F 5'-GCAGGGGC TTTGACCACGCAC-3', R 5'-AGACCGAGACCGCGGTP CCG-3'); GAPDH (F 5'-ACGGATTTGCTGTTATTTG-3'), with GAPOH (R 5'-CGCTCTGGAGATGTTGAT-3') as an internal control. The reaction conditions were 30 cycles at 94°C for 45 s, then subsequent cycles of 50°C, 52°C, 57°C, and 60°C for 45 s respectively, followed by 72°C for 45 s. The analysis was performed with the PCR auto-detection system and its data analysis software.

Fluorescence measurement of intracellular free calcium concentration in HeLa cells
Unexposed HeLa cells and those irradiated were loaded with 5 μM fura-2 (Sigma, St Louis, Missouri, USA) in Na–Hepes buffer containing (in mM): 140 NaCl, 4.7 KCl, 1 CaCl₂, 2 MgCl₂, 10 Hepes, 10 glucose (pH adjusted to 7.4) in 5% CO₂ at 37°C for 30 min, then washed twice to remove any remaining fura-2. When Ca²⁺ free conditions were applied, the Na–Hepes buffer contained no added Ca²⁺ and was supplemented with 0.5 mM EGTA. Fura-2 was excited by a xenon light source at 340 and 380 nm. Meanwhile, BAPTA/AM (the intracellular Ca²⁺ chelator, Sigma,USA) containing Ca²⁺ free extracellular solution was used. The [Ca²⁺] was determined by fluorescence measurement (Spectrofluorometer 850, HITACHI). The cells were excited with 340 and 380 nm wavelengths through 10 nm bandwidth and an emissive wavelength of 500 nm. [Ca²⁺] was calculated according to the following formula: [Ca²⁺] = [Ca] × (R – Rmin)/Rmax – R. Kd is 224 nmol/L, R was F340/380 and Rmax was the maximum value when 1% Triton X-100 was added while Rmin was the minimum value when 50 mmol/L EGTA was added.

Observation growth of tumors and immunohistochemical expression of VEGF in HeLa implanted BALB/c mice
The method of HeLa cell implantation mentioned in the
report by Faisst S et al. was used. BALB/c mice (6–8 weeks old, 20 ± 1.1 g), were obtained from the Experimental Animals Center of Tongji Medical College, Huazhong University of Science and Technology. The experimental protocols were approved by the Committee of Animal Care of Huazhong University of Science and Technology. HeLa cells (0.2 ml, 1 × 106 cells) were injected into the root of the right lower limb subcutaneously on day 0, and the mice were randomly divided into a control group and an irradiation group. On day 14, all HeLa implanted BALB/c mice were established successfully, and the irradiation group began to be irradiated daily for 2 h, as in Zaichkina’s study. The long and the minor axis of tumors in BALB/c mice were measured to calculate tumor volume. The process of RLED irradiation lasted for 54 days. The immunohistochemistry test was described in our previous study. On day 56, the mice were deeply anaesthetized with chloral hydrate and then perfused transcardially with saline followed by a phosphate buffer of 4% paraformaldehyde. Then paraffin-embedded coronal and serial sections (4 μm thick) were taken from each tumor. The expression of VEGF in the implanted tumors was measured by immunohistochemistry using a tissue STREPT-AVIDIN-BIOTIN-COMPLEX (SABC) kit (Boshide Company, Wuhan, China). Tissue fields with relatively complete cells were chosen for determining VEGF content. The presence of buffy granulosem cells was regarded as positive expression.

Statistical analysis
For statistical analysis, we used SAS 8.0 (SAS Institute, Cary, NC, USA) for a one-way analysis of variance. We used Student’s t test to test the mean difference between paired observations and the q test to determine interclass differences. A 95% confidence limit was taken to be significant, defined as $P < 0.05$.

RESULTS

MTT test and morphological Observation
To detect the inhibition rate of HeLa cells in RLED irradiation, an MTT assay was used. After 24 h of RLED irradiation, there was a statistically significant reduction in the proliferation of HeLa cells. The maximum inhibition rates were 61.9 ± 4.5% ($P < 0.05$) compared with the control group in 72 h constant irradiation, and the reduction was time dependent in Fig. 1A. Meanwhile, we found that the growth of HeLa cells in the 24 h irradiated group was initially depressed and its inhibition rates were 21.2 ± 4.1%. Through the Giemsa staining of morphology, we revealed that unirradiated HeLa cells after 24 h incubation grow in high density and were polygonal in shape. They had abundant cytoplasm, large nuclei and clear nucleoli, and adhered closely to the culture surface. However, 24 h irradiated cells had a much lower density and were arranged loosely, appeared rough, and concentrated around the edges of the culture surface, with noticeable apoptotic bodies developing (Fig. 1B). In addition, TUNEL labeling of these cells revealed that the cells after 24 h irradiation underwent induced apoptosis. (Fig. 1C).

HeLa cells apoptosis induced by RLED irradiation in flow cytometric analysis
To confirm the apoptosis rate of HeLa cells by RLED irradiation, annexin V flow cytometric experiments were performed. Apoptosis was detected in the cultured cells by staining them with PI and fluorescein-conjugated AnnexinV, which binds to phosphatidyserine residues, and the change in location of phosphatidyl-serine in the cell membrane during apoptosis can be detected with annexin V. Co-staining with annexin V and PI permits differentiation of viable cells (annexin V-negative, PI-negative) from early apoptotic cells (annexin V-positive, PI-negative) and late apoptotic cells (annexin V-positive, PI-positive). As shown in Fig. 2A, HeLa cells underwent induced apoptosis at 24 h, 48 h, and 72 h irradiation periods and the percentages of both early (bottom right quadrant) and late (top right quadrant) apoptotic cells increased time-dependently. In Fig. 2B, the histogram parallels the result in Fig. 2A. We found that in Fig. 2B the early apoptotic rates determined by flow cytometry after 24 h, 48 h, and 72 h of RLED irradiation were 14.9 ± 2.5%, 47.6 ± 2.4%, and 26.0 ± 7.9%, respectively. On the contrary, in the unexposed control groups, the early apoptotic rates were 4.7 ± 1.1% and the differences between the control and the 24 h, 48 h, and 72 h irradiation groups were significant ($P < 0.05$). Otherwise, early apoptotic rates in 72 h irradiation groups seemed to be less than in 48 h irradiation, because in 72 h irradiation groups, the majority of HeLa cells were in the late apoptotic/necrotic stage in Fig. 2B.

Cell cycle arrest induced by RLED
In order to find the cell-cycle distribution of RLED inducing apoptosis, we treated HeLa cells in the presence or absence of 24 h RLED irradiation. G0/G1, G2/M and S indicated the cell phase, and sub-G1 DNA content referred to the rate of apoptotic cells. Sub-G1 phase was the first blue peak and G0/G1 phase was the high red peak in Fig. 3A. G2/M phase was the short red peak, but in the right-hand figure of Fig. 3A the red peak was hidden and could not be seen because its value was only 1.4%. S phase was the inclined stripe in Fig. 3A. Flow cytometric analysis revealed that the unirradiated cells showed a typical distribution in G0/G1, G2/M and S phases, as shown in the left-hand figure of Fig. 3A. However, the population of cells cultivat ed with 24 h RLED irradiation in G0/M phase was decreased in the right-hand figure of Fig. 3A, and density in G2/M phase was 1.4 ± 1.0%, compared with 21.8 ± 2.3% in the control group in Fig. 3B ($P < 0.05$). Meanwhile, an increase in cell density was seen
Fig. 1. Inhibition rate and morphological changes were induced by RLED in HeLa cells. Fig. 1A RLED decreased the growth rate of HeLa cells at different irradiation time. Each column and bar represented Mean ± SE. *P < 0.05 (n = 24): compared with control group. Fig. 1B Morphological changes of HeLa cells in Giemsa staining were mediated by 24 h irradiation by RLED under a Light Microscope (× 400). Fig. 1C HeLa cell apoptosis induced by 24 h irradiation was detected by the TUNEL assay under a Light Microscope (× 400).

Fig. 2. HeLa cells apoptosis was detected by flow cytometry with Annexin V-FITC/PI double staining. Fig. 2A Representatives of HeLa cells apoptosis induced by RLED at 24 h, 48 h, 72 h irradiation time. Fig. 2B Percentage of HeLa cells apoptosis were shown by RLED at 24 h, 48 h, 72 h irradiation time. Each column and bar represented Mean ± SE. *P < 0.05: compared with control groups.

J. Radiat. Res., Vol. 50, No. 2 (2009); http://jrr.jstage.jst.go.jp
in S phase, accompanied by a marked increase of a sub-G1 population after 24 h RLED irradiation. Furthermore, the density of S phase was 50.5 ± 6.7% in the 24 h irradiated group relative to 10.9 ± 6.9% in unirradiated groups, and sub-G1 population was 18.7 ± 1.6% in the irradiated group, compared to 5.6 ± 1.3% in the unirradiated group in Fig. 3B (P < 0.05). These results indicated that RLED might induce HeLa cell apoptosis and influence G2/M and S phase in the cell-cycle.

Caspase 3, 6, 8 mRNA levels in RLED irradiation HeLa cells

To elucidate the molecular mechanism responsible for RLED inducing HeLa cell apoptosis, we measured caspase 3, 6, and 8 mRNA in the HeLa cells by semi-quantitative RT-PCR. We found that caspase 3, 6, and 8 mRNA expression increased and their bands brightened compared to the control group after 12 h and 24 h RLED irradiation. Particularly, caspase 6 and 8 mRNA expression was markedly up-regulated during 24 h RLED irradiation. What’s more, caspase 6 and 8 mRNA expression in the 24 h irradiation group was much brighter than in the 12 h irradiation group. This may suggest that caspase 3, 6, and 8 were involved in the apoptotic process induced by RLED and their increased expression was time-dependent.

RLED irradiation increased the intracellular calcium concentration

To detect the effects of RLED on intracellular calcium, cells were exposed to different irradiation periods by RLED and then measured for intracellular calcium concentration. As shown in Fig. 5, compared with the extracellular solution containing Ca2+ and the Ca2+ free control group, intracellular calcium concentration markedly increased 40.3 ± 1.3% and 43.1 ± 0.8% at 24 h irradiation by RLED (P < 0.05, n = 24), and intracellular calcium concentrations were 119.0 ± 17.0 nmol/L and 109.8 ± 15.6 nmol/L respectively. In contrast, in the presence of 10 μM BAPTA/AM (the intracellular Ca2+...
Ca\textsuperscript{2+} free extracellular solution, intracellular calcium concentration significantly decreased, compared with the control group, \( (P < 0.05, n = 24) \) and was 29.1 ± 9.8 nmol/L. This suggested that intracellular calcium concentration increased through the release of Ca\textsuperscript{2+} from the endoplasmic reticulum.

**Decreased growth of tumors and expression of VEGF in HeLa implanted BALB/c mice**

As shown in Fig. 6A, we found HeLa-implanted tumor growth after RLED irradiation was attenuated in a time-dependent relation. The HeLa-implanted tumor growth was inhibited 37.4 ± 3.3% relative to control groups \( (P < 0.05, n = 10) \) on day 50. To investigate the action pathway of decreasing tumor growth after RLED irradiation, we used an immunohistochemistry assay to find that VEGF was expressed primarily in the cytoplasm of cells in the HeLa-derived tumors, as dark brown granules, and also in the tumor vessel walls, which were of high-density in the tumors. In contrast, VEGF expression was much lower in irradiated tumors and was not detected in low-density tumor vessel walls (Fig. 6B). These results implied that RLED irradiation might reduce both the expression of VEGF and the growth of new vessels in HeLa-implanted solid tumors.

**DISCUSSION**

In the present study, we found that RLED did significantly induce apoptosis of HeLa cells through mediating morphological changes, arresting S phase, increasing intracellular calcium and activating caspases-3,-6,-8 in *in-vitro* experiments. Meanwhile, in *in-vivo* tests, RLED may decrease the growth of tumors and VEGF expression in HeLa cell-implanted BALB/c mice.

Apoptosis is a complex mechanism of cell death that includes a network of metabolic events activated by a variety of biological and physical stimuli. The death process is characterized by selective proteolysis of cytoplasmic and nuclear substrates that disable homeostatic and repair processes and mediate morphological changes and structural disassembly. Thus, the morphologic methods were used in
this study and we demonstrated that HeLa cells were arranged loosely and at low density, accompanied with noticeable apoptotic bodies after 24 h of exposure to RLED. In addition, we also detected DNA damage by the TUNEL method, which quantifies DNA strand breaks, and found that TUNEL-positive cells were shown in the 24 h-irradiated group. From these results, we initially infer that RLED irradiation might induce apoptosis of HeLa cells. To further investigate whether RLED would induce apoptosis in HeLa cells, flow cytometry was used. We found a significant difference in early apoptotic rates between cells irradiated for 24 h and unexposed cells. Although early apoptotic rates of cells irradiated for 48 h and 72 h markedly increased, compared with the control group, the total and late apoptotic rates were not yet notably increased. Due to the elevation of the late apoptotic rates, which couldn’t be completely differentiated from necrosis, 48 h and 72 h irradiation groups were not suitable to be reliably used as an apoptosis-induced index. What’s more, long-time irradiation may affect growth of normal cells around irradiated regions and induce latent side effects. So we presumed that a 24 h irradiation period of the HeLa cells would be adequate and optimal for inducing apoptosis.

Based on a 24 h irradiation of low intensity RLED, we conducted a series of experiments on apoptosis distribution of the HeLa cell cycle. We observed that there was a significant decrease of G2/M phase cells with a significant increase of S phase cells. It was possible that RLED could arrest the dividing cells at the onset of mitosis and interfere with S phase cell cycle progression. This also suggested HeLa cells in S phase might be more sensitive to RLED and RLED might be a new method to be combined with other cell cycle agents in antitumor treatment. Furthermore, these findings are consistent with previous studies. So apoptosis of HeLa cells induced by RLED had been further supported.

It is well known that the apoptosis pathway is closely connected with caspases and intracellular calcium. The caspase family exists in cells as inactive proenzymes and can be activated by apoptosis signals. According to the classic apoptosis theory, increased expression of caspases suggests a caspase dependent pathway is activated. Thus, in our study the initiator caspase-8, and the executioner caspase-3,-6 in HeLa cells were measured by RT-PCR, and these caspases all expressed at higher levels after RLED irradiation compared to the controls. Based on the apoptotic phenomenon detected by morphological observation and flow cytometric analysis mentioned above, here, it was further shown that the cells were prompted into apoptotic procedure through the activation of the caspase family. Additionally, in another classic apoptotic pathway, Ca2+ has played an important role and the involvement of Ca2+ signaling in apoptosis has been implicated in a number of recent studies. In a previous study, the characteristics of the Ca2+ signals associated with apoptosis were partly demonstrated and some studies also showed that Ca2+ signaling was truly involved in apoptosis. Thus, according to the Ca2+ signaling role in apoptosis, the changes in intracellular Ca2+ concentration were also observed. The main source of intracellular calcium depended on the activation of voltage-gated channels and the release of calcium from the endoplasmic reticulum (ER). We discovered that intracellular Ca2+ concentration was increased both in calcium-containing and calcium-free extracellular solution during RLED irradiation. Here, we presume that the elevation of intracellular calcium might be caused by intracellular reasons. While HeLa cells were incubated in 10 μM BAPTA/AM (the intracellular Ca2+ chelator) containing calcium-free extracellular solution, the elevation of intracellular calcium level was suppressed. These results implied that elevation of cytosolic Ca2+ induced by RLED irradiation would be caused by the release of Ca2+ from the ER to the cytosol. Furthermore, the biological information transformation model of low intensity laser (BIML) states that calcium release from intracellular stores was aroused by warm color exposure, such as red, orange, or yellow, to activate G-protein coupled receptors and receptor-related proteins with enzyme activity. Otherwise, Höger U found that the changes in intracellular calcium concentration could be induced by LED at different wavelengths, and it might be related to the rise of spider mechanoreceptor neurons during mechanical stimulation. These results suggested that the light receptor and mechanoreceptor might be activated while HeLa cells were irradiated by RLED so that they might contribute to the release of calcium from the ER by the calcium-inducing-calcium releasing manner, which could sensitize the cell to apoptosis. As a result, HeLa cell apoptosis induced by RLED may be related to increasing intracellular calcium and activating caspases-3,-6,-8.

In the in-vivo experiment, HeLa cells were implanted into the BALB/c mice and we directly found that the growth of HeLa cells had been depressed after irradiation of RLED. Meanwhile, expression of VEGF in the irradiated HeLa derived tumors decreased. As an important angiogenesis factor, vascular endothelial growth factor (VEGF) may promote tumor angiogenesis directly, and could participate in the migration of vascular endothelial cells, as well as tumor metastasis. Decreased VEGF expression suggests that tumor angiogenesis and the metastasis by vessels in the whole organism could be partly inhibited by RLED. Therefore, decreased VEGF expression might be contributed to relieve the growth of tumors after RLED irradiation.

As a cold light source, with little or no heat production, RLED is a promising technology for medical applications. However, at present, we do not have clinical evidence of antitumor effects after treatment of RLED, and the specific molecular pathways by which RLED induces HeLa cell apoptosis are still unknown, although we have proven that several possibilities exist. Further studies are being conducted in our laboratory.
ACKNOWLEDGEMENT

This work was supported by grants from the Huazhong University of Science and Technology Technology-Medicine Joint Foundation. We thank Miss. Chenchen Shen for carefully reading the manuscript.

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Received on January 10, 2008
1st Revision received on June 16, 2008
2nd Revision received on July 18, 2008
Accepted on July 29, 2008

J-STAGE Advance Publication Date: January 22, 2009