NITROGEN & He-Ne LASER EXPOSURE INCREASES APOPTOTIC DEATH RATE FOR MONOCYTES FROM HUMAN BLOOD

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Apoptosis is a physiological process of cell death. Activation of macrophages by lipopolysaccharides delays macrophage apoptosis. However, the extended life of macrophages further stimulates inflammation and results in local tissue damage. Exposure to low level lasers is also known to activate mononuclear phagocytes. Therefore, we determined if monocytes exposed to low level lasers (He-Ne & Nitrogen lasers) exhibit delayed apoptosis. Monocytes from 5 healthy volunteers were isolated from peripheral blood and cultured in microwells in-vitro. Replicate wells containing macrophages were exposed to He-Ne and Nitrogen lasers thrice on alternate days and the percentage of apoptotic cells were counted after staining with acridine orange stain. Apoptosis was observed in 0.52 to 12 % of the non-irradiated control cells, compared with 22 to 99 % of cells exposed to Nitrogen laser. He-Ne laser exposed cells showed a greater percentage of apoptotic cells than controls, but less so than Nitrogen laser exposed cells. The present observations revealed increased apoptotic death rates for monocytes exposed to He-Ne and Nitrogen lasers, suggesting that both lasers may reduce inflammation in diseases such as tuberculosis.

Key Words: Apoptosis, Monocytes, Laser Therapy, Inflammation.

Introduction

In 1972, Kerr, Currie and Wyllie⁽¹⁾ proposed the term apoptosis to describe a common series of morphological changes that accompanied the death of cells from a wide variety of tissue sources. Apoptosis is a physiological process of selective cell deletion that occurs during embryogenesis, metamorphosis, tissue atrophy and tumor regression^(2,3). As cells undergo apoptosis, the surface membrane 'boils' and pinches off to produce blebs, referred to as 'apoptotic bodies' which are usually phagocytized by other cells⁽⁴⁾.

Low level lasers like Nitrogen laser and He-Ne laser are being used as an adjunct to chemotherapy for the treatment of tuberculosis at our medical center. Monocyte-macrophage system forms the

key system in the pathology of tuberculosis, and is also considered important in immunity to the disease.

Earlier we showed⁽⁵⁾ that He-Ne laser exposure leads to increased cell size, DNA, RNA content, increased proliferation and increased cytokine release by mononuclear-phagocytic cells. These changes were suggestive of cell activation. It was also documented⁽⁶⁾ that these monocytes activated by exposure to He-Ne laser accentuate the killing of phagocytized mycobacteria. Macrophages activated by agents such as by lipopolysaccharides (LPS) show delayed apoptosis. Hence, we determined whether monocytes exposed to low level lasers show delayed apoptosis as well.

Methods

Blood Sample Collection

Ten mL. of heparinized blood was collected in sterile siliconized tubes from five healthy volunteers (Age 21 to 30 yrs.). Mononuclear cells separation, monocytes separation, viability testing and cultivation of cells were carried out as described previously⁽⁵⁾. Briefly, mononuclear cells were isolated from the heparinized blood by Ficoll - Hypaque gradient centrifugation. The mononuclear cells were washed three times with RPMI-1640 medium (Sigma, USA) without fetal bovine serum (FBS). The cells were suspended in 4 ml RPMI medium supplemented with 50% FBS (Hi-Media, India) and 30µg/ml Netilemycin (Fulford, India). Two hundred µL of the suspension was added to each of the microwells in the tissue culture plates (Nunc, Denmark). The plates were incubated overnight at 37°C in CO₂ incubator (NUAIRE, INC, USA) under 5% CO₂ and 95% humidity for adherence of macrophages.

The wells were washed three times with plain warm RPMI medium to remove non-adherent lymphocytes. The adherent monocytes were removed by briskly rinsing the wells with cold RPMI medium and the density was adjusted to approximately 10⁵-10⁶ / ml. The separated monocytes suspended in 200µl of RPMI supplemented with 50% FBS were transferred to new microwells. The plate was then incubated in a CO₂ incubator

Laser Exposure

He-Ne laser ('LGN' Model No. III, Russia) with 632.8nm wavelength and power output of 3mW and Nitrogen laser 337nm wavelength and power output 5 mW (fabricated by the Center for Advanced Technology, Indore, India) were used to stimulate the monocytes in the wells. The time exposure used for each well was 10 mins (duplicate wells in each set) and 5 min. for triplicate wells in each set. Three exposures were given on

alternate days starting on the third day of culture. Six to eight wells in each set were not irradiated. They served as controls.

Morphological Assessment of Apoptosis by Acridine Orange and Ethidium Bromide (AO + EB) Staining

Morphological study was done on the seventeenth day on individual wells after mixing the cells thoroughly with pipettes. The procedure for enumerating apoptotic cells has been described⁽⁷⁾. Briefly, 100 µg each of acridine orange and ethidium bromide (Sigma, USA.) were dissolved in phosphate buffered saline (PBS) and 20 µl of the cell suspensions were mixed with 2 µl of the dye on a microscopic glass slide. The coverglass preparations were examined under a fluorescent microscope and a minimum of 200 cells scored from each preparation. Cells were scored as live non-apoptotic (apple green nuclei), live apoptotic (apple green but grossly condensed nuclei or with multiple apoptotic bodies), dead (orange nuclei), or dead apoptotic (nuclei with orange apoptotic bodies).

Statistical Analysis

The percentage of apoptotic cells from He-Ne and Nitrogen lasers exposed wells were compared to the percentage of apoptotic cells from control unexposed wells by Wilcoxon's paired rank sum test using GB-Stat, USA (version-4.0) software. Inter group comparison of the percentage of apoptotic cells in wells exposed to Nitrogen laser & He-Ne laser was also carried out using the same statistical test. Similarly, comparison of apoptotic events for 10 min and 5 min exposure was carried out for sets exposed to He-Ne and Nitrogen laser.

Table 1: Apoptotic Changes in Monocytes Exposed to Nitrogen and He-Ne Lasers

No. of Sets	Exposure time (Minutes)	Nitrogen laser % Apoptotic Cells	He-Ne laser % Apoptotic Cells	Control unexposed % Apoptotic Cells
1	10 min	98.75 ± 0.35	32.5 ± 0.71	12.075 ± 2.99
	5 min	98.75 ± 0.35	20.7 ± 1.56	
2	10 min	98.05 ± 0.07	31.05 ± 0.07	9.48 ± 2.14
	5 min	98.05 ± 0.07	14.1 ± 3.48	
3	10 min	60.5 ± 0.70	11.8 ± 0.28	5.87 ± 0.53
	5 min	52.5 ± 0.70	7.05 ± 0.07	
4	10 min	37.5 ± 7.77	11.8 ± 4.27	7.5 ± 1.91
	5 min	22.2 ± 10.41	6.3 ± 0.88	
5	10 min	38.0 ± 0.07	12.0 ± 1.2	6.0 ± 0.52
	5 min	25.3 ± 0.70	6.2 ± 0.07	

p < 0.01 for Nitrogen laser exposed wells (10 & 5 min exposure)

p < 0.05 for He-Ne laser - 10 min exposure.

P < 0.05 for He-Ne laser - 5 min exposure.

P = 0.005 for Nitrogen vs He-Ne laser.

P = 0.10 for 10 min vs 5 min Nitrogen laser.

P = 0.04 for 10 min vs 5 min He-Ne laser.

Results

Data on the percentage of apoptotic and normal monocytes, before and after laser exposure, are shown in Table 1, and the apoptotic nuclear changes as evident in AO stained monocytes are depicted in the photomicrographs.

Monocytes exposed to Nitrogen laser for 10 min. showed faster apoptosis than the control unexposed cells ($p < 0.01$). Most of the cells were empty (without nuclear contents) and a few cells showed nuclear condensation or apoptotic bodies. With 5 min. exposure the majority of monocytes showed nuclear condensation or formation of apoptotic bodies and a few cells were empty. In the case of He-Ne laser exposure for 10 min., the monocytes showed a greater number of apoptotic cells than the control unexposed wells ($p < 0.05$), but the num-

ber of apoptotic cells were less when compared to nitrogen laser exposed monocytes ($p = 0.005$). Further, the number of empty cells was fewer in He-Ne laser exposed wells, as compared to the Nitrogen laser exposed wells. With 5 min. He-Ne laser exposure, there were fewer number of apoptotic cells, as compared to 10 min. exposure ($p = 0.04$).

The differences in the number of apoptotic cells from the Nitrogen laser exposed wells and unexposed wells were found to be highly significant ($p < 0.01$). The differences in the number of apoptotic cells in He-Ne laser exposed wells when compared with unexposed wells were statistically significant ($p < 0.05$ for 10 min exposure and $p = 0.05$ for 5 min exposed wells).

Photomicrograph showing multiple apoptotic bodies in macrophages after exposure to He-Ne laser. (Acridine orange stained cells under fluorescent microscope; Magnification = 400X).

Macrophages exposed to Nitrogen laser showing late apoptotic phase with hardly any nuclear contents left in the two macrophages and a smaller cell in the right corner with apoptotic bodies; Magnification = 1000 X.

Discussion

The monocyte-macrophage system plays an important role in host immunological response, antimicrobial, tumoricidal activity and wound healing⁽⁸⁾. In each of these roles, macrophages must be recruited effectively from the circulation to the site of inflammation. This recruitment may be achieved via chemotactic factors resulting from the inflammation or through complement activation following immunological reactions⁽⁹⁾. These processes also attract a large number of Polymorphonuclear (PMN) cells at the site⁽¹⁰⁾. PMN cells and mononuclear-phagocytic cells play an important role in the removal of invading microbes through phagocytosis. The PMN cells have a very short half-life. However, death due to necrosis will trigger the release of noxious cell contents outside the cell, thus giving further stimuli for local inflammation and tissue damage^(11,12). Death of phagocytic cells like PMN cells or mononuclear phagocytic cells through apoptosis does not trigger further inflammatory signals, and does not enhance further tissue damage caused by the inflammatory processes^(13,14,15).

Agents, such as LPS, are known to delay the apoptotic process in PMN cells & macrophages^(10,16). This delay may help maintain the inflammatory process. Thus, the increased life span of phagocytic cells by LPS-like agents is undesirable during inflammation. The present experimental data suggests that both He-Ne and Nitrogen laser accelerate the apoptotic process in monocytes. On the seventeenth day of culture, only 5-12 % of the non-irradiated monocytes had shown apoptotic changes, but exposure to Nitrogen laser resulted in 37-98% of cell death through apoptotic process. He-Ne laser also brought about an increase in apoptotic cells, and more so during 10 min. exposure. However, the percent of apoptotic monocytes was much less for He-Ne laser exposed cells. Further, the Nitrogen laser exposure resulted more often in an 'empty' cell appearance of the exposed monocytes. During the course of apoptosis, condensation

of the nucleus occurs first, followed by formation of apoptotic bodies which are released through the cell membrane. Emptying of the cells with regard to nuclear material occurs as a late event in the course of apoptosis. Phagocytic cells then easily remove these empty cells. It needs to be mentioned here that He-Ne laser exposure resulted in fewer empty cells (not shown in results) and the apoptotic cells were by and large with the apoptotic bodies inside. These observations correlate with faster apoptosis for Nitrogen laser exposed cells than for the He-Ne exposed monocytes. Faster apoptosis with Nitrogen laser exposure, as compared to He-Ne laser exposure, could be due to the shorter wavelength for Nitrogen laser since UV rays are also known to be classical agents which induce apoptosis⁽¹⁷⁾.

Previous studies⁽⁵⁾ suggest that laser exposure brings about an increase in cell size, nuclear size, DNA & RNA content, replication of cells and secretion of cytokines like GM-CSF & TNF- alpha. All these factors suggest an activated state for the macrophages. Moreover, the irradiated macrophages also show acceleration in phagocytosis, especially with reference to intracellular killing of Mycobacterial cells⁽⁶⁾. The increased rate of apoptosis could be viewed as a beneficial effect, since the apoptotic death would prevent a vicious cycle of inflammation.

The cytokines like GM-CSF and TNF-alpha released through the activated macrophages could foster the proliferation of macrophages and the recruitment of other macrophages at the infection site⁽⁸⁾. GM-CSF production in PMN from the inflamed area has been shown to delay the apoptotic process in human PMN cells⁽¹⁸⁾, but this does not appear to be true for macrophages as seen from the present data. Moreover, we have observed that patients with tuberculosis show a marked reduction in inflammatory reaction when treated with low-level laser therapy.

Conclusion

Our findings indicate, for the first time, that nitrogen laser and He-Ne laser accelerate the apoptotic process in monocytes. This effect may be of value in containing inflammation in diseases like tuberculosis.

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