MACROPHAGE RESPONSIVENESS TO LIGHT THERAPY WITH VARYING POWER AND ENERGY DENSITIES

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Macrophages are a source of many important mediators of wound repair. Cells of an established macrophage-cell line (U-937) were exposed *in vitro* to an 820 nm light source which was both coherent and polarized, the power densities used being either 400 mW/cm^2 or 800 mW/cm^2. The irradiation times were such that the energy densities to which the cells were exposed were 2.4 and 7.2 J/cm^2 for both probes. Twelve hours after exposure the macrophage-conditioned medium was removed and placed on 3T3 fibroblast monolayers. Fibroblast proliferation was assessed over a four-day period. By four days after the addition of medium conditioned by macrophages exposed to an energy density of 2.4 J/cm^2, there was a statistically significant difference in fibroblast number between the 400 mW/cm^2 and 800 mW/cm^2 treatments, 800 mW/cm^2 producing greater cell proliferation. However, there was no significant difference between the effects of sham irradiation and 400 mW/cm^2. In contrast, after the addition of medium conditioned by macrophages exposed to an energy density of 7.2 J/cm^2, 400 mW/cm^2 treatment produced a significantly greater increase in fibroblast number than sham irradiation. There was no significant difference in cell number between the sham irradiated and 800 mW/cm^2 irradiated samples, although there was a significant difference between the 400 mW/cm^2 and the 800 mW/cm^2, 400 mW/cm^2 producing greater cell proliferation.

KEY WORDS Macrophages (U-937) Energy density Power density Fibroblast proliferation

Introduction

Growth factors synthesized and secreted by macrophages can either inhibit or enhance the activity of many types of cells.[1-4] It has been shown that light of different wavelengths and energy densities can either encourage macrophage-like U-937 cells to release soluble factors that stimulate fibroblast production or inhibit the release of these factors.[5-7] The aim of this study was to determine the effect of varying the power and energy densities of the irradiation on these activities of macrophages. Earlier work[6] has shown that the optimum energy density required to stimulate factor release was 7.2 J/cm^2, and that an energy density of 9.6 J/cm^2 was significantly less effective, possibly due, at least in part, to damage to various light receptors in the U-937 cell at this higher energy level. Cytotoxic products could also have been formed in the cell as a result of an increase in energy absorbed. Another possibility is that the higher energy densities may have stimulated the release of fibroblast-inhibitory growth factors.

Although macrophages cannot be grown as a cell line in culture, the macrophage-like cell line U-937 can.[8] These cells have been shown to produce soluble factors which stimulate mitogenic activity in cultured fibroblasts.[9] The cell has a fast population doubling time (20–48 h), so large numbers can be acquired rapidly, and they provide an homogeneous cell population. In addition, very large volumes of conditioned medium can be obtained inexpensively and rapidly. The U-937 cells exist in an arrested yet pliant state of maturation. Although phenotypically they resemble monoblasts, histochemically they show monocyte characteristics.

The U-937 cell can be induced to mature by exposure to a number of chemicals such as TPA (tetramyristic phorbol acetate), and DMSO (dimethyl sulphoxide). Maturation of these cells is characterized usually by quantitative rather than qualitative changes.[10] The cytochemical changes in the U-937 cells show that they are a good model of the macrophage. One of the most important markers is the cell's ability to synthesize and secrete lysozyme, a property shared by monocytes and macrophages.[11] Although lysozyme is synthesized by other cell types such as neutrophils, release of the product can only be achieved by cell disintegration and not, as in monocytes and macrophages, by the merocrine secretory process.[12] Lymphoid cells do not produce

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lysozyme.[13] Lysozyme synthesis and secretion, therefore, seem to be other definitive characteristics of the monocyte–macrophage series. Cytochemical findings confirm that the U-937 cell line is closely related to macrophages. Esterase stainings are strongly positive; in particular, the naphthol AS-D acetate esterase reaction is positive with marked inhibition by sodium fluoride, a reactivity only noted in monocytes.[14] Surface marker studies have revealed the presence of both C3 and Fc receptors (detected with the rosette technique EA 7s).[15] This pattern of activity has been found in normal macrophages.[16,17]

Because the U-937 line shows many characteristics of the human macrophage, it was considered appropriate to use it as a model in investigations aimed at improving understanding of the mechanism of the effect of low level lasers on macrophage response to injury.

Materials and Methods

The phototherapeutic device used was a Biotherapy 3ML (Omega Universal Technologies, London), fitted with two interchangeable 820 nm probes.

Physical Characteristics of the Light Source

(a) Wavelength : 820 nm.
(b) Active medium : gallium aluminium arsenide (GaAlAs).
(c) Average power output : 50 mW, 100 mW.
(d) Spot size at 1 cm distance : 0.125 cm^2.
(e) Power density : 400 mW/cm^2 and 800 mW/cm^2.
(f) Source spectral band width at 100% intensity: 0.01 nm.
(g) Total angle of divergence: 23° parallel to junction plane, 16° perpendicular to junction plane.
(h) Pulsing frequency : 5000 Hz.
(i) Pulse width : 180 μs.

Dosimetry

The two 820 nm probes were calibrated with a Photodyne flat response meter (PX16E). They were found to have power densities of 400 mW/cm^2 and 800 mW/cm^2 respectively. To ensure that the energy densities were maintained constant at 2.4 and 7.2 J/cm^2, the exposure times used were as follows: 400 mW/cm^2—6 s, 800 mW/cm^2—3 s (energy density: 2.4 J/cm^2); 400 mW/cm^2—18 s, 800 mW/cm^2—9 s (energy density: 7.2 J/cm^2).

Cell Culture

U-937 Cells. The U-937 cells were grown in RPMI 1640 (Life Technologies) [LT], supplemented with 1% penicillin/streptomycin [LT] and heat-inactivated fetal calf serum [FCS] [LT]. The cells were subcultured approximately every 2 days. The cells were grown at 37°C with a 5% CO_2 atmosphere in 50 ml culture flasks, each flask containing 9 ml of medium plus cells. When confluent, each flask contained approximately 9×10^6 cells.

Fibroblasts. The fibroblasts used were Swiss 3T3 (supplied by the European Collection of Animal Cell Cultures [ECACC No 85022108] Porton Down). They were grown in Dulbecco's Minimum Essential Medium (DMEM) [LT], supplemented with 10% fetal calf serum (FCS) [LT] and 1% penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere. The cultures were confluent by three days after subculture. When confluent, each culture flask contained approximately $3–4 \times 10^6$ cells. The excess medium was poured out of each flask, and the cells were rinsed with phosphate buffered saline (PBS). Five ml of 0.25% trypsin was added to each flask to cover the layer of cells and left for 30 s. The excess trypsin was then poured off and the flasks replaced in the incubator in an inverted position so that the cell-covered surface was uppermost and any trypsin remaining in the flask was not in contact with the cells. This prevented excess damage being caused to the cells by the trypsin remaining in the flasks. After 10 min the cells detached. Three ml of DMEM was added to each flask and the resulting cell suspension was poured into a universal container. The cells were pipetted in 50 μl volumes into the wells of a microplate, each well thus containing approximately 1000 cells. The culture medium in which the cells were suspended was a 1:1 mixture of DMEM and the supernatants taken from the various U-937 treatment groups (see below); this provided suboptimal conditions in which stimulation of proliferation was possible.

Macrophage Viability Assay

Twelve hours after treatment, samples of macrophages suspended in culture medium in which they had either been irradiated or sham-irradiated, were mixed thoroughly. The suspension (0.5 ml) was then removed and transferred to a glass vial, a drop of trypan blue (0.4% w/v) being added to the suspension. A drop of the suspension was then added to a Neubauer counting chamber. In this viability test[18] all the dead cells are stained blue while viable cells, being able to exclude the dye remain colourless. The number of viable cells was calculated as a percentage of the total cell number.

U-937 Irradiation

When confluent, the U-937 cells were transferred to sterile 24-well culture plates (Flow Laboratories) in 1 ml volumes, at a cell density of 10^6/ml. The

dilutions were made up with fresh RPMI. Each well was then exposed to either a sham irradiation or light irradiation using a coherent, polarized light source (Figure 1.). Immediately after irradiation the cells were transferred to a 37°C incubator for 12 h, then centrifuged for 5 min at 500 rpm, immediately after which the macrophage-conditioned supernatant, which contained soluble factors released by the macrophages, was collected and stored at −20°C.

Fibroblast Cell Proliferation Bioassay

All the supernatants were assayed for their activity on fibroblast proliferation using a methylene blue assay described by Martin *et al.*[19] and reviewed by Oliver *et al.*[20] One microplate was set up for each of the following time periods: time 0, 24 h, 48 h, 72 h and 96 h post plating. Each microplate was set up as in Figure 2. At the end of each time period one of the five plates set up was prepared for reading on a spectrophotometer as follows:

(1) The cell medium was removed.
(2) The cells were rinsed with borate buffer (pH 8.6, 0.01 M).
(3) One hundred μl of 100% methanol was added to each well to fix the plates.
(4) The excess methanol was removed and the wells allowed to dry.
(5) One hundred μl of 1% methylene blue was added to each well for 30 min.
(6) The excess methylene blue was removed and each well was rinsed three times with borate buffer.
(7) One hundred μl of acid alcohol was added to each well and the microplate agitated to ensure complete mixing of the eluted stain and the acid alcohol.

The microplates were then placed in an eight-channel Anthos spectrophotometer (TechGen) and the absorbance of the contents of each well was read at 650 nm.

After the microplates had been read on the spectrophotometer, the absorbance was compared

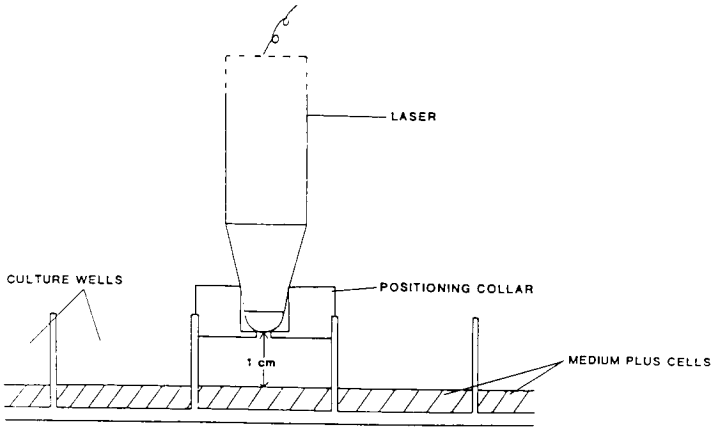

Figure 1. Experimental arrangement for the exposure of U-937 cells in suspension to light therapy

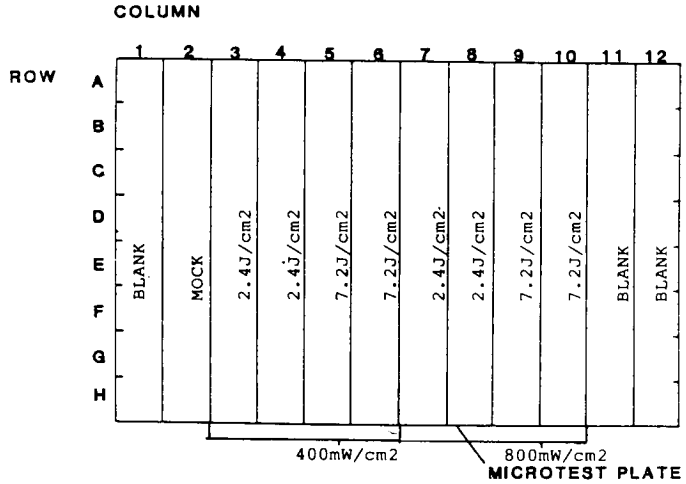

Figure 2. Diagram of the 96-well microtest plate showing the arrangement of the different test groups

against a standard curve to find the actual cell number per treatment per time period.

Results

U-937 Cell Viability

The results of the trypan blue test for viability showed that by 12 h after treatment the number of U-937 cells remaining viable in all the groups ranged from 94–97% of the initial number per well. This did not represent a statistically significant fall in cell numbers as a result of the treatment regime to which they were exposed.

Fibroblast Proliferation Assay

The results of the fibroblast proliferation assay are shown in Tables 1 and 2 and expressed graphically in Figures 3–6. The statistical analyses are shown in Tables 3 and 4. After 96 h post-plating, at an energy density of 2.4 J/cm^2, there was no statistically significant difference between the effect of mock irradiated samples and the 400 mW/cm^2 irradiated samples, however there was a significant difference in fibroblast number between the mock and 800 mW/cm^2, the latter producing greater cell proliferation, and also between the 400 and 800 mW/cm^2. Again, the 800 mW/cm^2 produced greater cell proliferation. In contrast, at an energy density of 7.2 J/cm^2, there was a significant difference in fibroblast number between the mock irradiated samples and the 400 mW/cm^2, the 400 mW/cm^2 producing greater proliferation, but not between the mock and 800 mW/cm^2 irradiated samples. Between the 400 and 800 mW/cm^2 samples there was a significant difference in fibroblast numbers, 400 mW/cm^2 producing greater cell proliferation.

Table 1. Fibroblast number per treatment with macrophage-conditioned medium per time period. Energy density to which the marcophages were exposed = 2.4 J/cm^2

Hours post plating	Fibroblast number per well		
	Mock	400 mW/cm^2	800 mW/cm^2
0	1000	1000	1000
24	1847 ± 31	2139 ± 44	2482 ± 49
48	2320 ± 116	3353 ± 146	2854 ± 106
72	3722 ± 76	4486 ± 68	6490 ± 209
96	9428 ± 128	10259 ± 244	13128 ± 250

± = Standard error of mean (SEM).

Table 2. Fibroblast number per treatment with macrophage-conditioned medium per time period. Energy density to which the macrophages were exposed = 7.2 J/cm^2

Hours post plating	Fibroblast number per well		
	Mock	400 mW/cm^2	800 mW/cm^2
0	1000	1000	1000
24	1847 ± 31	2427 ± 84	2622 ± 75
48	2320 ± 116	5544 ± 191	3653 ± 192
72	3722 ± 76	4878 ± 53	4959 ± 90
96	9428 ± 128	12172 ± 179	8914 ± 247

± = Standard error of mean (SEM).

Table 3. Results of the statistical analysis Student's *t*-test

	Mock	400 mW/cm^2	800 mW/cm^2
Energy density = 2.4 J/cm^2; 96 h post plating			
400 mW/cm^2	NS	–	$P < 0.05$
800 mW/cm^2	$P < 0.05$	$P < 0.05$	–
Energy density = 7.2 J/cm^2; 96 h post plating			
400 mW/cm^2	$P < 0.05$	–	$P < 0.05$
800 mW/cm^2	NS	$P < 0.05$	–

NS — No statistical difference.

Table 4. Results of the statistical analysis (Student's *t*-test) comparing 2.4 and 7.2 J/cm^2 at 96 h post plating

7.2 J/cm^2	2.4 J/cm^2	400 mW/cm^2	800 mW/cm^2
400 mW/cm^2		$P < 0.05$	NS
800 mW/cm^2		NS	$P < 0.05$

NS — No statistical difference.

Discussion

As has already been reported,[5] *in vitro* irradiation of U-937 cells with light at specific wavelengths can modify their ability to affect fibroblast proliferation. The type and degree of modification was found to be dependent both upon the wavelength and energy density used. In this study, the effect of exposure to different power and energy densities was examined. It was found that at an energy density of 2.4 J/cm^2, found in earlier work[6] to be suboptimal, there was a greater increase in fibroblast proliferation when using a probe with a higher power density (800 mW/cm^2) than one of 400 mW/cm^2: in contrast, at a higher energy density (7.2 J/cm^2), found in earlier work to be optimal, fibroblast proliferation was greater using a probe with a lower power density (400 mW/cm^2). This observation is of considerable interest in that it suggests that higher power densities are less effective in the *in vitro* system used here. Earlier work[6] has shown that an optimum energy density of 7.2 J/cm^2 is required to

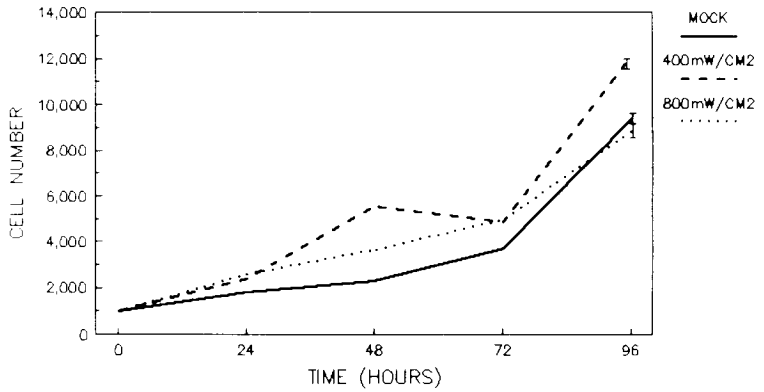

Figure 3. Graph plotting fibroblast cell number against time post plating for each test group: (2.4 J/cm^2)

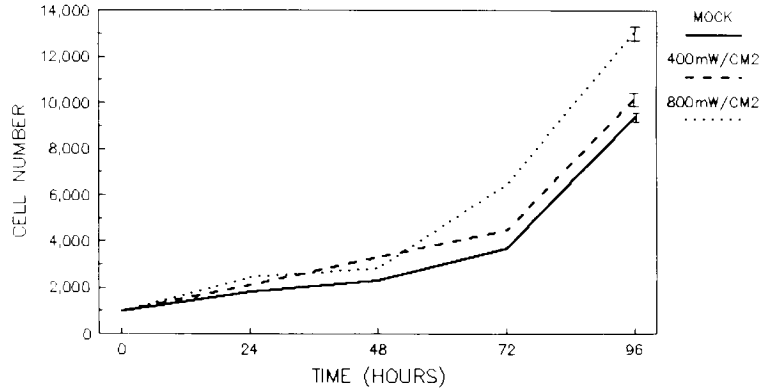

Figure 4. Graph plotting fibroblast cell number against time post plating for each test group: (7.2 J/cm^2)

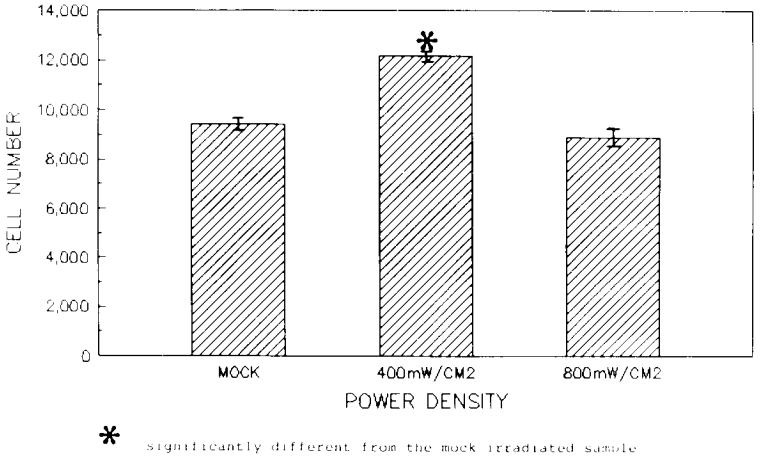

Figure 5. Histogram showing final fibroblast cell number against power densities: (2.4 J/cm^2)

elicit maximum response in this model, however, it is clear that these results also indicate that the response is also power density dependent. Essentially, these results show that at a low energy density (2.4 J/cm^2), a high power probe (800 mW/cm^2) produces similar results to that of a low power probe (400 mW/cm^2) at a higher energy density. The results obtained also indicate that using a high power density and a high energy density together, fibroblast proliferation is reduced. As indicated earlier this could be due, in part, to changes to various light receptors in the U-937 cell at this higher level. Cytotoxic products could also be formed in the cell as a result of an increase in the amount of energy absorbed by the cell in a relatively short time period; this may alter cellular respiration. Alternatively, the higher power density, coupled with the higher energy density, may stimulate the release of fibroblast-inhibitory growth factors without damaging the cells; if correct, this could be

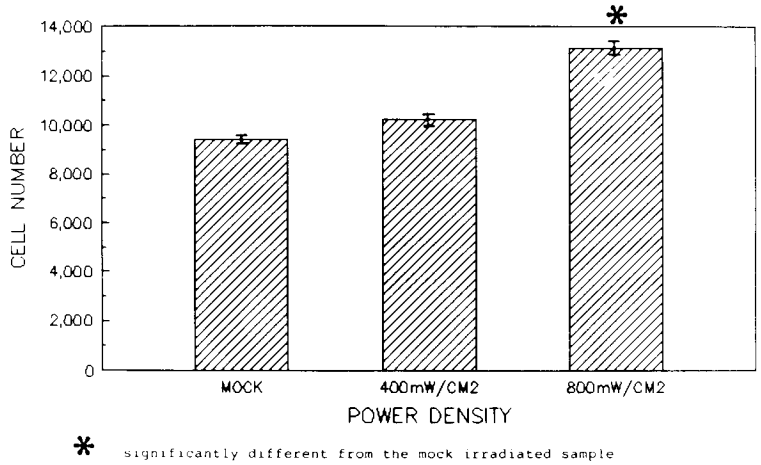

Figure 6. Histogram showing final fibroblast cell number against power densities: (7.2 J/cm^2)

of clinical significance in situations where fibroblast growth is excessive.

When these, and previous, results[5–7] are considered collectively, they support the hypotheses that there are (1) energy density and (2) power density dependent responses of the U-937 cell's ability to synthesize and secrete soluble factors in response to light therapy, and thus be able to modify fibroblast proliferation. The nature of these factors remains to be determined.

When considered clinically, the treatment of injuries is costly. Tissue injuries occur frequently and it is in the interests of both the community and the individual that their rate of repair be optimized. The stimulatory effects of light have been observed *in vitro* in a number of studies,[5–7,21] and there have also been reports of its effectiveness *in vivo*.[21–27] Although the optimal *in vivo* treatment parameters have yet to be determined, *in vitro* studies suggest the importance of considering both energy density and power density since thresholds appear to exist for both above which damage may ensue. From the clinical perspective, it is of paramount importance to optimize the required dosage in treatment of tissue injuries and, in doing so, reduce expenditure, length of hospital stay and discomfort to the patient. The results of these studies provide information which may well be of value in optimizing and maintaining the safety of these procedures.

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