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Macrophage Responsiveness to Light Therapy

Steve Young, PhD, Peter Bolton, BSc, Mary Dyson, PhD, Wil Harvey, PhD, and Costas Diamantopoulos, BSc

Anatomy Department, United Medical and Dental Schools, Guy's Hospital, London SE1, 9RT (S.Y., P.B., M.D.); Oral Surgery Department, Eastman Dental School, London WCI (W.H.) and Omega Research Laboratories, London N16UT(C.D.)

Macrophages are a source of many important mediators of wound repair. It was the purpose of this study to see if light could stimulate the release of these mediators.

In this study an established macrophage-like cell line (U-937) was used. The cells were exposed in culture to the following wavelengths of light: 660 nm, 820 nm, 870 nm, and 880 nm. The 820-nm source was coherent and polarised, and the others were non-coherent. Twelve hours after exposure the macrophage supernatant was removed and placed on 3T3 fibroblast cultures. Fibroblast proliferation was assessed over a 5-day period.

The results showed that 660-nm, 820-nm, and 870-nm wavelengths encouraged the macrophages to release factors that stimulated fibroblast proliferation above the control levels, whereas the 880-nm wavelength either inhibited the release of these factors or encouraged the release of some inhibitory factors of fibroblast proliferation.

These results suggest that light at certain wavelengths may be a useful therapeutic agent by providing a means of either stimulating or inhibiting fibroblast proliferation where necessary. At certain wavelengths coherence is not essential.

Key words: fibroblasts, laser biomodulation, wound repair

INTRODUCTION

Macrophages can inhibit or enhance the activity of many types of cells [1–4]. If light of different wavelengths can affect the ability of macrophages in vitro to release factors that cause these opposing effects, then light therapy could have great potential as a modulator of wound repair. Wounds that may be prone to hypertrophy or to keloid formation could, perhaps, be treated with wavelengths stimulating the release of inhibiting factors (e.g., prostaglandins) that suppress fibroblast activity, whereas in cases such as varicose ulcers where the problem is one of delayed repair, wavelengths enhancing the release of stimulating factors (e.g., monokines) could be given to encourage fibroblast activity and the development of granulation tissue.

It was the purpose of this study to find out if the macrophage could be encouraged to synthesize and release these factors that modulate wound repair by treating macrophage cultures with coherent and noncoherent light of various wavelengths. Although macrophages cannot be grown as a cell line in culture the macrophage-like cell line U-937 [5] can. These cells have been shown to stimulate mitogenic activity in cultured fibroblasts [6]. This cell line has a fast population doubling time (20–48 hr), so large numbers can be acquired rapidly, and they provide a homogeneous cell population. In addition, very large volumes of conditioned medium can be inexpensively and rapidly obtained.

The U-937 cells exist in an arrested yet pliant state of maturation. Phenotypically, they re-

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Address reprint requests to Steve Young, Anatomy Department, United Medical and Dental Schools, Guy's Hospital, London SE1, 9RT, England.

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semble monoblasts; however, histochemically, they show monocyte characteristics. The U-937 cell can be induced to mature using a number of chemicals such as TPA (tetramyristic phorbol-acetate), and DMSO (dimethyl sulfoxide). Maturation of these cells is characterised usually by quantitative rather than qualitative changes [5].

The cytochemical findings 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, which is a property of monocytes and macrophages [7]. Although lysozyme is synthesized by other cell types such as neutrophils, release of the product can only be achieved by cell disintegration and not by the secretory process [8]. Lymphoid cells do not produce lysozyme [9]. Lysozyme synthesis and secretion, therefore, seems to be a reliable characteristic of cells in the monocyte-macrophage series.

The cytochemical findings in the U-937 cell line also shows that they are closely related to macrophages. Esterase stainings are strongly positive; in particular, the Naphthol AS-D acetate esterase reaction is positive with marked inhibition by NaF, which is a reactivity only noted in monocytes [10].

Surface marker studies have revealed both C3 and Fc receptors (detected with the rosette technique EA 7s) [11]. This pattern of activity has been found in normal macrophages [12–14].

Because the U-937 shows many characteristics of the human macrophage it was considered appropriate to use the cell line as a model to understand better the mechanisms of the macrophage response to injury and the subsequent effect of light on this response.

MATERIALS AND METHODS

Cell Culture

U-937 Cells. The U-937 culture was grown in RPMI 1640 (Flow Laboratories). The medium was supplemented with 1% penicillin and streptomycin (Gibco) and 10% heat-inactivated fetal calf serum (Gibco). Subculturing was approximately every 2 days. The cells were grown in 50-ml Nunc culture flasks. Each flask contained 9 ml of medium + cells. The cells were grown at 37°C in an incubator with a 5% CO_2 atmosphere. Each confluent flask contained approximately 9 $\times 10^6$ cells.

Fibroblasts. The fibroblasts used were isolated from Swiss 3T3k mouse kidney (supplied by the Imperial Cancer Research central services). They were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penstrep. The incubator was maintained at 37°C and had 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 milliliters of trypsin was added to each flask to cover the layer of cells and left for 30 sec. The excess trypsin was then poured off, and the flasks were placed back into the incubator inverted so that the cell-covered surface was uppermost and any trypsin remaining in the flask was not in contact with them This prevented any excess damage being caus[illegible] by the trypsin remaining in the flasks. After [illegible] minutes the cells detached. Three milliliters [illegible] DMEM was added to each flask, and the resu[illegible] cell suspension was poured into a universal [illegible]tainer. The cells were pipetted in 50 μl vol[illegible] microplate so that each well of the micropla[illegible]tained 1,000 cells. The culture medium in [illegible] the cells were suspended was a 50:50 [illegible] DMEM and the supernatants taken from [illegible]ious U-937 and RPMI treatment groups [illegible]low); this was a suboptimal medium so tha[illegible]ulation was possible.

U-937 Irradiation

When confluent, the cells were transferred to sterile 24-well culture plates (Flow Laboratories) in 1-ml volumes, with cell densities of 1 million/ml. The dilutions were made up with fresh RPMI.

Each well was then exposed to either a sham irradiation for 20 sec or light irradiation for 20 sec (see Fig. 1), using a Biotherapy 3ML device (Omega Universal Technologies). Four different light wavelengths were used: 660 nm, 820 nm, 870 nm, and 880 nm. The physical parameters of the light source are listed below.

Noncoherent sources (Omega Superluminous diodes):

1. Wavelength: 660 nm, 870 nm, 880 nm.
2. Active medium: gallium aluminium arsenide (GaAlAs).
3. Average power output: 15 mW.
4. Spot size at 1 cm distance: 0.125 cm^2.
5. Power density: 120 mW/cm^2.

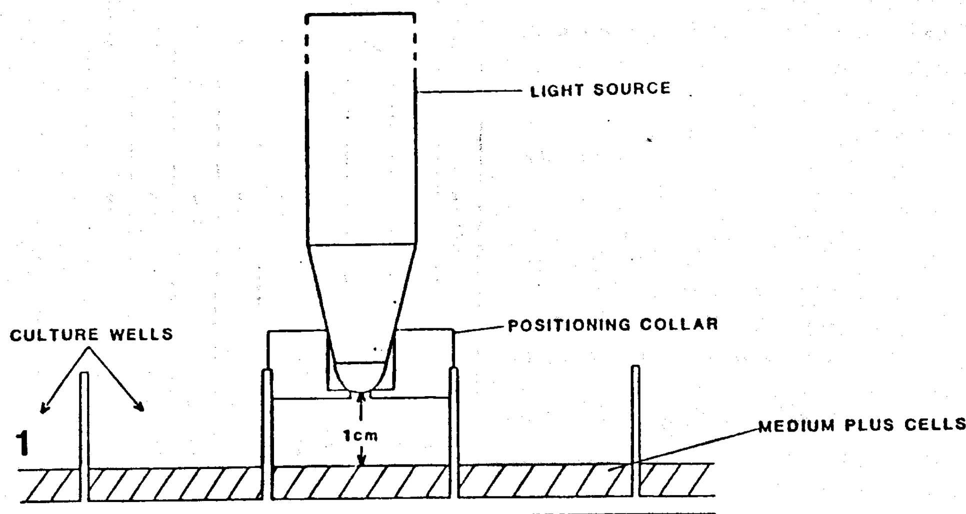

Fig. 1. Experimental arrangement for the exposure of U-937 cells in suspension to light therapy.

6. Source spectral band width at 100% intensity (15 mW): 5 nm.
7. Total angle of divergence: 10°.
8. Pulsing frequency: 5,000 Hz.
9. Pulse width: 18 μsec.

Coherent source (Omega Laser diode):

1. Wavelength: 820 nm.
2. Active medium: GaAlAs.
3. Average power output: 15 mW.
4. Spot size at 1 cm distance: 0.125 cm^2.
5. Power density: 120 mW/cm^2.
6. Source spectral band width at 100% intensity (15 mW): 0.01 nm.
7. Total angle of divergence: 23° parallel to Junction plane, 16° perpendicular to Junction plane.
8. Pulsing frequency: 5,000 Hz.
9. Pulse width: 18 μsec.

Dosimetry:

1. Exposure time: 20 sec.
2. Energy density: 2.4 J/cm^2.

The sources were calibrated with a Photodyne flat response power meter (PX16E).

After irradiation the cells were left for 30 min, then centrifuged for 5 min at 500 rpm, resuspended in fresh RPMI, and left in the incubator for 12 hr. The cells were then again centrifuged for 5 min at 500 rpm, immediately after which the supernatant was collected and stored at −20°C.

RPMI Irradiation

In order to be able to determine whether the proliferative response to light was a direct effect on the cells or was mediated through the culture medium, the RPMI only was irradiated in exactly the same way as described for U-937 cells. Also U-937 cells were irradiated in PBS, then transferred into fresh RPMI and left in the incubator overnight; the irradiated media were collected and stored at −20°C.

Macrophage Viability Assay

Samples of macrophages, suspended in the culture fluid in which they had been either irradiated or sham-irradiated, were mixed thoroughly, then 0.5 ml of the suspension was removed and transferred to a glass vial. A drop of Trypan blue (0.5% w/v) was added to the suspension. A drop of the suspension was then transferred to a Neubauer counting chamber. In this viability test [15] 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 number.

Fibroblast Proliferation Assay

All the supernatants were then assayed for their activity on fibroblast proliferation using a modification of the methylene-blue assay described by Martin et al. [16]. Each 96-well microplate was set up as follows: 1) 50 μl of DMEM (containing approximately 1,000 fibroblasts) were placed in each of the wells to be assayed using an

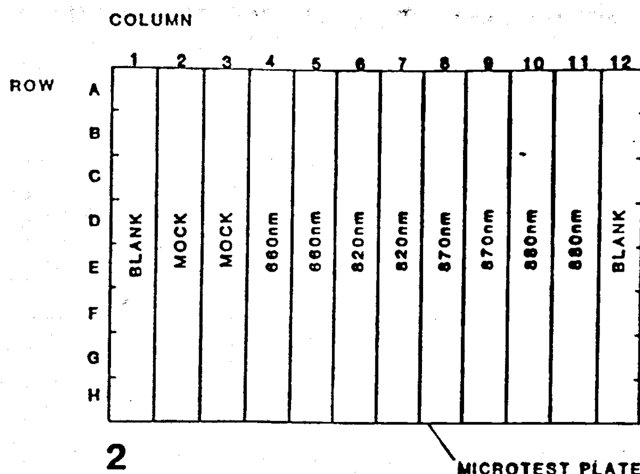

Fig. 2. Diagram of the 96-well microtest plate showing the arrangement of the different test groups.

eight-channel multipipette; 2) 50 μl of each of the supernatants were then added to the cells as in Figure 2.

This, therefore, gave two eight-well columns of mock, 660 nm, 820 nm, 870 nm, and 880 nm: a total of 16 wells per supernatant.

One microtest plate was set up for each time period, i.e., time 0, 12 hr, 36 hr, 60 hr, 84 hr, 108 hr, and 132 hr after macrophage irradiation. This gave a total of seven time periods.

At the end of each time period the corresponding plate was prepared for reading on a spectrophotometer as follows:

1. The medium covering the fibroblasts was removed.
2. The cells were rinsed with borate buffer.
3. One hundred microliters of 100% methanol was added to each well for 4 min to fix the cells.
4. The excess methanol was removed, and the wells allowed to dry by exposure to air.
5. One hundred microliters of methylene blue was added to each well for 30 min.
6. The excess methylene blue was removed, and each well was rinsed 3 times with borate buffer.
7. One hundred microliters of acid-alcohol was added to each well, and the microplate was agitated to ensure complete mixing of the eluted stain and the acid-alcohol.

The microplates were then placed in an eight-channel Multiskan spectrophotometer 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 read against a standard curve to obtain the cell number per treatment per time period.

The results were analysed statistically using the Student's t-test.

RESULTS

The results of the trypan blue exclusion test for viability showed that 12 hr after treatment the number of U-937 cells remaining viable in all groups ranged from 93 to 96%. This did not represent a statistically significant fall in cell number owing to the treatment regime.

The effect on fibroblast proliferation of supernatants obtained from U-937 cells irradiated in RPMI with light of different wavelengths is summarized in Table 1 and illustrated in Figure 3. The maximum stimulation was obtained with 660-nm noncoherent light. It was found that 820 nm (coherent and polarized) and 870 nm (noncoherent) were also stimulatory, although to a lesser extent. In contrast, the supernatant obtained after irradiation with 880 nm noncoherent light had an inhibitory effect on fibroblast proliferation. The results of a statistical analysis of the comparative effects of each irradiation are to be found in Table 2a–2f.

The effect of supernatants obtained from light-treated U-937 cells irradiated in PBS (Fig. 4) was not significantly different from that of supernatants obtained from U-937 cells irradiated in RPMI.

It was observed that RPMI treated with light in the absence of U-937s also affected fibroblast proliferation, although to a lesser extent than when U-937 cells were present (Fig. 5).

The absorption characteristics of RPMI are illustrated in Figure 6. Virtually no absorption occurred within the range of wavelengths used in the experiments described here (i.e., 660–880 nm).

The effect of light treatment on macrophage proliferation is summarized in Figure 7. There was no significant difference in macrophage number between groups exposed to any of the wavelengths of light examined and the sham- or mock-irradiated control group at 12 hr after irradiation, the time period at which the supernatants were obtained, although by 36 hr after irradiation all the wavelengths of light used were temporarily stimulatory.

TABLE 1. Fibroblast Number per Treatment per Time Period

	Fibroblast no. per supernatant				
Hours after plating	Mock	660 nm	820 nm	870 nm	880 nm
0	1,000	1,000	1,000	1,000	1,000
12	3,200 ± 85[a]	3,350 ± 69	3,200 ± 103	3,200 ± 94	2,850 ± 67
36	3,750 ± 188	4,100 ± 121	4,100 ± 77	3,750 ± 94	3,400 ± 107
60	4,800 ± 197	5,800 ± 348	6,800 ± 366	6,300 ± 372	5,950 ± 266
84	6,300 ± 145	12,400 ± 236	11,000 ± 275	9,150 ± 151	6,300 ± 107
108	7,000 ± 114	13,400 ± 291	12,400 ± 360	9,250 ± 305	6,200 ± 236
132	9,250 ± 227	12,500 ± 284	12,300 ± 424	9,900 ± 176	6,900 ± 119

[a]± standard error of mean (SEM).

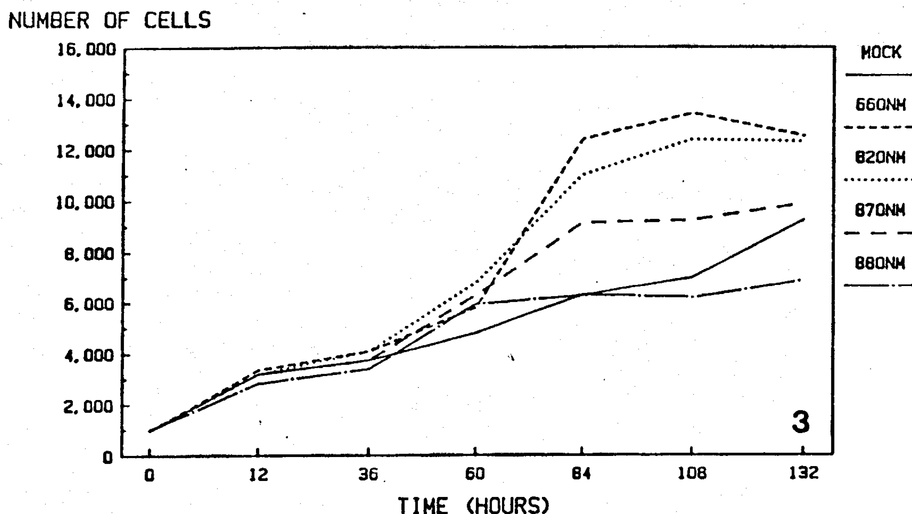

Fig. 3. Graph plotting fibroblast cell number (thousands) against time (hours) after plating, for each test group: mock-treated, 660 nm, 820, 870 nm, and 880 nm.

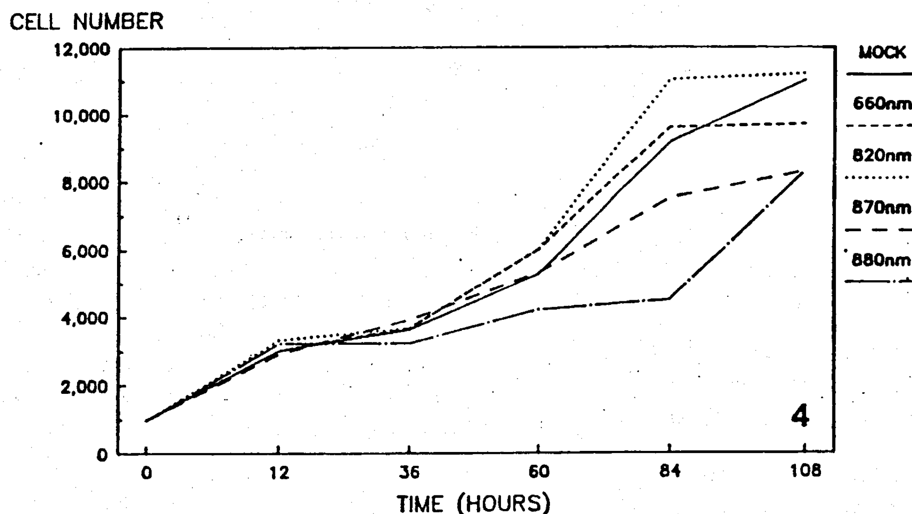

Fig. 4. Graph plotting fibroblast cell number (thousands) against time (hours) after plating, for each test group. (U-937s irradiated in PBS).

DISCUSSION

These results indicate that 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 upon the wavelength of light used. Irradiation of U-937 cells in RPMI with noncoherent light at a wavelength of 660 nm produced the maximum stimu-

TABLE 2. Results of the Statistical Analysis (Student's t-test)

	Mock	660	820	870
a. 12 Hr after plating				
660	No			
820	No	No		
870	No	No	No	
880	$P < .05$	$P < .05$	$P < .05$	$P < .05$
b. 36 Hr after plating				
660	No			
820	No	No		
870	No	No	No	
880	No	No	No	No
c. 60 Hr after plating				
660	$P < .001$			
820	$P < .001$	No		
870	$P < .001$	No	No	
880	$P < .001$	No	No	No
d. 84 Hr after plating				
660	$P < .001$			
820	$P < .001$	$P < .001$		
870	$P < .001$	$P < .001$	$P < .001$	
880	No	$P < .001$	$P < .001$	$P < .001$
e. 106 Hr after plating				
660	$P < .001$			
820	$P < .001$	No		
870	$P < .001$	$P < .001$	$P < .001$	
880	$P < .001$	$P < .001$	$P < .001$	$P < .001$
f. 132 Hr after plating				
660	$P < .001$			
820	$P < .001$	No		
870	No	$P < .001$	$P < .001$	
880	$P < .001$	$P < .001$	$P < .001$	$P < .001$

latory effect on fibroblast proliferation, 820 nm (coherent and polarised) and 870 nm (noncoherent) also producing stimulatory effects, but to a lesser degree. In contrast, irradiation of U-937 cells with 880-nm (noncoherent) light produced an effect on the U-937 cells that resulted in an inhibition of the proliferation of fibroblasts to below the levels produced by sham-irradiated U-937 cells (Fig. 3). The observation that coherence is not always essential for laser biomodulation supports the findings of Karu [17].

The changes observed in fibroblast proliferation followed their exposure to supernatant obtained from the U-937 cell cultures 12 hr after their irradiation in RPMI, suggesting that the synthesis and secretion of growth factors into the RPMI by the U-937 cells could have been modified by the irradiation procedure. It has been shown that macrophages [1–4], of which U-937s can be considered a type [5], contain and secrete such factors during tissue repair. This preliminary observation (Fig. 3) has a number of possible explanations. It could, for example, indicate that exposure to light had affected

1. The U-937s *directly,* altering their ability to synthesize and liberate growth factors,
2. The U-937s *indirectly,* via changes induced in the RPMI,
3. The U-937s *both directly and indirectly,*
4. The RPMI in a manner affecting its ability to modify fibroblast proliferation.

These possibilities were investigated experimentally.

U-937s Irradiated in PBS

The observation that the supernatant of U-937s irradiated in PBS had a basically similar

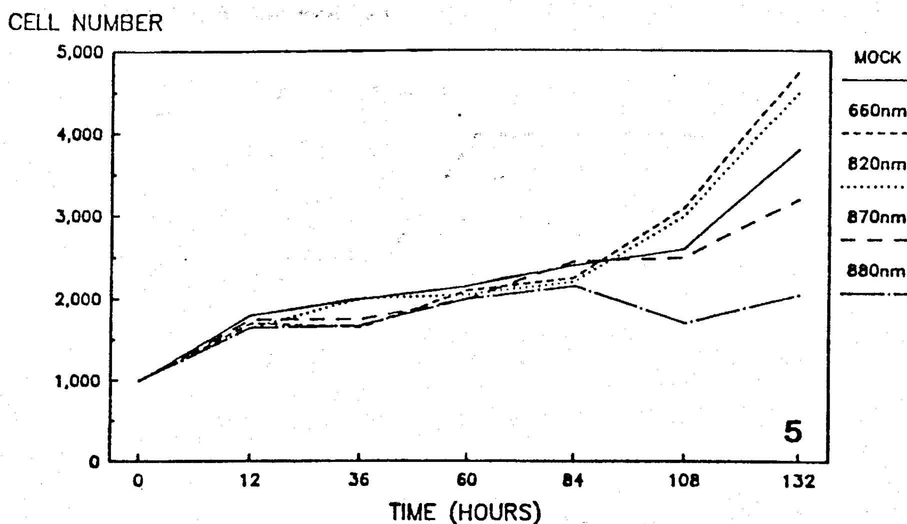

Fig. 5. Graph plotting fibroblast cell number (thousands) against time (hours) after plating, for each test group (RPMI 1640 irradiated).

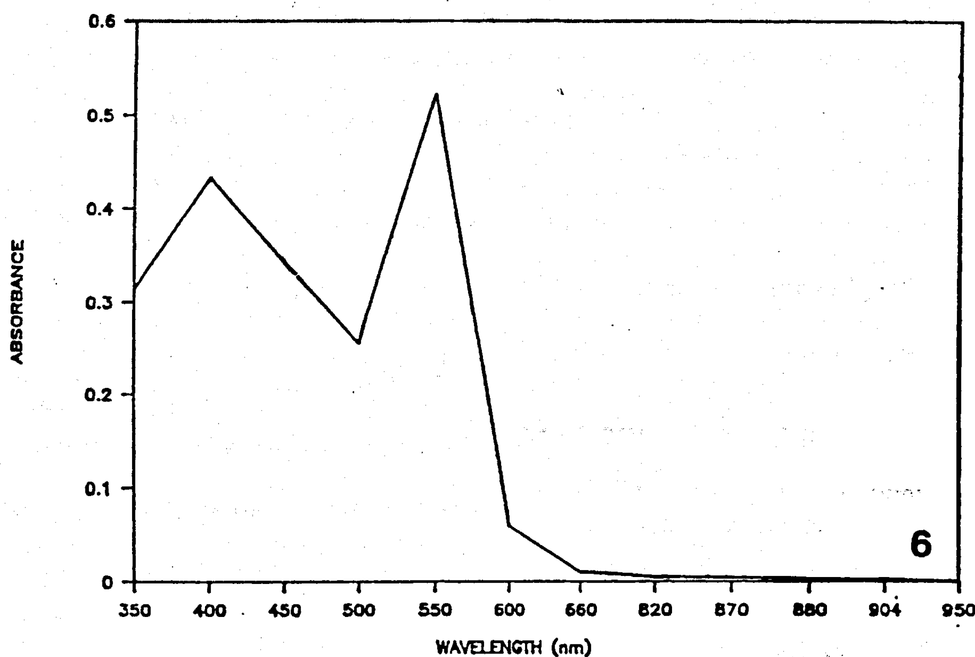

Fig. 6. Absorption spectrum for RPMI 1640.

effect on fibroblast proliferation to that of U-937s irradiated in RPMI suggests that main effect(s) of the irradiation were on the components which these two targets had in common, i.e., the cells and/or the buffered saline (Fig. 4).

RPMI-Only Irradiated

Since the addition to fibroblasts of RPMI that had been irradiated with light in the absence of macrophages also affected the proliferation rate of the fibroblasts, it can be inferred that light induces changes in the RPMI that can affect these cells directly, i.e., without the mediation of the U-937 cells. The observation, however, that the effect of irradiated RPMI was less than that of the supernatant from U-937s irradiated while maintained in RPMI supports the hypothesis that irradiation induces changes in both the U-937 cells and the medium, and that both these changes can affect the ability of fibroblasts to proliferate (Fig. 5).

The observation that irradiation of RPMI at different wavelengths produced different effects on the RPMI in the absence of cells led us to con-

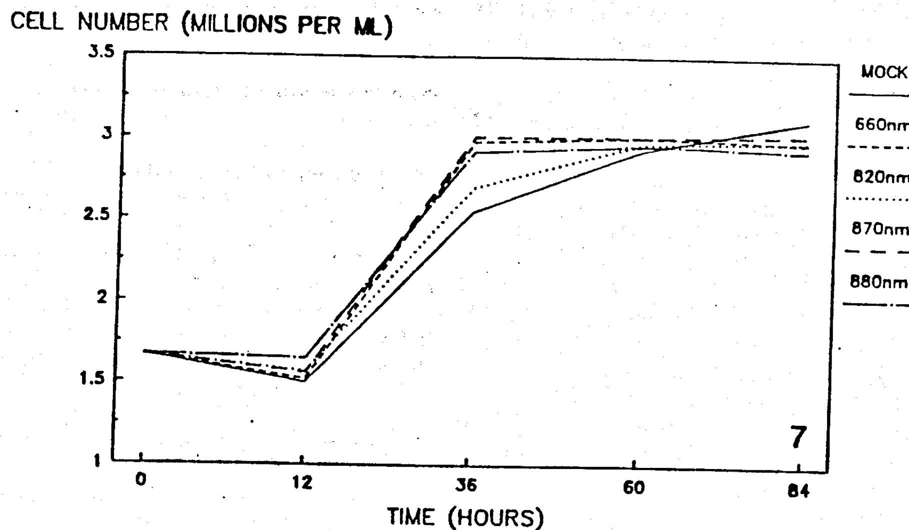

Fig. 7. Graph showing macrophage proliferation (millions per ml) against time (hours).

sider the possibility that chromophores present in this medium might absorb light at these wavelengths. However, there was no significant absorption at the wavelengths used (660–880 nm) (Fig. 6). Results such as these, which support those of other investigators [17], throw into doubt the validity of the chromophore theory of photobiomodulation [18]. Further investigations into the interaction between light of different wavelengths and tissue culture media are clearly indicated.

Considered collectively, these results support the hypothesis that exposure of U-937s in vitro to light affects both the cells and the medium, and in doing so modifies their influence on fibroblast proliferation. It is presumed that the ability of the U-937 cells to synthesize and secrete growth factors is affected, and there appears, in addition, to be a direct effect on the inorganic component of the tissue culture medium that further modifies fibroblast proliferation.

Since by 12 hr after irradiation, i.e., the time at which the supernatant was taken and added to the fibroblast cultures, there was no significant difference in U-937 number between the sham-irradiated controls and any of the light-irradiated groups (Fig. 7), it can be inferred that the light-induced change in the ability of individual U-937 cells to synthesize and secrete growth factors is not dependent on light-induced changes in the number of cells available.

Clinically, the treatment of injuries is costly in terms of expenditure, length of hospital stay, reduced availability of beds and staff, and pain and incapacity to the individual affected. Since tissue injuries occur so frequently it is in the interests of the individual and the community that their rate of repair be optimized. By increasing fibroblast production, the proliferative phase of repair could be accelerated. Should the stimulatory effects observed in vitro in the experiments described above also occur clinically, and provided that no adverse side effects are found, then low-level laser therapy could be of considerable benefit in the treatment of tissue injuries.

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