BIOSTIMULATION OF PHOTOSENSITIZED FIBROBLASTS BY LOW INCIDENT LEVELS OF VISIBLE LIGHT ENERGY

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It was long assumed that visible ($\approx 380 \sim 650$ nm) and near infrared (I.R.) (≈ 650 nm $\sim$ 1000 nm) light does not interact with tissue. In the past three decades, however, it has been found that lasers in the visible and near I.R. cause biostimulation of injured tissues at very low levels of incident energy. We therefore studied the effect of various light sources in the visible (540 nm, 630 nm,) and near I.R. (660 nm, 780 nm, 940 nm,) on fibroblasts and keratinocytes. We found that at certain low energy doses there is an enhancement in their proliferation. Since these two radiation regions differ dramatically in their photochemical and photophysical properties, we share the opinion that visible light starts the cascade of metabolic events after being absorbed by endogenous porphyrins in the mitochondria, whereas infrared radiation starts the cascade of metabolic events by activating enzymes in the membranes. In order to examine our hypothesis that biostimulation in the visible is a photosensitization process, we measured the proliferation rate of fibroblasts enriched with very small amounts of hematoporphyrin derivatives (HPD) after HeNe (633 nm) irradiation and 940 nm irradiation. We have found that the effect of HeNe light on fibroblast proliferation was dependent on exogenous HPD while that of 940 nm was not. These results have important implications in both phototherapy and photodynamic therapy (PDT).

Key words: Photosensitization, photochemical reaction, photophysical reaction, phototherapy, photodynamic therapy

Introduction

There has been much interest in the biological effects of low energy visible and near infrared lasers. Most of the experiments show that low energy visible and near infrared lasers do have specific bioeffects which seem to change from stimulatory to damaging with increasing doses. In a previously reported study,⁽¹⁾ we irradiated fibroblasts with various light sources and found that at specific, relatively low energy doses there is an accelerated cell mitosis, while at higher doses cell proliferation is inhibited. Moreover, we have found that the effect is not only dose dependent but also intensity dependent.⁽²⁾

In the present work we irradiated keratinocytes or fibroblast with light sources in the visible, 540nm and 630nm and with light sources in the near I.R., 660nm, 780nm and 940n. we have found that at certain energy doses there is an enchantment in their proliferation. Since these two radiation regions differ dramatically in their photochemical and photophysical properties,⁽³⁾ we believe that visible light starts the cascade of metabolic events by formation of photosensitized reactive oxygen species (ROS) which stimulate the redox activity of the respiratory chain (RC), whereas the infrared radiation reactivates the ATPase ion pumps in the cell membrane. In order to examine the hypothesis that biostimulation in the visible is a photosensitized process, we measured the proliferation rate of fibroblasts

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enriched with very small amounts of hematoporphyrin derivatives (HPD) after 663 nm irradiation and 940 nm irradiation, we have found that the effect of HeNe light on fibroblast proliferation was dependent on exogenous HPD while that of 940nm was not.

Materials and Methods

Materials

Culture media, fetal calf serum, trypsin and antibiotics were purchased from Biological Industries Co. (Beit Haemek, Israel). Tissue culture dishes were from Costar (Cambridge, Mass., USA)

Fibroblasts

Skin fibroblasts were established from the dermis of a four year old foreskin. The cells were grown in DMEM containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μ g/ml), gentamycin (50 μ g/ml) and glutamine (2 mM) at 37°C and 8% CO. They were split for seeding for experiments at 70-80% confluence. In some experiments NIH/3T3 fibroblasts were used.

Keratinocytes

Normal human keratinocyte cultures were initiated from young foreskins according to the methods of Rheinwald and Green^(4,5) on feeder layers of mitomycin C-treated 3T3 fibroblasts. Cultures were fed every 48 hours with keratinocyte growth medium formulated according to Green *et al*, comprised of DMEM:F12 (3:1), 8% FCS, antibiotics, glutamine, adenine (0.81 mM), hydrocortisone (0.4 μ g/ml), insulin (4 μ g/ml), transferrin (5 μ g/ml) and T3.

Experimental Conditions

Cells were seeded in 24- or 96-multi-well clusters, according to the area irradiated by each source, at 2.5×10^4 or 8×10^3 cells/well, respectively. 48 hours later the cells were washed and exposed to light for various periods, in triplicate while in PBS. In some experiments, haematoporphyrin derivative (HPD, 0.25 μ g/ml) was added to half of the wells for 30 min in the dark, and after washing the cultures with PBS the cells were irradiated as described. Following irradiation, cultures were replenished with fresh growth medium and were further incubated for 24 to 72 hours. At the end of the incubation the cultures were washed with PBS and trypsinized. The percentage of dividing cells (two un-separated daughter cells) 24 hrs after irradiation and the total cell number after 48 or 72 hrs post irradiation were determined by an inverted microscope (CK2-TRC, Olympus, Japan). Sham irradiated wells served as controls. Each experiment was repeated three times in triplicate.

Irradiation

The light sources were: 1) a 10 mW HeNe laser, $\lambda = 633$ nm (Uniphase model 1135); 2) a 6-13 mW 780 nm diode laser (manufactured in our own laboratory); 3) an Xe lamp with an interference filter emitting 540 nm light at an output power of 5-10 mW; 4) A 7 mW light-emitting diode (LED) at 660nm; and 5) a 7 mW 940 nm LED (Amcor, Israel). The laser beam area covered the cells in the well. The intensity of the light sources was determined by an Ophir (model PD2-A) power meter.

Results

The irradiation of normal human keratinocytes (NHK) at 540 nm (Figure 1), 633 nm (Figure 2) and 780 nm (Figure 3) resulted in a significant 1.5- to 2-fold increase in the percentage of dividing cells 24 hrs after irradiation.

Exposure of NIH fibroblasts to 633 nm (Figure 4), 660 nm (Figure 5) and 940 nm (Figure 6) increases the percentage of dividing cells 24 hrs post irradiation to a similar extent. The energy densities needed for enhancing proliferation were 1-10 J/cm² for visible light and 0.2-2 J/cm² for near I.R. light (660 nm, 780 nm, and 940 nm)

In Figure 4 it can be seen that introducing a small concentration of HPD (0.25 mg/ml) to fibroblast cultures before exposure to 633 nm light promotes the effect of the irradiation in the range of 1.5-4 J/cm². The HPD- enriched cells

Fig 1: Percentage of dividing cells of NHK 24 hours after exposure to 5mW 540nm light.

Fig 2: Percentage of dividing cells of NHK 24 hrs post irradiation at 10 mW 633 nm HeNe light.

Fig 3: Percentage of dividing cells of NHK 24 hrs post irradiation at 6.5 mW 780 nm light.

did not change their proliferative profile, however, when 940 nm light was used (Figure 6).

Discussion

The data obtained show that light in the visible wave-band (540nm and 633nm, Figures 1, 2, and 4), and light in the near infrared, (660nm, 780nm and 940nm, Figures 5, 3, and 6) enhance proliferation of fibroblasts and keratinocytes. Since these two radiation regions differ dramatically in their photobiological properties⁽³⁾

we would like to discuss their possible stimulation on biological activities. As was mentioned previously by Karu⁽⁶⁾ and by us,⁽⁷⁾ light in the visible is responsible for photomodulation of the redox activity of the respiratory chain. The far red light is assumed to reactivate the ATPase ion pumps in the cell membrane.^(8,9)

We believe that the stimulating action of the visible light is by an electronic excitation of the endogenous photosensitizers, porphyrins or cytochromes, in the mitochondrial respiratory chain, producing ROS such as singlet oxygen or superoxide ion.^(6,7) The ROS acts as a potent oxidizer stimulating the redox activity of the respiratory chain, enhancing ATP production,⁽¹⁰⁾ accompanied by changes in Ca^{++} transport⁽¹¹⁾ in the cytoplasm, thus promoting mitosis. The far red radiation acts on the cell through a different mechanism. Only vibrational overtone excitation of enzymes can be considered. We assume that vibrational excited enzymes (veENZ) undergo a conformational transformation. According to Bolognani *et al.*,⁽⁹⁾ this transformation constitutes a reactivation of the enzyme.

Reactivated calcium-ion and sodium potassium ion ATPase pumps enhance the calcium-ion gradient and the cell-membrane potential. Both the increase in the calcium ion gradient across the cell membrane and the cell membrane potential increase the calcium electrochemical gradient across the cell membrane.

The enhanced calcium electrochemical gradient is the activated driving force for calcium-ion influx back into the cytosol. The enhanced calcium-ion influx into the cytosol proceeds via gated ion channels which allow the build-up of the electrochemical gradient and produce oscillations in the calcium-ion concentration in the cytosol. Influx of calcium and other ions presumably enhances mitosis.

In order to examine our hypothesis that visible light, in contrast to far red light, biostimulates the cell through endogenous porphyrins, we introduced small amounts of HPD to fibroblasts and measured the effect of visible light (HeNe, 633 nm) and near infrared light (940 nm) on proliferation of the enriched cells.

Anionic lipophilic sensitizers such as HPD partition into the cytoplasmic membrane as well as in intracellular membranes. It is well known in the literature

Fig 4: Percentage of dividing fibroblasts relative to control 24 hrs post irradiation.
 ● 8 mW HeNe light ▲ 8 mW HeNe light, cells enriched with HPD.

Fig. 5: Percentage of dividing fibroblasts 24 hrs post irradiation at 7mW 660nm light.

Fig 6: Percentage of dividing fibroblasts relative to control 24 hrs post irradiation. ● 7mW 940 nm light ▲ 7mW 940 nm light, cells enriched with HPD.

that high concentrations of porphyrins and visible light cause cell destruction, a phenomenon which is exploited in photodynamic therapy (PDT).

Data of the present work show that very small concentrations of porphyrins and low intensity visible light (644 nm) can promote cell proliferation (Figure 4). As expected, near infrared light, 940 nm has no effect on HPD- enriched fibroblasts (Figure 6). The fact that external porphyrins (at a very small concentration) combined with low energy visible light enhance proliferation must be taken into account when using PDT. Cancerous cells enriched with small amounts of photosensitizers (like those at the borders of a malignant tumor) may proliferate better after irradiation.

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