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Effects of low-power 632 nm radiation (HeNe laser) on a human cell line: influence on adenylnucleotides and cytoskeletal structures

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Abstract

HeNe (632 nm) irradiation (5, 15 and 30 min) of an embryonal human cell line (EUE) was used to study the short-term effects on energy charge and the rapid, energy-dependent, remodelling processes of cytoskeletal and adhesion structures. The adenosine triphosphate (ATP) concentration, tested by luminometric and high performance liquid chromatography (HPLC) procedures, is constant after 15 and 30 min of HeNe treatment; the lower phosphorylated nucleotides, i.e. adenosine diphosphate (ADP) and adenosine monophosphate (AMP), change after 30 min in opposite directions: the ADP concentration decreases by 39% whilst that of AMP increases about sixfold. The adenylate energy charge (AEC) decreases by 21.7% in treated EUE cells (AEC = 0.65) in comparison with untreated EUE cells (AEC = 0.83). In HeNe-treated cells, the remodelling of cytoskeletal and adhesion molecules becomes evident after 15 min of treatment. The following events are important: (1) modification of stress fibre assembly and increase in vinculin-containing adhesion plaques; (2) assembly and bundling of intermediate filaments; (3) increase in laminin and L-cell adhesion molecules (L-CAM) expression. The lowered energy charge in irradiated cells is related to the increase in AMP production at the expense of ADP. ATP is dynamically constant despite its requirement in short-time remodelling processes of the cytoskeletal network which are enhanced in irradiated cells.

Keywords: HeNe laser; Adenylnucleotides; Cytoskeleton; Adhesion molecules; Immunocytochemistry

1. Introduction

HeNe laser effects on human and bacterial cells have been studied in different experimental conditions with contradictory results [1–6]. Non-linear effects have been observed depending on the wavelength and dose [1,7,8]. Coherent (HeNe laser at 632 nm) and incoherent radiation have been compared in terms of their capacity to enhance DNA and protein synthesis with the aim to provide an explanation for the increase in cell growth in cultures exposed to visible (red) light at low energy [2–5]. These effects depend on the available energy, and the mechanisms involved in the absorption of energy in the transduction of radiation into chemical energy, i.e. adenosine triphosphate (ATP), have been studied [9]. Attention has been directed to the target of red light at the subcellular and molecular level. Of the subcellular organelles involved in explaining the increase in ATP in irradiated heart cells, Salet et al. [10] and Passarella et al. [11,12] have focused their attention on mitochondria. Possible candidates which absorb radiation are considered to be the different aromatic rings belonging to the "respiratory chain" in mitochondria [9,13]. The mechanisms of transduction of radiation into chemical energy is still a matter of debate [6,14].

In addition to the molecules involved in mitochondrial structures, other enzymatic systems may be involved directly or indirectly in explaining laser effects [13,15,16]. Lubart et al. [17] outlined the possible interaction of laser radiation at 632 and 780 nm with Ca^{2+} regulatory mechanisms to explain the laser-induced motility in bull spermatozoa, possibly related to ATPase regulation [18]. Attention was focused on myosin ATPase. This

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enzyme, in the presence of protons and adenosine diphosphate (ADP), can produce ATP (reverse reaction), and this process is enhanced by laser irradiation [19].

The specific demonstration of the influence of lasers on mechanochemical effectors in cytoplasm is lacking; laser effects on several organelles, whose importance is well established in cell biology, have been reported [20,21]. The repair of stress fibres and intermediate filaments following laser cutting has been studied [22].

These observations have led us to investigate a human cell line (Epiteliali Umane Embrionali, EUE) exposed to HeNe laser radiation (10, 15 and 30 min). The short-time effects on the adenylnucleotide concentration (chosen as an indication of the available energy for measuring energy charge) and the cytoskeletal components and adhesion molecules (chosen as a signal of the mechanochemical response and short-term adaptive mechanism) were studied.

The EUE cells have been thoroughly investigated by our group in terms of the response to short and prolonged adaptation to a hypertonic medium [23] by morphological and cytochemical tests [24,25]. Biochemical changes [26–28] and kinetic variations [29–31] have also been reported, as well as variations in the protein pattern [32]. The EUE cell line is suitable for testing the remodelling of cytoskeletal structures during adaptation to osmotic stress [25].

2. Materials and methods

2.1. Cell culture

All experiments were carried out on EUE cells, a human heteroploid cell line with epithelioid morphology. EUE cells were cultured in 50 ml Falcon flasks in Eagle's medium supplemented with 12% calf serum, 100 U penicillin and 100 mg ml^{-1} streptomycin. Cells grown in flasks were transferred onto glass coverslips in 24-well dishes (diameter, 15 mm) at a density of about 5×10^4 cells per well.

2.2. Instrumentation and laser irradiation

An "Oriel" optical bench with the following set-up was used: (a) an HeNe continuous wave laser ("Space Laser", Valfivre, Firenze) at 632 nm with a nominal power of 5 mW and a spot diameter of 0.8 mm; (b) a micrometric laser beam aligner; (c) a beam expander; (d) a detector diode; (e) a computer-aided electronic system (MERLIN). The power delivered, after the beam expander (spot diameter, 2 cm), was considered to be that reaching the surface of the sample; scattering and reflection inside the plastic container were omitted. The laser energy was 14.84 cm^{-2} and the energy density was 0.64 mW cm^{-2}.

For cytochemical studies, cells on round-shaped coverslips (diameter, 12 mm) covered by culture medium were exposed to the HeNe laser for 5, 15 and 30 min.

For the determination of ATP, ADP and adenosine monophosphate (AMP), 10^6 cells were submitted to laser treatment in 2 ml of phosphate-buffered saline (PBS, Sigma, code P-4417) for 15 and 30 min.

2.3. ATP determination

The amount of ATP was determined by a luminometric procedure [33] according to Lundin et al. [34] on EUE cells submitted to laser treatment for 15 and 30 min at 21 °C and controls (untreated) using the ATP monitoring kit (D.I.D., code 1243-102). The reagent was reconstituted with 10 ml of 0.1 M, pH 7.75 Tris–HCl (Tris, tris(hydroxymethyl)aminomethane) buffer containing 2 mM of ethylenediaminetetraacetic acid (EDTA). Portions (20 μl) of reagent stable at 25 °C and containing *Photinus pyralis* luciferase, D-luciferin, 50 mg of bovine serum albumin, 0.5 mmol of magnesium acetate and 0.1 μmol of inorganic pyrophosphate were used. After laser irradiation, 10^6 treated and untreated (controls kept in a dark room) cells were added to 300 μl of 0.4 N $HClO_4$ and kept at 0 °C for 10 min. After centrifugation at 5000 rev min^{-1} for 3 min, 150 μl of supernatant was neutralized with 75 μl of 0.8 M K_2CO_3. The ATP concentration was calculated by converting the millivolt signal into picomoles of ATP using a calibration curve (ATP standard, D.I.D., code 1243-201, from equine muscle) in the range 0.1–50 pmol. The ATP concentration was reported for 10^6 EUE cells and expressed as the number of picomoles of ATP per 10^6 cells.

A Bio Orbit 1251 luminometer was used to evaluate the light emission. An MS-DOS computer equipped with dedicated software was connected to monitor the electric trace derived during the reaction.

2.4. Separation of adenylnucleotides

Treated and untreated cells (10^6) were added to 300 μl of 0.4 N $HClO_4$ and kept at 0 °C for 10 min. After centrifugation at 5000 rev min^{-1} for 3 min, 150 μl of supernatant was neutralized with 75 μl of 0.8 M K_2CO_3. After filtration through a Millipore filter (code SN 180407), 100 μl was injected into a JASCO high performance liquid chromatography (HPLC) equipment (system controller, model 801-SC; UV–visible detector, model 875-UV; ternary gradient unit, model 880-02; HPLC pump, model 880-PU; Rheodyne injector equipped with 100 μl loop; Hypersil 5 ODS column, 250 mm × 5 mm). Adenylnucleotides were eluted with phosphate buffer (0.2 M, pH 6.0) according to Rottland et al. [35]. The absorbance was monitored at 254 nm (AUFS 0.01) by dedicated software installed on an MS-DOS computer. The concentrations of ATP, ADP and

AMP were calculated by fitting the area under the curve of each nucleotide to specific calibration curves (from 0.5 to 3 mmol) and expressed as nanomoles per 10^6 cells.

The adenylate energy charge (AEC) was calculated according to Atkinson [36]

$$AEC = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}$$

2.5. Morphological and cytochemical stains

The plating efficiency was calculated by comparing untreated and laser-treated (5, 15 and 30 min) cultured cells. In order to identify cell changes induced by laser irradiation, some treated and untreated coverslips were stained with haematoxylin–eosin, perodic acid Schiff (PAS) reaction, Alcian blue 8GX pH 2.5, Bromophenol blue and Oil Red O stains (for histochemical procedures, see Bancroft and Stevens [37]).

2.6. Immunocytochemical procedures

Treated and untreated cells were fixed in 4% *para*-formaldehyde in PBS (pH 7.4) for 5 min. The following reagents and primary monoclonal (mAb) and polyclonal (pAb) antibodies were used: fluorescein isothiocyanate (FITC)-labelled phalloidin (2.5 $\mu g\ ml^{-1}$, Sigma Chemical Company, USA), mouse anti-vinculin mAb (1–10 $\mu g\ ml^{-1}$, Boehringer Mannheim Biochemia, Germany), mouse anti-cytokeratin mAb (20 $\mu g\ ml^{-1}$, Boehringer), mouse anti-β-tubulin mAb (2 $\mu g\ ml^{-1}$, Boehringer), a mixture of mouse anti-α-tubulin mAb (1:1000, Amersham International, UK) and mouse anti-β-tubulin mAb (1:1000, Amersham), anti-microtubules associated proteins $(MAP)_1$ mAb (10 $\mu g\ ml^{-1}$, Boehringer), mouse anti-laminin mAb (10 $\mu g\ ml^{-1}$, Boehringer), goat anti-fibronectin pAb (1:20, Sigma), mouse anti-cellular fibronectin mAb (1:100, Sigma), rabbit anti-vitronectin pAb antibody (1:40, Telios Pharmaceutics Inc., San Diego, CA, USA) and rat anti-uvomorulin mAb (1:1000, Sigma). For the detection of microtubules, cells were dehydrated in cold absolute methanol (−20 °C, 5 min) followed by cold acetone (−20 °C, 5 s) and air dried, whereas for the detection of the other antigens, the cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min or non-permeabilized for extracellular detection of antigen. Incubation with primary antibodies was carried out for 30–60 min at 37 °C. After rinsing in PBS, cells were incubated for 30–60 min at 37 °C with the specific secondary antibodies (Boehringer) labelled with FITC or rhodamine (1:30). In some cases F-actin was simultaneously detected by adding 2.5 $\mu g\ ml^{-1}$ fluorescein-labelled phalloidin in the second incubation. After rinsing in PBS, coverslips were mounted in 50% PBS–glycerol and were observed and photographed with a Zeiss Photomikroskop III equipped with fluorescence illumination (band pass filter 485/20 and suppression filter 520 for FITC-labelled antibodies; band pass filter 546/12 and suppression filter 590 for rhodamine-labelled antibodies). The detection of fibronectin and uvomorulin was also performed by the immunoperoxidase technique using avidin–biotin-peroxidase complex (Peroxidase Vectorstain ABC Kit, Cat. no. PK-4000, Vector Laboratories, Burlingame, CA, USA) [38] with diaminobenzidine (DAB, Sigma) as substrate. The specificity controls were carried out using non-immune serum.

Image processing and analysis of treated and untreated cells after immunocytochemical staining were performed with a densitometer (Macintosh IIsi computer interfaced with a Microtek colour scanner from Microtek International Inc., Hsinchu, Taiwan). The image processing and analysis program (version 1.41) was obtained from Jet Propulsion Laboratory, NASA, FL, USA.

2.7. Statistical analysis

The significance of the differences between the groups of untreated and treated cells was evaluated by the *t*-test and two-way completely randomized variance analysis (ANOVA).

3. Results

3.1. Effect of low-power laser (LPL) irradiation on EUE ATP concentration and adenylate energy charge

The amount of ATP was evaluated by chromatographic and luminometric techniques; its concentration of EUE is about 4–6 nmol per 10^6 cells, with small differences between the two analytical methods (Table 1). The ATP concentration is unaffected by 15 and 30 min LPL treatment (Table 1). The amount of ADP (evaluated by a chromatographic technique) is about 1.4 nmol per 10^6 EUE cells. Laser treatment for 15 min does not affect ADP concentration, whilst after 30 min the amount of ADP decreases by about 39% ($p < 0.05$). The AMP concentration of EUE is around 0.30 nmol per 10^6 cells. After 15 min of LPL treatment, no significant differences were evaluated, whilst after 30 min the AMP concentration increases about sixfold over that of the controls.

The adenylate energy charge (AEC), evaluated as reported by Atkinson [36], is about 0.83 for the control, 0.84 after 15 min LPL treatment and 0.65 after 30 min (Table 1). The significant ($p < 0.05$) difference (−21.7%) between the control and 30 min laser treatment is a sensitive index of the metabolic (energetic) changes of EUE cells.

Table 1
Adenylnucleotide concentration in EUE cells (untreated cells, 15 min and 30 min treated cells) expressed as nanomoles per 10^6 cells ± standard error (s.e.) and AEC values ± s.e. Amount of ATP was evaluated by luminometric and chromatographic (HPLC) techniques

Treatment	ATP (luminometric) (nmol per 10^6)	ATP (HPLC) (nmol per 10^6)	ADP (nmol per 10^6)	AMP (nmol per 10^6)	AEC
Control	5.73 ± 0.14	4.24 ± 0.12	1.42 ± 0.15	0.31 ± 0.02	0.828 ± 0.01
Laser (15 min)	5.34 ± 0.21	4.14 ± 0.06	1.24 ± 0.05	0.30 ± 0.02	0.836 ± 0.01
Laser (30 Min)	5.67 ± 0.17	4.15 ± 0.05	0.86 ± 0.24	1.98 ± 0.17	0.655–0.01

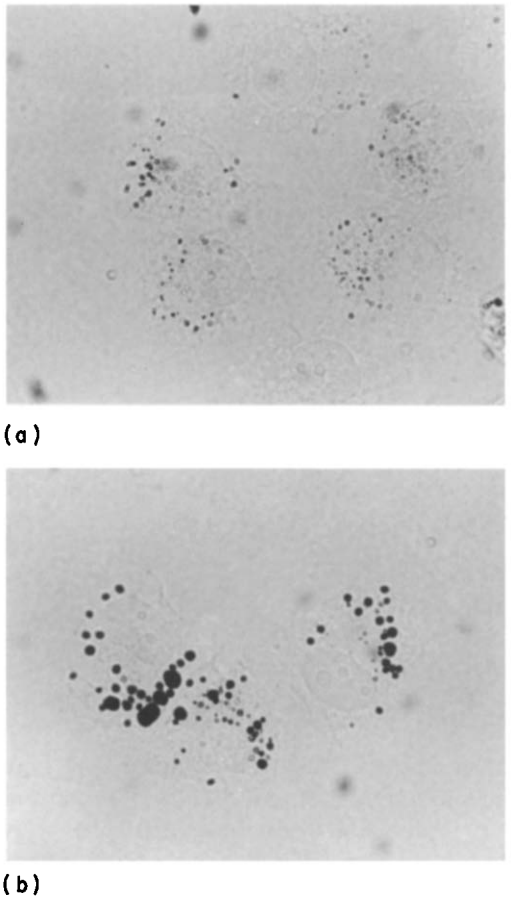


Fig. 1. (a) Untreated cells; (b) 30 min treated cells. Oil Red O stain. Note the increase in number and size of lipid droplets after irradiation.

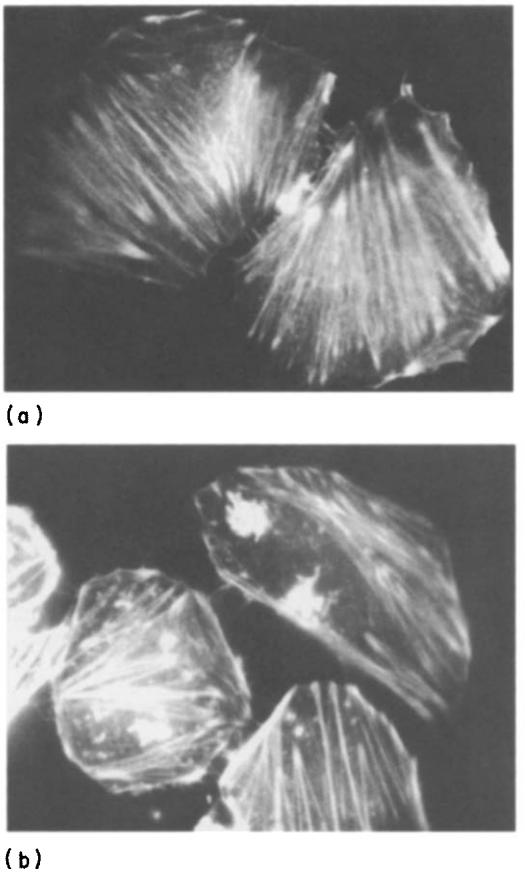


Fig. 2. (a) Untreated cells; (b) 30 min treated cells. Immunofluorescence stain with phalloidin. After 30 min of irradiation the stress fibres are thicker and disassembled.

3.2. *Effect of LPL irradiation on EUE cytoskeletal structures and adhesion molecules*

Laser irradiation for 5 min did not affect the cell structures in our experimental conditions. Modifications appeared after 15 min of treatment and became more evident after 30 min of laser irradiation. The plating efficiency was decreased by 20% only after 30 min of irradiation.

Morphological changes were not detected in cells after laser irradiation by the stains performed; a shift towards cytoplasmic acidophilia (eosinophilia) was observed in long-time treated cells, as well as less fibrillar aspects of chromatin in interphase cells. Modifications of the cytoplasmic glycoconjugate or protein components were not evident (PAS reaction, Alcian blue 8GX and Bromophenol blue stains). A small increase in lipid droplets was observed in treated cells (Oil Red O stain), where the lipid component appeared to be stored particularly in the innermost area of the cells (Fig. 1).

The cytoskeletal structures of EUE cells have been studied previously [25]. They show bundles of microfilaments of the stress fibre type that often span the whole width of cells and end at areas of focal contacts enriched in vinculin. An array of microtubules with monocentric distribution and intermediate filaments of

the keratin and vimentin type as wavy slender fibres are also present.

After 15 min of laser irradiation, the microfilaments show thicker stress fibres; after 30 min, the thickness is irregular along the fibres and in some cells the stress fibres are disassembled (Fig. 2). The areas of focal contacts revealed with vinculin antibody are more numerous and larger that those of the controls and are grouped at the cell periphery (not shown). The microtubules revealed with both anti-α- and anti-β-tubulin antibodies are increased slightly in number and give rise to thin waves arranged in a more compact network after 15 min of irradiation (Fig. 3). Some aggregation centres are also evident. No other changes are observed in cells irradiated for longer times. The most marked effect is on the intermediate filaments of the keratin type which form thick, packed bundles around the nucleus (Fig. 4). This feature starts after 15 min of irradiation and is more evident after prolonged treatment, since analysis indicates an increase in fluorescence in laser-treated cells compared with controls. The immunofluorescence stain for MAP_1 gives slightly positive results in untreated cells and is unchanged in treated cells.

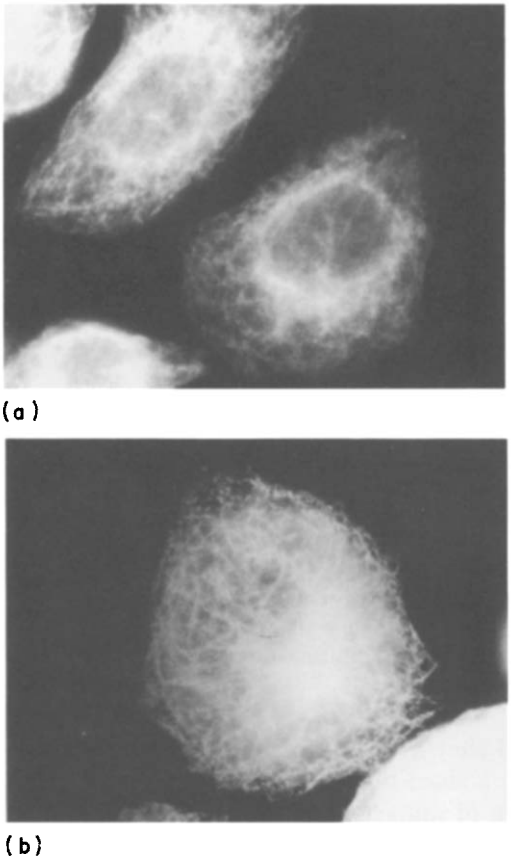


Fig. 3. (a) Untreated cells; (b) 15 min treated cells. Immunofluorescence stain with anti-β-tubulin antibody. After irradiation a more compact network of microtubules is evident.

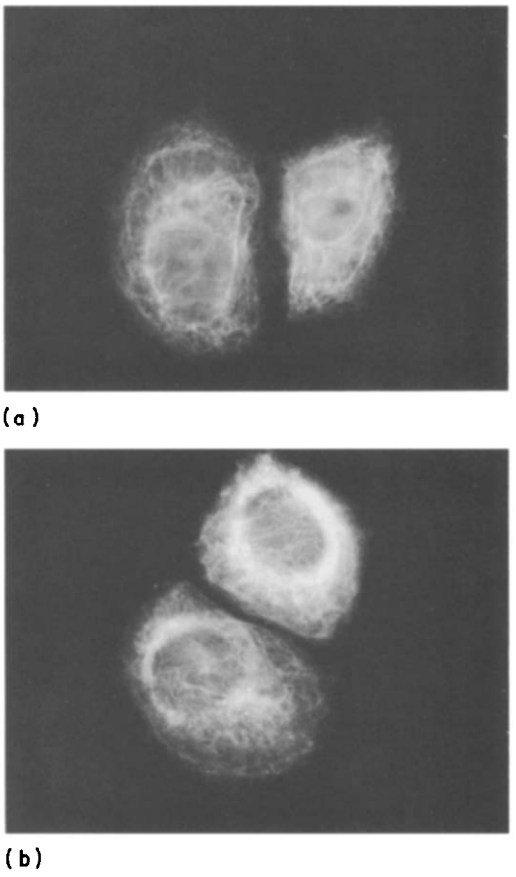
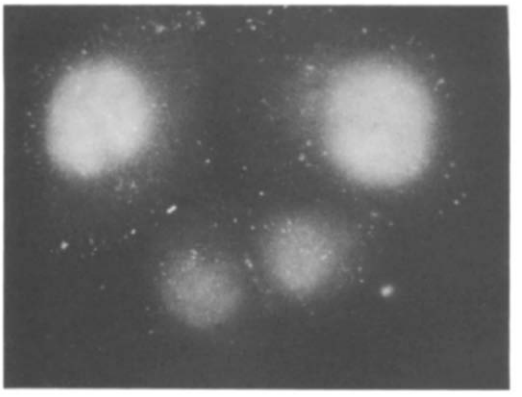


Fig. 4. (a) Untreated cells; (b) 30 min treated cells. Immunofluorescence stain with anti-keratin antibody. After irradiation the intermediate filaments of the keratin type form thick, packed bundles around the nucleus.

Several adhesion molecules can be detected on the plasma membrane of EUE cells by immunofluorescence or ABC methods with punctate staining: cellular fibronectin, laminin and uvomorulin (corresponding to L-cell adhesion molecules (L-CAM)). Negative results are obtained using the vitronectin antibody. A thin cytoplasmic network of fibronectin fibrils appears with specific monoclonal antibody when permeabilization is performed before the reaction.

An increase in laminin and uvomorulin expression on the plasma membrane is evident after 30 min of laser treatment (Figs. 5 and 6). This effect is not uniform on the plasma membrane but is particularly evident in some areas. No modifications are observed in fibronectin distribution. A marked increase in pixels around the nucleus and at cell borders appears in laser-treated cells submitted to image analysis. Figure 7 illustrates the photodensitometric analysis of untreated and treated cells for keratin evaluated by anti-cytokeratin mAb. The arrows indicate the peaks detected by densitometric analysis of treated cells, corresponding to the thick, packed bundles around the nucleus (see also Fig. 4).



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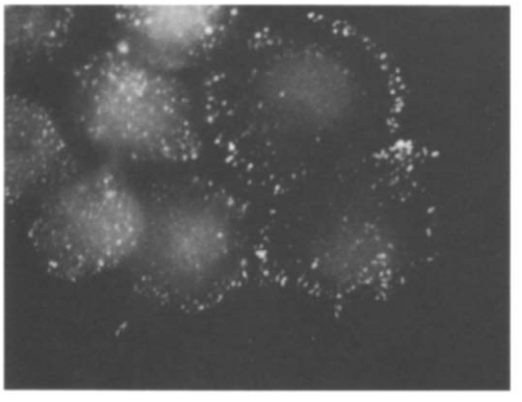
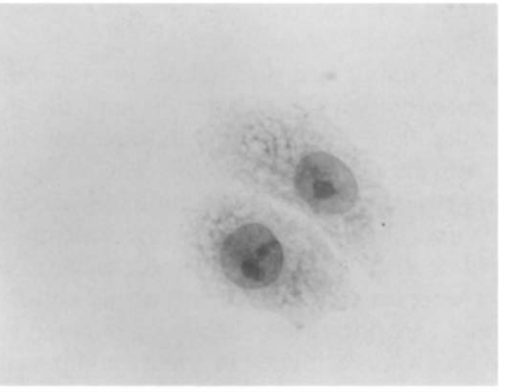


(b)

Fig. 5. (a) Untreated cells; (b) 30 min treated cells. Immunofluorescence stain with anti-laminin antibody performed without permeabilization. An increase in laminin expression is evident after irradiation.



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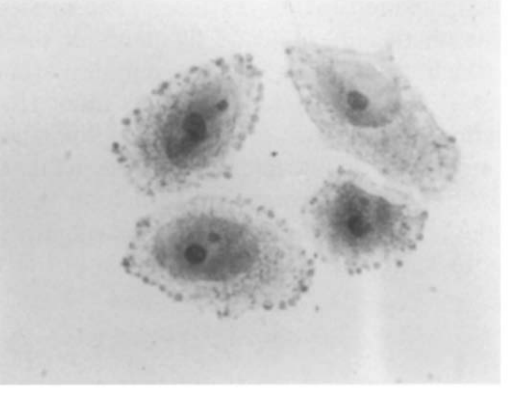

(b)

Fig. 6. (a) Untreated cells; (b) 30 min treated cells. Immunoperoxidase stain with anti-uvomorulin (L-CAM) antibody. An increase in L-CAM expression is evident after irradiation.

Figure 8 shows the analysis of untreated and treated cells for laminin evaluated by anti-laminin antibodies. The arrows indicate the peaks detected by densitometric analysis of treated cells, corresponding to the increase in laminin expression at the cell borders (see also Fig. 5).

4. Discussion

In the present research, the effects of red light (632 nm HeNe laser) on EUE cells were studied, focusing on the ATP balance with dephosphorylated nucleotides (ADP and AMP) and taking into account that ATP is involved in the activation of cytoskeletal precursor molecules.

The adenylate energy charge was also evaluated as a sensitive index of the energetic change in the cells [36]. It is modified by laser irradiation which affects the energetic cell balance. Laser irradiation causes the activation of de novo ATP synthesis in bacterial cells and isolated mitochondria [12,39]. In our experimental conditions, ATP is maintained almost constant despite its utilization in the activation of molecules involved in cytoskeletal polymerization processes, as clearly observed after 15 and 30 min of irradiation. ATP synthesis within a short time is probably supplied by laser stimulation of mitochondria [12].

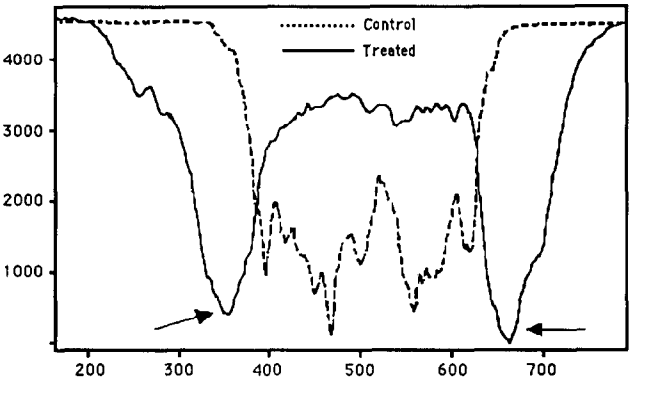

Fig. 7. Photodensitometric processing of untreated and treated EUE cells for keratin detected by anti-cytokeratin mAb. The abscissa indicates in arbitrary units the direction of scan along the longer axis of the cells. The ordinate expresses in arbitrary units the grey levels.

Adenylate kinase may also be involved in supplying ATP [36] according to the reversible reaction: $2ADP \rightleftharpoons AMP + ATP$. In fact, we observed, in treated cells, a marked decrease in ADP (39%) and an increase

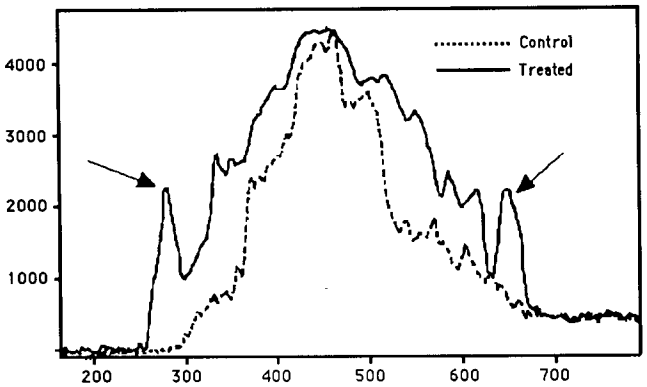

Fig. 8. Photodensitometric processing of untreated and treated EUE cells for laminin detected by anti-laminin antibodies. The abscissa indicates in arbitrary units the direction of scan along the longer axis of the cells. The ordinate expresses in arbitrary units the grey levels.

in AMP (about sixfold) indicating movement to the right in this reaction.

EUE cells submitted to laser treatment reorganize their cytoskeletal structures; this requires ATP supply as demonstrated above [40]. It is also known that energy is required to reach a more ordered state as a response to laser irradiation [41]. LPL (632 nm) irradiation of EUE cells leads to the remodelling of their cytoskeleton and some adhesion molecules. Three events are important: (1) the modification of the stress fibre assembly and the increase in vinculin-containing adhesion plaques; (2) the assembly and bundling of intermediate filaments; (3) the increase in laminin and L-CAM expression.

We suggest that the increase in cytoskeletal components is due to a new polymerization process stimulated by laser irradiation. The increase in microfilaments and intermediate filaments is prominent with respect to that of microtubules. Comparable modifications in cytoskeletal components are described in EUE cells adapted to a hypertonic medium [25]. It can be argued that LPL irradiation is effective in interacting with a homeostatic device involved in monomeric to polymeric transition processes controlling cytoskeletal structures.

The expression of adhesion molecules, such as laminin and L-CAM, is probably related to the epithelial nature of the cells, whilst the expression of fibronectin may be related to the activation of genes silent in the original phenotype. The increased expression of some adhesion molecules following laser treatment may be due to the interaction with regulatory systems involved in balancing intracellular and extracellular homeostasis of osmotic pressure. Moreover, it should be stressed that EUE cells submitted to long-lasting LPL irradiation develop large, peripheral, vinculin-containing adhesive plaques and large, numerous laminin patches which suggest that the capacity to adhere to the substratum is increased.

Moreover, ATP is utilized for the organization of intracellular and plasma membrane cell structures (in biosynthetic processes including nucleotide synthesis) and, in these conditions, ATPases must be stimulated, as previously demonstrated [42].

5. Conclusions

We can argue that LPL treatment can produce the modification of macromolecular and molecular cellular structures by acting on energetic processes. In addition, LPL treatment induces a modification of adhesion molecules with a remarkable increase in the capacity of the cells to adhere to the substratum.

It is evident that more ordered structures are derived by red light irradiation. This is demonstrated by the appearance of more ordered cytoskeletal structures in epithelioid cells (such as EUE). Similar cytoskeletal remodelling was observed in EUE cells adapted to hypertonic medium [25]. Important modifications were described in the cytoskeletal network for survival in hypertonic conditions: the stress response involved morphological and chemical changes.

It still remains to be proved whether red light irradiation is effective in eliciting a chemical response after an adaptation to thermal stress (note that thermal stress is associated with previous red and IR irradiation). It seems that 632 nm irradiation is effective in promoting a variation in energy charge and cytoskeletal changes as a "conditioned response" for cell defence against "thermal stress", although true thermal stress is prevented in our experimental conditions.

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