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Photobiological modulation of cell attachment *via* cytochrome c oxidase

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The number of cells attached to glass substrates increases if HeLa cell suspensions are irradiated with monochromatic visible-to-near infrared radiation (600–860 nm, 52 J m^{-2}) prior to plating. The well-structured relationship between this biological response and the radiation wavelength (action spectrum with maxima at 620, 680, 760, and 820 nm) suggests the existence of a photoacceptor responsible for the enhancement of attachment (presumably cytochrome c oxidase, the terminal enzyme of the respiratory chain) and, secondly, the existence of signaling pathways between the mitochondria, the plasma membrane, and the nucleus of the cell. Treating the cell suspension with ouabain (a Na^+, K^+-ATPase inhibitor), amiloride (an inhibitor of N^+/H^+ exchangers), or sodium azide (a cytochrome c oxidase inhibitor) prior to irradiation significantly modifies the action spectrum of cell attachment enhancement. The action of the chemicals under study also depends on their concentration and radiation fluence. Our results point to the existence of at least three signaling pathways (reaction channels) relating together the cell attachment, the respiratory chain, and the Na^+, K^+-ATPase and N^+/H^+ exchanger activities.

Introduction

Research into mechanisms of cell–cell and cell–matrix interactions is motivated by the involvement of the cell surface and its adhesive interactions in regulation of such processes as growth, differentiation, morphogenesis, wound repair, and formation of metastases, to name but a few. Cells from a freshly resuspended monolayer require energy to initiate and maintain their attachment to glass,[1] as well as to collagen-coated surfaces.[2] It was found that conditions which inhibit ATP synthesis (treatment of cells with respiratory chain inhibitors) reduce the ability of cells to attach to substrates. The inverse is also true, cell attachment can be increased in conditions where ATP extrasynthesis occurs, *e.g.* activation of the respiratory chain by irradiation of cells with monochromatic light in the visible-to-near infrared (NIR) spectral region.[3]

Comparative analysis of the dependence of cell attachment, as well as of DNA and RNA synthesis rates, on the wavelength of light used for irradiation (action spectra) and the available spectroscopic data for biomolecules absorbing in this region led to the proposition that the photoacceptor in mammalian cells is a redox intermediate of the terminal enzyme of the respiratory chain, cytochrome c oxidase.[4] Coincident action spectra for processes in the nucleus (DNA, RNA synthesis) and the plasma membrane (attachment) suggests that the photoacceptor is the same and implies the existence of signaling pathways between the photoacceptor located in the mitochondria and the nucleus, as well as between the photoacceptor and the plasma membrane.[5]

Early research into the photobiology of the interaction of monochromatic visible light with cells was initiated by the successful use of lasers in medicine, mainly for the improvement of tissue repair.[6] Recent research results evidence that improving cell metabolism by irradiation with monochromatic light provides benefits in the treatment of infraction,[7] the repair of spinal cord nerves,[8,9] the elimination of the toxic effects of chemicals,[10–12] and the promotion of cell survival.[13]

The latest developments in cellular signaling research have provided evidence that the plasma membrane enzymes Na^+, K^+-ATPase and Na^+/H^+ exchangers (NHEs), which were for a long time thought to be only ion pumps, play important roles in gene regulation and signal transduction as well.[14–16] The involvement of Na^+, K^+-ATPase and NHE in cell signaling cascades that are initiated by activation of the respiratory chain by laser radiation, leading to increased DNA and RNA synthesis rates, was first suggested more than a decade ago.[5]

The NHEs are a group of integral transmembrane proteins found in all mammalian cells which play a role in the regulation of intracellular pH (pH_i), cell volume, vectorial ion transport, and cell proliferation.[17] NHE activity and pH_i are believed to mediate some of the effects of adhesion on cellular proliferation.[18–20]

The signal-transducing role of Na^+, K^+-ATPase is now considered to be its standard function in most, if not all, cells.[15] Using partially inhibitory concentrations of a specific ligand of Na^+, K^+-ATPase ouabain, the signal transduction from Na^+, K^+-ATPase to the gene regulation has been shown to occur in HeLa cells as well.[21] HeLa cell attachment is initiated by both the non-specific substrate glass and the specific substrates gelatin and collagen, and follows patterns similar to those seen in cells cultivated *in vitro*.[22,23] Our previous research on cell attachment, DNA, RNA, and ATP synthesis enhancement, and on optical spectroscopy has been performed with HeLa cells.[4,5,10] It should be emphasized that the effects of irradiation on cellular metabolism are not specific to HeLa cells. All the cells studied so far respond to radiation in the visible-to-NIR region of certain wavelengths and fluences. The possibility of enhancing the metabolic process in cells, as well as the magnitudes of the irradiation effects, depend on the initial physiological state of the irradiated cells.[4,5]

In the present work, we use inhibitors of Na^+, K^+-ATPase (ouabain), NHEs (amiloride), and the respiratory chain (sodium azide) in combination with visible-to-NIR radiation of various wavelengths and fluences to study cellular signaling pathways that include cytochrome c oxidase. It will be shown that cell attachment can be modulated *via* at least three signaling pathways connecting the respiratory chain and the plasma membrane.

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Materials and methods

Cells

HeLa cells, obtained from the Institute of Virology, Moscow, Russia, were cultivated as monolayers in closed Carrell vials (diameter 45 mm) at 37 °C in 5 ml of RMPI-1640 (Flow Lab) with 10% fetal bovine serum (Flow Lab) and 100 units ml^{-1} of penicillin and streptomycin. 1.5×10^6 cells were planted per vial and grown for 72 h (middle-log phase). The HeLa culture used is characterized by anchorage-dependent growth and forms confluent monolayers. Characteristics of the growth of this culture can be found elsewhere.[24]

Cells were harvested using 0.02% Versene solution (37 °C) and suspensions for irradiation were prepared in RMPI-1640 medium containing 10% bovine embryo serum. Cell culture processing was performed in darkness or under dim natural light. Extraneous illumination (sunshine or artificial light) was avoided.

Light sources

Monochromatic radiation was obtained by means of a monochromator designed by Dr A. Lifshits at the Institute of Spectroscopy, Russian Academy of Sciences, Troitsk, to have bandwidths of 400–700 and 540–1050 nm. The monochromator was operated in accordance with an autocollimation scheme. A single slit 1.2 mm wide was used to direct radiation both into and out of the monochromator. Dispersion amounted to 8 nm mm^{-1}. Thus, the exit radiation bandwidths amounted to 10 nm (full width at half-maximum). The dispersive element used was a diffraction grating of 1200 lines mm^{-1}. To obtain radiation in the region 600–860 nm, the second order of the grating, a thermal filter, and a 600 nm cut-off glass filter, placed between the radiation source (a stabilized 250 W high-pressure xenon arc lamp) and the monochromator, were used. The source radiation was focussed with a positive lens and delivered by an optical fiber light guide to the upper part of the slit. After diffraction of the beam, the monochromatic radiation exited from the lower part of the slit and was transmitted by another light guide. In the wavelength range 600–860 nm, the output power of the monochromator and the power measured after the sample were constant. A Coherent Fieldmaster power meter (suitable for measuring powers in the microwatt range) was used for the power measurements. The light intensity in experiments was 1.3 W m^{-2}, the dose 52 J m^{-2}, and the irradiation time 40 s. The light spot fully covered the vial containing the cell suspension (0.38 cm^2).

Radiation at 820 nm was obtained from a GaAlAs light diode (Polyus). The technical characteristics of this light source are as follows: $\lambda = 820 \pm 10$ nm, pulse repetition frequency 10 Hz, pulse width 20 ms, and duty factor (ratio of pulse duration to pulse period) 20%. Irradiation was performed through an optical fiber so that a homogenous light spot covered the exposed suspension surface (0.38 cm^2). The radiation intensity at the suspension level was 3.54 W m^{-2}, and the radiation dose varied from 8 to 120 J m^{-2} (exposure time from 10 to 170 s). Intensity measurements were made using a Spectra-Physics M404 power meter.

Irradiation

The samples of cellular suspension were irradiated in special glass sample cells. These cuvettes were made as follows: two glass rings with ground-in edges (inner diameter 0.7 cm, height 0.4 cm, volume 130 μl) were stuck on a microscope slide 5 cm apart. Both wells were filled with cell suspension (85000 cells per vial). In all cases, one of the vials was irradiated and another one was used as the control. Optimal irradiation conditions (shape and dimensions of the vial, number of cells per vial) were developed in a special series of experiments. Irradiation was performed at room temperature in darkness. The control well was specially protected from light during the irradiation experiment. A new pair of sample cells was used for every measurement.

Measurement of cell–glass adhesion

The criterion by which changes in the adhesion properties of the cell membrane were judged was the number of cells that attached themselves to the bottom of the cuvette in the course of 30 min at 37 °C. This time was chosen according to experiments describing the time-course of attachment of HeLa cells to the glass substrate in our experimental conditions.[3] In the control culture, 42.5 ± 2.5% of cells adhered to the surface after 30 min incubation. Thus, in these conditions, the stimulatory and inhibitory effects of the irradiation and chemicals could be measured under the same conditions. After incubation, the nutrient medium was removed and the cuvettes were washed with warm (37 °C) Hanks' solution to remove non-attached cells. The attached cells were trypsinized and counted with a haemocytometer. Each data point represents the mean of at least 10 independent measurements.

Inhibitors

Amiloride, ouabain, and sodium azide were purchased from Sigma. 10 μl of a freshly prepared solution of an inhibitor in non-colored Hanks' solution (37 °C) was added to each cuvette; 10 μl of Hanks' solution without the inhibitor was added to the control cuvettes. The toxicity of the chemicals was assessed using the Trypan Blue exclusion test: all concentrations used were found to be non-toxic under our experimental conditions (viability of cells >95%). The solutions of inhibitors did not show any absorption bands in the optical region under study (checked by recording the absorption spectra).

Statistical analysis

The results obtained were statistically processed by means of the STATISTICA V.4.5 and GraphPad program packages, and expressed in terms of the mean value ± standard deviation over 10 measurements. $p < 0.05$ was considered significant using the ANOVA and Student's *t*-tests.

Results

Inhibition of cytochrome c oxidase with sodium azide

The number of cells attached to the glass surface increases upon irradiating the cell suspensions with light at wavelengths of 620, 680, 760, and 820–830 nm (curve labeled *hv* in Fig. 1). Recall that a graph representing a biological response as a function of wavelength is called an action spectrum; the action spectrum coincides (some limits exist) with the absorption spectrum of the photoacceptor molecule.[25]

Addition of sodium azide to cell suspensions prior to irradiation modifies the action spectrum of attachment increase significantly in the red spectral region (curve labeled Azide + *hv* in Fig. 1). The bands with maxima at 620 and 680 nm are completely eliminated in azide-treated samples. The bands in the NIR spectral region with maxima at 760 and 820–830 nm are influenced only a little by azide in terms of their shape, the peak positions being the same as in the curve labeled *hv*.

Fig. 2 presents the dependence of the percentage of adhered cells on the concentration of sodium azide in the absence of irradiation (curve labeled Azide). Under our experimental conditions, azide concentrations of between 4×10^{-8} and 2×10^{-5} M do not influence cell attachment. Cell attachment decreases at higher concentrations of azide (from 4×10^{-5} to 4×10^{-4} M); azide concentrations of between 1×10^{-4} and 4×10^{-4} M inhibit cell attachment practically to the same extent (percentage of attached cells 23.6 ± 2.1 and 22.6 ± 3.3%, respectively). Such a saturation-type curve may indicate the existence of a subpopulation of cells which are insensitive to azide.

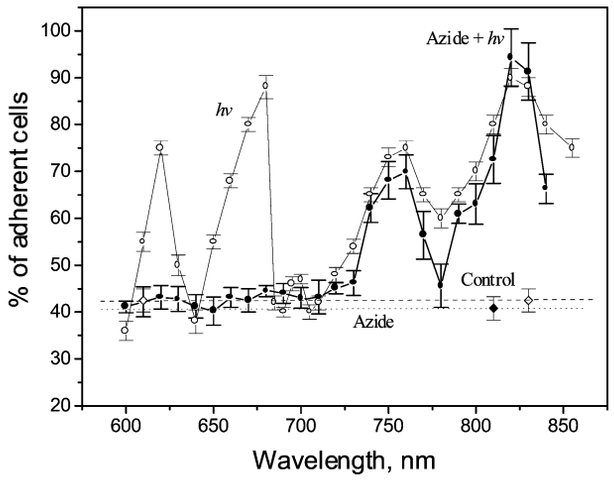

Fig. 1 Dependence of cell attachment on wavelength used for irradiation of cell suspensions without (curve labeled *hv*) and with sodium azide (2×10^{-5} M) added (curve labeled Azide + *hv*) prior to irradiation (CW light, fluence 52 J m^{-2}, intensity 1.3 W m^{-2}, irradiation time 40 s, adhesion measurements performed 30 min after irradiation). The dashed line indicates cell attachment in the non-irradiated suspension (control). The dotted line indicates the attachment of cells in the presence of azide (2×10^{-5} M) without irradiation.

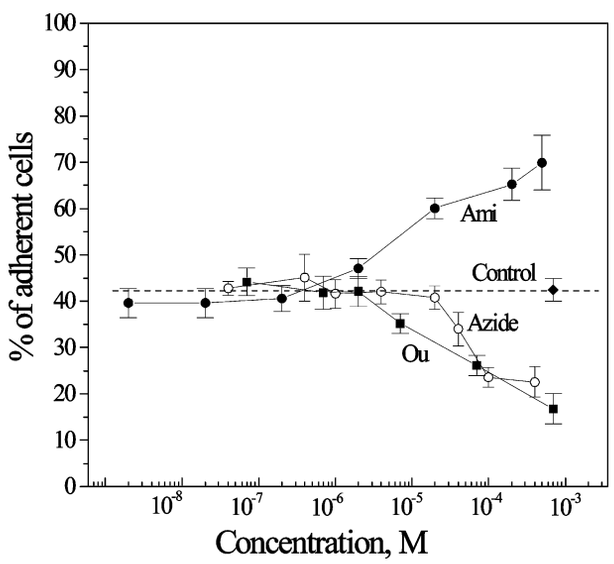

Fig. 2 Dependence of cell attachment on concentration of inhibitor added to the cell suspension in the absence of irradiation: sodium azide (Azide), ouabain (Ou), and amiloride (Ami).

For the action spectroscopy experiment (Fig. 1), sodium azide was used at a concentration of 2×10^{-5} M. This concentration of azide does not influence cell attachment (Fig. 2). In the next series of experiments, we examined a situation when azide is added in an inhibitive concentration (1×10^{-4} M) and the light fluence is varied from 8 to 120 J m^{-2}. The irradiation wavelength for this experiment was chosen as 820 nm, *i.e.* the maximum in the action spectrum, which was unaffected by azide in the previous experiment (Fig. 1). Furthermore, pulsed radiation was used. This type of radiation provides a less stimulative effect (64.5 ± 2.0%) when used in optimal fluence (60 J m^{-2}, maximum of curves labeled *hv* in Fig. 3) as compared with the continuous wave (CW) light used in action spectroscopy (Fig. 1).

A sodium azide concentration of 1×10^{-4} M strongly inhibits cell adherence (in the control suspension, 42.5 ± 2.5% of cells are attached; in the presence of azide, this value is 23.6 ± 2.1%). Irradiation increases the percentage of cells attached from suspensions pretreated with azide [curve labeled Azide + *hv* in Fig. 3(A)]. At a fluence of 60 J m^{-2}, the percentage of attached cells (38.7 ± 3.0%) is comparable to that in the control experiment (42.5 ± 2.5%), and with further increases in fluence, the

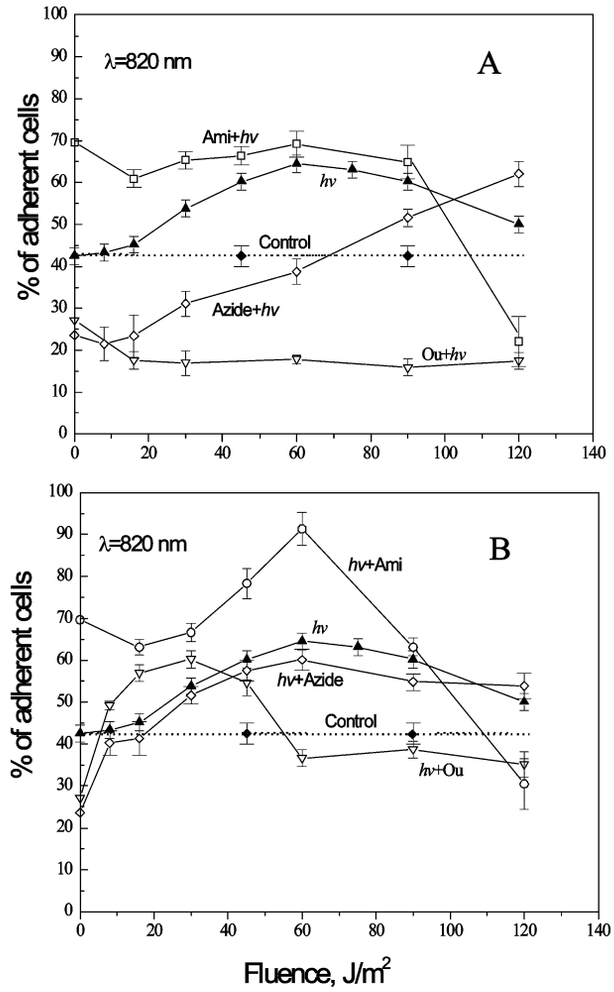

Fig. 3 Effects on cell attachment of sodium azide (1×10^{-4} M), ouabain (7×10^{-5} M), and amiloride (5×10^{-4} M) added to the cell suspensions before (A) or after (B) various radiation doses (pulsed radiation at $\lambda = 820$ nm, intensity 3.54 W m^{-2}, irradiation time 10–170 s). The dashed lines show the attachment of control cells; the curves labeled *hv* indicate the dose-dependence of light action.

percentage of attached cells increases as well. At a fluence of 120 J m^{-2}, the percentage of attached cells in this experiment (62.1 ± 3.0%) is comparable with percentage of attached cells for the 'irradiation only' experiment at 60 J m^{-2} [64.5 ± 2.0%; curve labeled *hv* in Fig. 3(A)].

Treatment of previously irradiated cell suspensions with sodium azide has only a non-significant effect on cell attachment [curve labeled *hv* + Azide in Fig. 3(B)] as compared to samples of irradiated cells with no azide added [curve labeled *hv* in Fig. 3(B)].

The two sets of experiments described above evidence that addition of sodium azide to cell suspensions causes changes in the absorption of the primary photoacceptor, as shown by the action spectra (Fig. 1). The complicated dependence of cell attachment on the radiation dose and azide concentration (Fig. 2 and 3) point to both factors having a subtle influence upon a particular reaction channel (cellular signaling cascade) between the photoacceptor and the response of cells to irradiation (cell attachment).

Inhibition of Na^+, K^+-ATPase with ouabain

Fig. 4 presents the action spectra for increases in cell attachment in suspensions without (curve labeled *hv*) and with ouabain (1×10^{-6} M) added (curve labeled Ou + *hv*) prior to irradiation. Ouabain modifies the action spectrum significantly in the far-red spectral region: two bands characteristic of the action spectrum at 680 and 760 nm (curve labeled *hv*) are completely

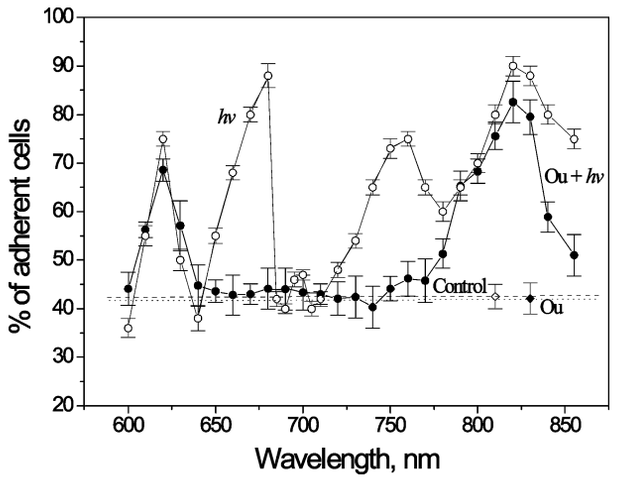

Fig. 4 Dependence of cell attachment on wavelength used for irradiation of cell suspensions without (curve labeled *hv*) and with ouabain (1×10^{-6} M) added (curve labeled Ou + *hv*) prior to irradiation (CW light, fluence 52 J m^{-2}, intensity 1.3 W m^{-2}, irradiation time 40 s, adhesion measurements performed 30 min after irradiation). The dashed line indicates cell attachment in the non-irradiated suspension (control). The dotted line indicates the attachment of cells in the presence of ouabain (1×10^{-6} M) without irradiation.

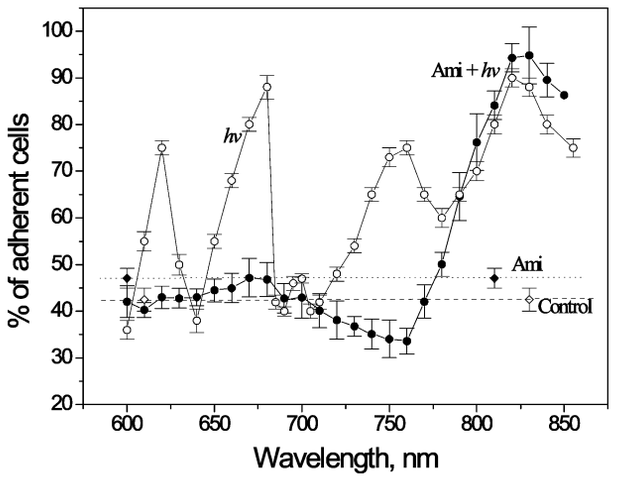

Fig. 5 Dependence of cell attachment on wavelength used for irradiation of cell suspensions without (curve labeled *hv*) and with amiloride (2×10^{-5} M) added (curve labeled Ami + *hv*) prior to irradiation (CW light, fluence 52 J m^{-2}, intensity 1.3 W m^{-2}, irradiation time 40 s, adhesion measurements performed 30 min after irradiation). The dotted line indicates cell attachment in the non-irradiated suspension (control). The dashed line indicates the attachment of cells in the presence of amiloride (1.7×10^{-5} M) without irradiation.

eliminated (curve labeled Ou + *hv*). The positions of the bands at 620 and 820–830 nm are unchanged and only minor changes in the shape of the NIR band are seen in the region 830–860 nm (curve labeled Ou + *hv*).

At the concentration of 1×10^{-6} M used in the action spectroscopy experiment (Fig. 4), ouabain does not influence cell attachment in non-irradiated samples (curve labeled Ou in Fig. 2). Increasing the concentration of ouabain decreases cell attachment practically linearly. When ouabain is added to cell suspensions in an inhibitive concentration of 7×10^{-5} M (26.1 ± 2.2% of cells attached) and irradiation is performed subsequently with various fluences from 16 to 120 J m^{-2}, the respective dependence is determined by the action of ouabain [curve labeled Ou + *hv* in Fig. 3(A)]. The situation is quite different when 7×10^{-5} M ouabain is added after irradiation [curve *hv* + Ou in Fig. 3(B)]. There is a stimulation of cell attachment at lower doses of radiation (8–48 J m^{-2}), which is statistically significantly higher than the stimulation caused by irradiation at these fluences. Slight inhibition of cell attachment at a level of 36–38% is seen at higher fluences (60–120 J m^{-2}). Recall that ouabain alone at this concentration inhibits cell attachment more strongly (26.1 ± 2.2%; Fig. 2).

So, the addition of ouabain to cell suspensions changes the absorption of the photoacceptor molecule in cells, as evidenced by the action spectra presented in Fig. 4. Different bands in this action spectrum are modified by ouabain (Fig. 4) as compared with those affected by the action of sodium azide (Fig. 1). This finding suggests that a different reaction channel (signaling pathway) between the respiratory chain and the plasma membrane is involved in this case.

Inhibition of Na^+/H^+ exchangers with amiloride

Fig. 5 presents the action spectra for increases in cell attachment in suspensions without (curve labeled *hv*) and with amiloride (2×10^{-5} M) added (curve labeled Ami + *hv*) prior to irradiation. Amiloride modifies the action spectrum of cell attachment significantly: three bands at 620, 680, and 760 nm are eliminated; the band at 760 nm is not only eliminated, but amiloride also causes a slight inhibition of cell attachment below the control level. The band with a maximum at 820–830 nm is practically unmodified in both position and shape.

Addition of amiloride to samples without irradiation stimulates cell attachment at concentrations higher than 2×10^{-6} M in a concentration-dependent manner (curve labeled Ami in Fig. 2). For example, at concentrations of 2×10^{-4} and 5×10^{-4} M, the percentage of adhered cells (65.2 ± 3.5 and 69.9 ± 5.9%, respectively) is comparable to that in samples irradiated at the optimal dose (60 J m^{-2}) at 820 nm (64.5 ± 2.0%). We did not find any inhibition of cell attachment by amiloride without irradiation in concentration range used (2×10^{-9}–5×10^{-4} M; curve labeled Ami in Fig. 2).

When amiloride (5×10^{-4} M) is added to a cell suspension before irradiation at 820 nm [Fig. 3(A)], irradiation at doses of 8–90 J m^{-2} does not appear to have any effect on cell attachment: the percentages of attached cells under combined action are comparable to the percentages of attached cells under the action of amiloride alone (69.9 ± 5.9%). In the fluence range 90–120 J m^{-2}, a sharp decrease in the percentage of adhered cells can be seen [curve labeled Ami + *hv* in Fig. 3(A)].

Irradiation of cells in various doses before treatment with amiloride [curve labeled *hv* +Ami in Fig. 3(B)] results in a rather similar dependence to that described above for irradiation after treatment with amiloride. But there is one difference; in fluence range 30–90 J m^{-2}, there is a zone of stimulation of cell attachment, which is specific for this treatment order [curve labeled *hv* +Ami in Fig. 3(B)].

The experiments with amiloride evidence that the addition of this inhibitor to cell suspensions prior to irradiation changes the absorption of the primary photoacceptor. These changes (Fig. 5) are different from those caused by azide (Fig. 1) and ouabain (Fig. 4), suggesting that a different reaction channel between the photoacceptor and the plasma membrane is involved.

Discussion

The number of HeLa cells attached to glass substrates increases after irradiation of cell suspensions with monochromatic visible-to-NIR radiation, which means that a new subpopulation of cells enter an adhesive state due to irradiation. The percentage of the subpopulation made capable of adhering to glass depends on the parameters of the radiation used, *i.e.* wavelength (existence of the action spectrum), fluence, and type (continuous wave or pulsed).[3,10] The action spectrum of cell attachment enhancement shown in Fig. 1, 4, and 5 (curves labeled *hv*) is believed to mirror the absorption spectrum of one (as yet unidentified) redox intermediate of cytochrome c oxidase.[4] The signaling pathways (reaction channels) that are involved in signal transduction between the respiratory chain

and the plasma membrane have not yet been established. The experimental data evidence that ouabain not only modifies cell attachment,[26–28,14] but also regulates respiration activity[29] and suppresses glucose-induced mitochondrial ATP production by generation of reactive oxygen species.[30]

The main finding in the present work that sodium azide, ouabain, and amiloride modify the action spectrum of cell attachment enhancement points to interference between the actions of radiation and chemicals on the level of cytochrome c oxidase. However, at this time there is no experimental evidence to show how the signals are transduced and it has not been established which particular pathways are involved. Note that direct action of radiation in the visible-to-NIR region on cell attachment cannot be considered because the cell–matrix and cell–cell attachment molecules isolated and characterized so far belong to the large family of glucoproteins which are not capable of absorbing light in this optical region.[31,32]

The bands in the action spectrum represented by the curve labeled *hv* in Fig. 1, 4, and 5 were identified by analogy with the absorption spectra of the metal–ligand systems.[4] The main contribution to the 820–830 nm band is believed to arise from the oxidized Cu_A, to the 760 nm band from the reduced Cu_B, to the 680 nm band from the oxidized Cu_B, and to the 620 nm band from the reduced Cu_A chromophores of cytochrome c oxidase. Recall that the redox-active Fe and Cu pairs of cytochrome c oxidase are situated in complex IV of the mitochondrial electron transport chain, where the electrons pass to molecular oxygen. However, the roles of each redox pair, and especially the mechanisms describing how the changes in the concentrations inside each pair drive ATP synthesis, remain elusive, mainly due to technical measurement limitations caused by extremely rapid electron and proton transfer in redox reactions under physiological conditions, as well as by the extreme complexity of the carrier molecules.

Under normal physiological conditions, fully oxidized or fully reduced cytochrome c oxidase is not present, it can be only more reduced or less reduced (less oxidized or more oxidized). During respiration, the Cu_A chromophore of cytochrome c oxidase (more exactly, a pair of Cu_A chromophores) accepts electrons from the cytochrome c molecule; internal electron transfer between Cu_A, heme *a*, and the heme a_3–Cu_B center causes reduction of molecular oxygen *via* at least seven redox intermediates.[33] The photoacceptor in our case is believed to be one (not yet identified) of those intermediates. The data on the absorption spectra of these transient intermediates is limited due to the technical difficulties associated with measurements (extremely fast electron transfer, broad and overlapping absorption bands). For these reasons, the exact contribution of the absorption of each chromophore to the broad absorption bands is not known. This circumstance makes it impossible evaluate exactly the contribution of the chromophores to the action spectrum. Redox absorbance measurements on cell monolayers under physiological conditions evidence, firstly, that four bands present in the action spectrum (curve labeled *hv* in Fig. 1, 4, and 5) are also observed in the absorption spectra and, second, irradiation of the cell monolayer with light can change both the absorption[34] and optical activity[35] of these bands.

Elimination of a band in the action spectrum to the control level by an inhibitor added before irradiation evidences that a particular reaction channel, which was activated by absorption of light, is not activated in the presence of this particular chemical. For this reason, the percentage of adherent cells remains at the control level. This means that the irradiation opens a new reaction channel(s) that allows more cells to be attached. In the presence of an inhibitor, this channel is closed. The percentage of this new subpopulation attached under irradiation depends not only on the wavelength of light used for irradiation (action spectrum labeled *hv* in Fig. 1, 4, and 5), but also on fluence (curve labeled *hv* in Fig. 3).

Comparison of action spectra recorded in the presence of sodium azide (Fig. 1), ouabain (Fig. 4), or amiloride (Fig. 5) indicates that three different types of modifications occur. Sodium azide (Fig. 1) eliminates the bands at 620 and 680 nm (thought to be due mostly to $Cu_{A_{reduced}}$ and $Cu_{B_{oxidized}}$). Ouabain (Fig. 4) eliminates the bands at 680 and 760 nm (due to $Cu_{B_{oxidized}}$ and $Cu_{B_{reduced}}$). Amiloride (Fig. 5) eliminates three bands at 620, 680, and 760 nm ($Cu_{A_{reduced}}$, $Cu_{B_{oxidized}}$, $Cu_{B_{reduced}}$). The band at 760 nm in this action spectrum is not only eliminated, but there is an inhibition of cell attachment below the control level. This finding indicates that amiloride also interferes with cells from the subpopulation that do not need light activation for attachment.

There are two common features in the action spectra that are modified by the inhibitors. First, in all three cases, the position of the band with a maximum at 820–830 is not changed at all and its shape is only altered slightly (Fig. 1, 4, and 5). This suggests that the inhibitors do not cause modifications near the Cu_A chromophore in the oxidized state. On the other hand, the neurohormone melatonin causes major changes in this particular band and not in bands in the red spectral region.[36] Secondly, all three chemicals eliminate the band at 680 nm (Fig. 1, 4, and 5). It can be suggested on the basis of these data that the absorption of the Cu_B chromophore in its oxidized state is changed in all cases. Recall that reaction with oxygen occurs in the a_3–Cu_B center of cytochrome c oxidase.[37] In other words, this center plays a decisive role in cellular energetics, ATP synthesis included.

Changes in absorption (reflected in the action spectra in our case) are commonly caused by structural and conformational changes. The type of interaction occurring between the inhibitors under study and cytochrome c oxidase cannot by evaluated by the present type of experiments. It is known that azide binds to cytochrome c oxidase bridging Cu_B and a_3.[37] The results of experiments looking at the combined action of sodium azide and irradiation (Fig. 3) suggest that irradiation facilitates the appearance of a product (reaction intermediate) that does not allow azide to block the reaction channel that is connected with attachment. In other words, this as yet unidentified product of irradiation can facilitate cell attachment by eliminating or bypassing the azide block.

Amiloride and ouabain, as higher molecular weight substances, probably cannot react with cytochrome c oxidase (and the a_3–Cu_B center in particular) in the same way as the small azide ligand does. Xie and Askari[15] suggested that signals to mitochondria are transduced *via* protein–protein interactions by ouabain binding to Na^+,K^+-ATPase. They believe that the activation of this regulation pathway is a common feature of the signal-transducing function of Na^+,K^+-ATPase in most cells, including HeLa.

Radiation with the appropriate parameters (wavelength, fluence) increases the percentage of cells capable of adhering to glass substrates (curves labeled *hv* in Fig. 1 and 3–5). This implies that irradiation makes a parameter of cellular homeostasis optimal for attachment in a new subpopulation. It is known that attachment of cells to solid substrates is accompanied by an increase in intracellular pH (pH_i) of 0.1–0.3 units.[18] An intracellular signaling pathway is believed to mediate the effects of cell adhesion and receptors of pH_i.[19] The increase in pH_i when the cells come into contract with glass and other substrates causes a short-term activation of NHE which can blocked by amiloride.[18,20] With this in mind, our result that amiloride increases cell attachment *without irradiation* (Fig. 2) is rather unexpected. Recall that our experimental conditions were chosen specifically to keep the number of cells attached reasonably low (42.5 ± 2.5% in control samples). It can be speculated that there is a subpopulation with elevated pH_i and amiloride helps those cells to achieve the optimal pH_i for attachment. The complicated dependencies arising from the combined action of radiation and amiloride (Fig. 3) lead to the

hypothesis that these two factors influence different signaling cascades with partly shared pathways.

Both amiloride and sodium azide[38] induce intracellular acidification, decreasing pH_i. But the action of these two chemicals (in the absence of irradiation) on cell attachment is different (Fig. 2), as is their action on light-induced increases in cell attachment (Fig. 1, 3, and 5). This comparison suggests that signal transduction pathways are more complicated than simple regulation of pH_i.

It was found in this work that the classical inhibitor of cytochrome c oxidase sodium azide, the classical inhibitor of Na^+,K^+-ATPase ouabain, and the classical inhibitor of Na^+/H^+ exchangers amiloride influence cell attachment *via* interaction with chromophores of cytochrome c oxidase. It is suggested that, for azide, this interaction is direct, *via* binding to the a_3–Cu_B center, and for amiloride and ouabain, it is a facilitated one. Three different as yet unidentified reaction channels between cytochrome c oxidase and cell attachment could be involved, but partly shared signaling pathways quite probably exist.

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