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Electric Discharge Plasmas Influence Attachment of Cultured CHO K1 Cells

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Non-thermal plasmas can be generated by electric discharges in gases. These plasmas are reactive media, capable of superficial treatment of various materials. A novel non-thermal atmospheric plasma source (plasma needle) has been developed and tested. Plasma appears at the end of a metal pin as a submillimetre glow. We investigate the possibility of applying the plasma needle directly to living tissues; the final goal is controlled cell treatment in microsurgery. To resolve plasma effects on cells, we study cultured Chinese hamster ovarian cells (CHO-K1) as a model system. When these are exposed to the plasma, instantaneous detachment of cells from the surface and loss of cell–cell interaction is observed. This occurs in the power range 0.1–0.2 W. Cell viability is assessed using propidium iodide (PI) and cell tracker green (CTG) fluorescent staining utilizing confocal laser scanning microscopy (CLSM). Detached cells remain alive. Use of higher doses (plasma power >0.2 W) results in cell necrosis. In all cases, plasma-influenced cells are strictly localized in submillimetre areas, while no reaction in surrounding cells is observed. Due to its extreme precision, plasma treatment may be applicable in refined tissue modification. Bioelectromagnetics 25:362–368, 2004.

Key words: cell adhesion; cell detachment; plasma needle

INTRODUCTION

Plasma Application

Plasma is a partially ionized gas, containing electrons, positive/negative ions, radicals, and various excited atoms and molecules. Plasma generated by an electric discharge can be non-thermal: electrons present in this active medium are highly energetic, with typical temperatures above 10 000 K, while ions and neutral species remain at (almost) room temperature. Such a non-equilibrium situation is achieved when only electrons are electrically heated. This is common in high frequency driven discharges, e.g., in the radio frequency (MHz) or microwave (GHz) range, because in these systems only the light and mobile electrons are able to follow rapidly oscillating electric fields. Energy transfer from electrons to heavy atoms and molecules is an inefficient process, so that the background gas remains relatively cold (<1000 K). The best known example is a low pressure plasma, sustained in a vacuum reactor. Non-thermal discharges can be also obtained at atmospheric pressure [Laroussi, 1996]. When the size of the discharge is below 1 mm, energy leaks by thermal diffusion prevent gas heating. A combination of two physical principles: high frequency excitation and spatial constriction of the discharge, has recently resulted in the development of a novel non-thermal atmospheric plasma source, the ''plasma needle'' [Stoffels et al., 2002].

Due to their unique reactivity and chemical interactions with surfaces, non-thermal plasmas are capable of virtually any surface treatment. Plasma etching, thin layer deposition, cleaning, and activation of surfaces are well established techniques in material science. Recently, plasmas are also applied in biomedical technology, e.g., in plasma coating of artificial implants to increase their biocompatibility [Klein et al.,

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1994], surface micropatterning of scaffolds to achieve controlled cell adhesion [Ohl and Schröder, 1999], and bacterial decontamination of medical/surgical equipment [Laroussi, 1996; Moisan et al., 2001]. The effects of plasma treatment on microorganisms under lethal and sublethal doses have been studied by Laroussi et al. [2002].

For interactions with living tissues, the choice of an adequate plasma source is crucial. It must operate under atmospheric pressure, be electrically and chemically safe, and may not cause any thermal damage to the living object. Most hitherto developed sources, although non-thermal, are still too hot or aggressive to be applied in vivo.

In this paper, we shall demonstrate that the plasma needle is suitable for treatment of biological materials. Our aim is to introduce this source as a novel tool for high precision treatment of pathological tissues. Desired effects are controlled cell removal without inflammatory reaction, damage to the surrounding (healthy) tissue, and possibly inducing apoptosis. At present, we conduct a fundamental study to identify and elucidate various cell responses. First, we perform temperature measurements to establish that plasma needle does not inflict thermal damage. Plasma–cell interactions are investigated using Chinese hamster ovarian cells (CHO-K1, ATCC number CCL-61). These fibroblasts make a good model system for a study of basic cell responses to a medium, which up till now has been unknown to living objects.

EXPERIMENTAL METHODS

Principle of Plasma Operation

The discharge type was an atmospheric radio frequency (RF) glow. In case of RF excitation, an active plasma zone is created only at the powered electrode, i.e., the electrode to which RF voltage is applied. A bipolar (two electrode) configuration is not necessary. In our arrangement, the powered electrode was a metal wire. The (remote) surroundings, like the walls of the plasma box, act as the counter electrode (ground). In this type of discharges, voltage fall occurs in the closest vicinity of the powered electrode; in the outer plasma zones, which make contact with samples, only small residual electric fields are present. Plasma appears as a faint glow with less than 1 mm diameter, located at the tip of the powered electrode (see Fig. 1). Depending on the desired chemical effect, various gases can be used, e.g., air, nitrogen, hydrogen, and many others. However, it is recommended that the buffer gas be helium, because the operating voltages are lower than in other gases and the gas temperature is low due to high thermal

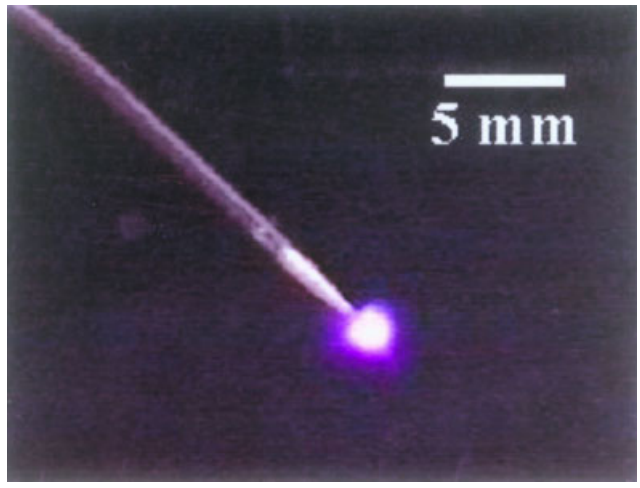

Fig. 1. Typical appearance of the plasma glow generated at the tip of a metal wire. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

conductivity of helium. Moreover, helium is inert and non-toxic. The latter feature allows keeping a good control of plasma chemistry, because active gases can be carefully introduced into the buffer gas.

Electric Apparatus

Plasma was generated using a Hewlett Packard (Palo Alto, CA) 33120A waveform generator in combination with an Amplifier Research (Souderton, PA) 75AP250RF amplifier and a home-built λ-type matching network (see Fig. 2). The powered electrode was a metal needle 5 cm long and 0.3 mm diameter; the discharge excitation frequency was about 10 MHz, and the peak-to-peak RF voltage was about 300 V. Both sine and square wave excitations could be applied;

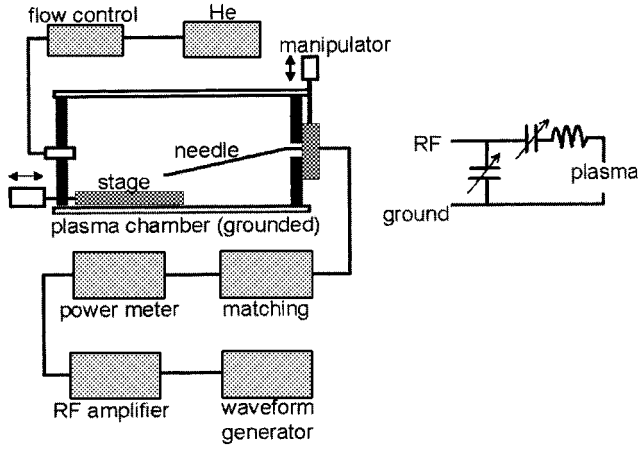

Fig. 2. Scheme of the experimental apparatus. The matching network is depicted at the right hand side. It consists of two variable capacitors (0–2 nF) and a coil (1.6 μH).

the latter had a somewhat lower ignition threshold voltage. Optimizing power dissipation in the plasma was performed either by varying capacitances in the matching network or by tuning the RF frequency to achieve resonance in this AC circuit. The latter method allowed for much finer tuning, because the circuit had a high quality factor (width of the resonance curve was in the kHz range) and the frequency of the waveform generator can be varied more accurately than the capacitances. The discharge power was monitored using an Amplifier Research PM 2002 power meter connected to an Amplifier Research dual directional coupler. The power input into the plasma was 0.1–0.3 W.

Plasma Chamber

The plasma chamber was a 10×10 cm stainless steel box, closed by removable plastic covers (see Fig. 3). The connection was not vacuum-tight. The-plasma chamber was filled with gas (inlet is visible at the left hand side in Fig. 3) with a flow rate up to 2 L/min, controlled by a Brooks series 5850E mass flow controller (Brooks Instrument, Hatfield, PA). Air contamination due to leakage is in the sub percent range. Striving for higher purities was not of much use, because the presence of air and water vapor is inevitable during treatment of the living cells. Samples for plasma treatment were placed on a moving stage, mounted at the bottom of the chamber. The samples could be moved up to 2 cm in the right–left direction. A similar manipulator was used to adjust the vertical distance between the needle and the sample. Both manipulators were operated externally with an accuracy of 0.01 mm.

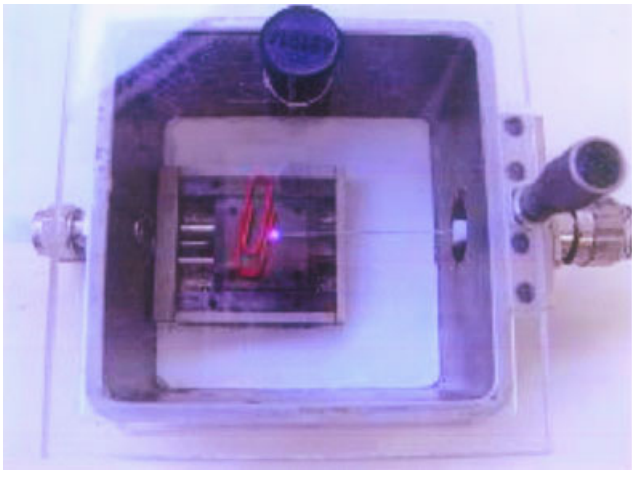

Fig. 3. Photograph of the plasma chamber from above. The plasma needle can be moved up and downwards. The samples are placed on the moving stage visible at the bottom. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Cell Culture

CHO-K1 cells were cultured in flasks containing Ham's F-12 medium with stable L-glutamin (Bio Whittaker Europe, Verviers, Belgium) containing 9.0% of fetal bovine serum (FBS, Biochrom AG, Berlin, Germany) and 0.45% of Gentamycin (10 mg/ml, Biochrom AG). The cells were maintained in an incubator at 37 °C with 5% CO_2. To prepare samples for plasma treatment, the cells were trypsinized (0.05% Trypsin/0.02% EDTA solution in PBS, Biochrom AG) and transferred onto sterilized object slides ($26 \times 10 \times 1$ mm) and placed in multiwell dishes. The cells were incubated in the multiwell dishes for 2 days prior to treatment.

Plasma Treatment

For the treatment, the samples were taken out of the incubator for 15–30 min. No indication was found in control samples that cells were affected by this. Just before treatment, the sample was put in the helium-filled plasma box. To prevent drying out of the cells, the sample was covered with a layer of phosphate buffered saline (PBS). The plasma needle was brought close to the sample; visually the glow was just touching the surface of the PBS solution. The treatment time was 1 min, during which the sample was moved by the manipulated stage over a distance of 1 cm. This produced a typical ''track'' of plasma-treated cells, which could be easily recognized under the microscope. Individual cells on this track were irradiated for 5 s. The total treatment time could not be varied in a broad range: it was limited by the (low) speed of the manipulator and by avoiding sample desiccation due to helium flow. The experiments were performed at least five times for a given condition. After exposure to the plasma, the samples were either immediately observed under the microscope (within 1–2 min) or returned to the incubator and observed several hours after treatment.

Visualization

Cells were studied with a light microscope and with a confocal laser scanning microscope (LSM 510 by Zeiss, Oberkochen, Germany). Trypan blue (0.5% in physiological saline, Biochrom AG) is a stain used to distinguish dead cells from living ones. The whole cytoplasm of a dead cell is colored blue, which is easily visualized by light microscopy. For more detailed observations, propidium iodide (PI, 10 μg/ml, Molecular Probes, Eugene, OR) and cell tracker green (CTG, 10 μM, Molecular Probes) were used in combination with the CLSM. PI penetrates cells with permeable membranes and binds to DNA and RNA in

the cell. It can be used to detect dead cells or to reveal the structure of cell nuclei. The He–Ne laser of the CLSM (wavelength 543 nm) excited the PI. A long pass filter at 585 nm was used, and red fluorescence around 617 nm was monitored. CTG was used to detect living cells. When irradiated by an argon ion laser (wavelength 488 nm), CTG produces green fluorescence in the cytoplasm of viable cells. To monitor this fluorescence, we used a band pass filter of 505–530 nm. CTG is traceable for 36 h and is inherited by daughter cells after cell division. Typically, we used dual staining (CTG and PI) about 2 h after plasma treatment to distinguish between living and dead cells. For a study of long term viability after treatment, we applied CTG and observed the cells for a few hours up to 1 day. After the incubation, the cells were fixed with buffered 4% formaldehyde and permeabilized with 0.1% Triton (Tx-100). They were counterstained with PI and the structure of the nuclei was examined. This allowed detection of possible abnormalities induced by the treatment, e.g., apoptotic cells.

Temperature Measurements

To ensure that cells did not suffer from hyperthermia during treatment, the gas temperature in the plasma was measured using a NiCr–Ni thermocouple (Hasco z251/1, Ludenscheid, Germany) with a temperature range up to 400 °C and resolution of 0.1 °C. The thermocouple was fastened to the moving stage and the temperature was determined as function of the distance between the needle and thermocouple head, in both dry and wet environments. In the latter case, the thermocouple head was immersed in 0.4 ml PBS solution.

RESULTS AND DISCUSSION

Thermal Properties of the Plasma Needle

Since even slight heating may be fatal to cells, it is very important to keep a good control of gas temperature during plasma treatment. Figure 4 shows the temperature determined by a thermocouple placed at various distances from the tip of the plasma needle. Only a slight temperature increase is observed at the distance of 2 mm, which is the normal working distance during cell treatment. In this situation, the sample is in direct contact with active plasma (glowing zone). The temperature recorded by the thermocouple is slightly higher when this metal device is dry. This is most likely due to lower heat capacity of the metal in comparison to water.

During cell treatment, a sample containing cells is always covered by a thin film of PBS. This serves not only to prevent the cells from drying out, but also to create a smooth conducting surface, which is beneficial for plasma stability. Moreover, it can divert any static electricity (surface charging) created by the plasma away from cells and conduct it to the ground (metal moving stage).

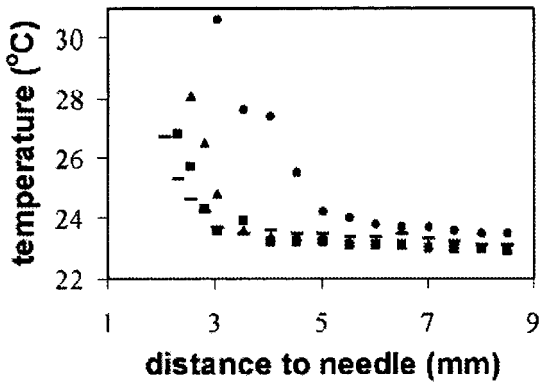

Fig. 4. The temperature determined by a thermocouple as a function of distance between the plasma needle and the surface for various conditions. Dry thermocouple: ● 0.3 W plasma in helium, ■ 0.15 W in helium, ▲ 0.15 W in helium with 3% air. Thermocouple immersed in PBS: – 0.15 W in helium. Indicated plasma powers are initial values at 9 mm distance. Temperature increase is directly related to plasma power. When the plasma is applied to a dry thermocouple, the temperature increase is slightly larger. The same is valid for air-contaminated plasma.

As expected, gas heating is directly related to plasma power, so using higher powers in combination with short distances should be avoided. Plasma with 0.3 W power input, applied at the working distance of 2 mm, causes total necrosis in the treated area after only a few seconds of treatment. We conclude that the thermocouple measurements show that heat generated by the plasma cannot cause damage to the cells when treatment is conducted under the following conditions: powers around 0.15 W or less, distances about 2 mm, and treatment time of about 5–10 s per cell.

Cell Responses

Treatment with the plasma needle induces cell responses in a restricted area. A typical "track" of the plasma left on the cell sample is merely 0.2 mm wide. This is the typical size of the influenced area, reproducible within 20%. It is also possible to create "plasma marks" of 100 μm size, as shown in Figure 5, but the treatment time must be very short (1 s per "spot") and the sample must be immediately brought out of the reach of the needle. In the present configuration, it is difficult to control such fast movements.

There is clear threshold behavior in cell responses. They occur only for a given range of parameters (e.g., plasma power), and within this range they are

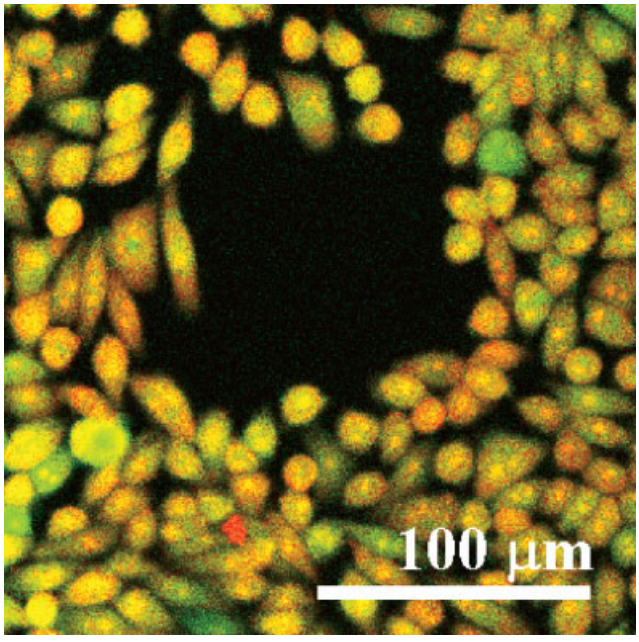

Fig. 5. Plasma-induced void of ca. 100 µm size on a sheet of cultured cells. This demonstrates high precision of plasma treatment. The cells have been treated with a very low power (<0.05 W) plasma, and after 2 h CTG has been applied. Prior to this observation (4 h after treatment), cells have been fixed with formaldehyde and treated with PI to reveal the internal structure, but no special features can be distinguished. The cells were alive after plasma treatment, but they did not reattach within 4 h. Objective lens: Zeiss 40×, NA 0.95 corr, resolution: 0.45 µm/pixel.

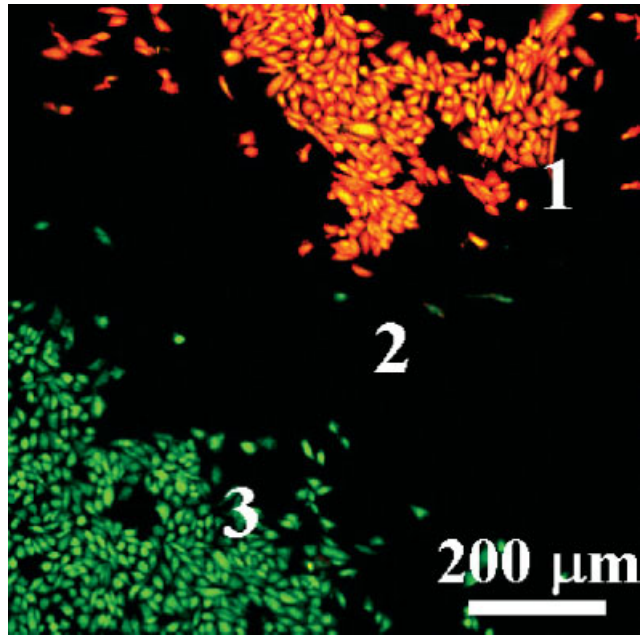

Fig. 6. Typical image of an area treated with a sufficiently high power to cause necrosis (ca. 0.2 W). Dual staining has been applied. The necrotic zone (indicated by 1), the void (indicated by 2), and the living cells (indicated by 3) are visible. A number of the living cells is detached; they form clusters and are stained green by CTG. Objective lens: Zeiss 10×, NA 0.3, resolution: 1.8 µm/pixel.

insensitive to parameter variation. All cells in the treated area are influenced in the same way, the borders between various regions are very sharp and no "mixing" of intact and altered cells is observed. A typical image of the treated area is shown in Figure 6. In this case, a relatively high power plasma (0.2 W) was applied and a dual staining assay was performed 2 h after treatment. In Figure 6, three zones can be distinguished: in the top right of the picture a necrotic zone (PI positive cells exhibiting red fluorescence), in the middle a typical void, and in the lower left a cluster of normal and rounded cells, which are detached from the surrounding ones. Below, we shall describe these zones in more detail.

The necrotic zone was closest to the plasma needle. Cells, which are not viable after plasma treatment, seem to preserve their shape and internal structure. However, they display an abnormal DNA distribution in their nucleus (see Fig. 7). This type of cell death is not due to ordinary thermal damage, as verified by comparing with cells destroyed by heating. At present, we cannot decide whether this plasma induced necrosis is a useful method of disposing of unwanted cells in a tissue. Studying long term reactions of a tissue may supply an answer. In this work, we attempt to observe and classify as many plasma induced cell responses as possible, and therefore we mention necrosis as one of the options. However, cell necrosis is

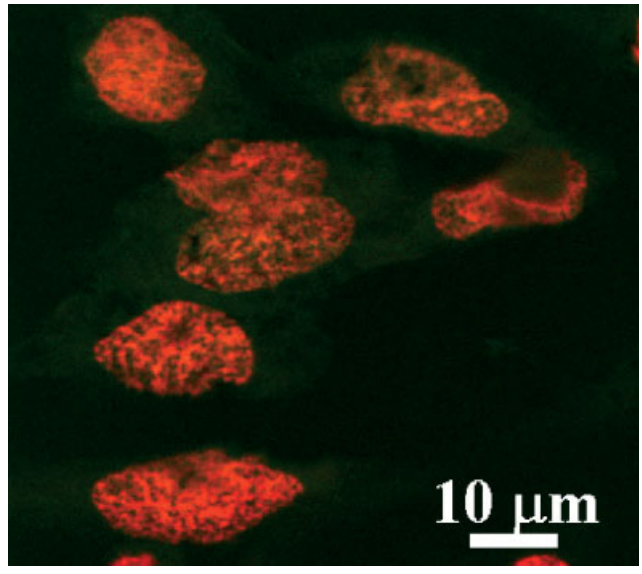

Fig. 7. Close-up of necrotic cells from Figure 6. Objective lens: Zeiss 40×, NA 0.95 corr, resolution: 0.15 µm/pixel.

an a priori undesired effect and from now on we shall concentrate on other reactions.

As said before, cell necrosis occurs at powers higher than 0.2 W (threshold process). At lower powers, necrotic zones like in Figure 6 are absent in the treated sample. However, voids and rounded cells can still be observed. A global feature of plasma treatment is cell detachment, both from the bottom and from the surrounding cells. The first process is responsible for the formation of the void; the detached cells are floating in the liquid (PBS) and can be transferred to another Petri dish. The loss of cell–cell contact is reflected by rounding of cells. These cells are still attached to the bottom. Loss of cell contact appears even more readily than cell detachment from the surface. In some cases (about 20%) no voids, but areas filled with rounded cells, are present. Cell viability after detachment was verified using CTG staining. Rounded cells were always stained green by CTG, like in the ''clustered cells'' zone visible in Figure 6. The threshold power for cell detachment is about 0.1 W; all cells in the treated area are detached.

Long term behavior of detached cells deserves to be studied in detail. Information on cell condition and long term viability may allow us to manipulate cells with plasmas in vitro and possibly also in a living tissue. In our in vitro study we placed the treated CHO-K1 samples in cell culture medium and stored them in the incubator. Loose cells suspended in medium were collected separately. Figure 8 shows the development of the CHO-K1 culture after plasma treatment. The viability of these cells is evident: only 1 h after exposure to plasma, cells start to move around and attach. The halos around the rounded cells disappear. After 4 h, a sheet of cells is formed and within 24 h confluence is attained, as in normal untreated samples. In case of fully detached cells suspended in medium after treatment, reattachment is somewhat slower. Typically, cell–cell interaction is fully restored within 24 h.

At higher doses of plasma treatment, reattachment is inhibited. This occurs within the power range of 0.1–0.2 W, but at a total treatment time of 2 min (10 s per treated spot). Cells retain their rounded shape up to several days after treatment. Cell activities like proliferation continue, but at a slower rate than in normal cells. A typical treated area after 24 h is shown in Figure 9. Some cells are in advanced phase of cytokinesis. Before disposing of this sample the cells were stained with trypan blue, but no dead cells were found.

Under moderate conditions, plasma treatment leads predominantly to loss of contact between individual cells and often to cell detachment from the surface. The mechanism of this process is not yet completely clear however, it is expected to be of (plasma)chemical nature. The influence of other plasma-related factors was checked separately. The effect of UV radiation was studied by irradiating cells with excimer lamps described elsewhere [Tarasenko et al., 1998]. Exposure to UV did not cause detachment, but necrosis appeared above a certain threshold of irradiance. RF electric fields applied to the needle in absence of discharge induced no reaction in cells, even at relatively high amplitudes (1000 V peak-to-peak) and distances shorter than 1 mm. Therefore we tentatively conclude that species emitted from the plasma are

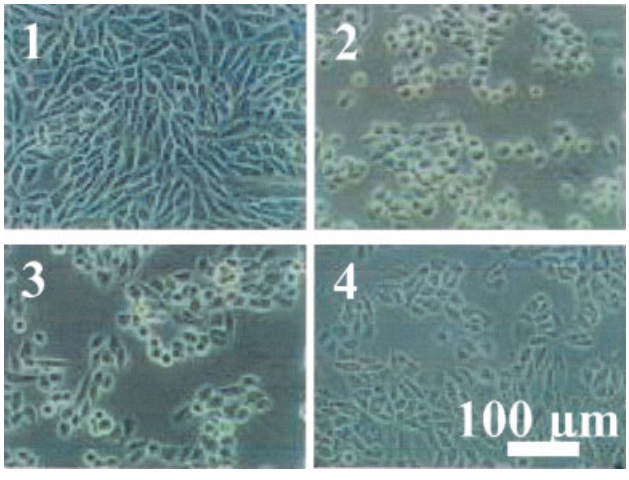

Fig. 8. Phase-contract microscope photographs of cells treated with a 0.1 W plasma. Cells have been observed for several hours, and reattachment of detached cells has been established: 1—before treatment, 2—after 15 min, 3—after 1 h, 4—after 4 h. Objective lens: Zeiss 10×, NA 0.25.

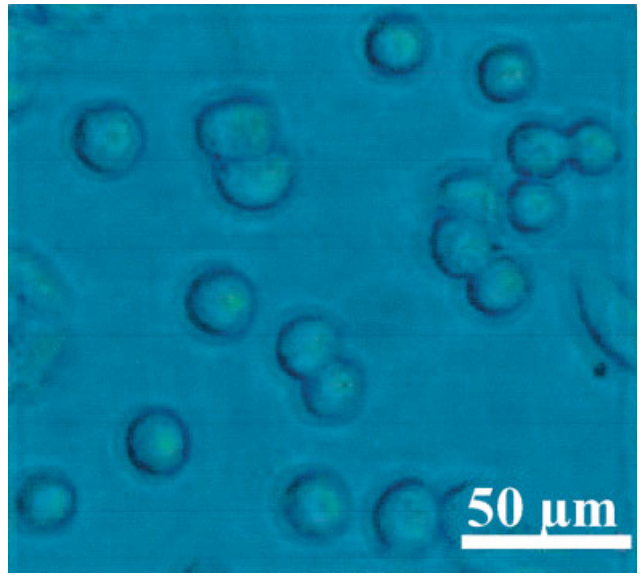

Fig. 9. In the case of a higher exposure time to the plasma, cell reattachment is inhibited even after 24 h. Objective lens: Zeiss 20×, NA 0.3.

responsible for cell detachment. From all these species, including electrons, ions and radicals, only the latter can penetrate under water and reach the cells. Cell detachment can be a consequence of destruction (e.g., oxidation) of cell adhesion molecules (CAMs): cadherins and integrins. The first of these seem to be destroyed more readily: loss of cell contact is easier to induce than total cell detachment from the bottom. On the other hand, penetration of plasma species under water is limited. Densities of active particles may decrease drastically with depth, with a gradient length in the order of cell thickness. Integrins connect the cells to the bottom of the sample, so they cannot be reached by reactive species so easily as cadherins.

CAMs in CHO-K1 are restored on a time scale of a few hours. This is also the typical observed time for cells to reattach and restore a sheet structure after plasma treatment. This supports the hypothesis of plasma induced damage to CAMs. Further studies will be carried out to identify specific chemical interactions that are responsible for this damage.

CONCLUSIONS

For the first time, interaction of an electrically generated plasma with single cells has been described. A CHO-K1 culture has been used as a model system to observe cell responses to a medium, which has not been applied to living objects before. We have verified that our newly constructed non-thermal atmospheric plasma source does not cause thermal nor electric damage to the cells. Cell reactions induced by plasma treatment are of more sophisticated nature. When the plasma power is in the range of 0.1–0.2 W, only cell adhesion is affected. Cell detachment can be achieved with a high precision in an area as small as 0.1 mm diameter. Detached cells remain viable; they typically restore contacts with other cells and reattach to the bottom within a few hours. Based on this observation, we conclude that plasma action is limited to the cell exterior, so that only CAMs are destroyed. A possible mechanism responsible for cell detachment may be damage of cadherins and integrins due to interactions with active radicals (oxygen and nitrogen species) emitted from the plasma. We suppose that the plasma conditions for cell detachment have the least intrusive effect on the cells, since the treated cells do not seem to be altered in any other way. Of course, applying harsh conditions (plasma powers higher than 0.2 W) leads to severe damage and necrosis.

The final aim of this research is high precision, controlled cell manipulation in tissue. Tissue modification without necrosis would not lead to inflammation and unnecessary damage. Cell detachment is a promising method because it is easier to induce than apoptosis. The plasma effect on cell adhesion is potentially applicable in refined cell removal. Cell detachment in a living tissue will be investigated in the near future.

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