Reattachment and Apoptosis After Plasma-Needle Treatment of Cultured Cells

Ingrid E. Kieft, Moamina Kurdi, and Eva Stoffels

***Abstract*—Nonthermal plasmas can be used to locally influence cell adhesion: cells can be removed from their surroundings without causing necrosis. In fact, cells remain alive and can reattach within hours. This phenomenon may, in the future, be used for microsurgical procedures. Another method to remove cells is to induce apoptosis or programmed cell death. This type of cell death is preferred above necrosis, which may cause inflammation reactions. When the detached cells are allowed to reattach and grow, it is important to know their condition. Therefore, long-term effects of plasma-needle treatment were assessed, with special focus on reattachment and apoptosis. The cells were treated using a plasma needle. This device generates a small (1-mm diameter) plasma at atmospheric pressure. To avoid any heat effects, it is important that the plasma temperature is at or below physiological temperature. This is the case for the plasma needle.**

***Index Terms*—Apoptosis, cell attachment, plasma needle.**

I. Introduction

THE PLASMA needle is a tool to generate a nonthermal plasma at atmospheric pressure [1], [2]. This plasma has unique properties that make it suitable for biomedical applications. For example, the needle is not enclosed in a reactor, and the plasma can thus be applied to any surface. In the future, the plasma may even be generated inside a catheter [3]. Furthermore, the gas temperature in the plasma remains below body temperature, which ensures treatment without thermal damage. Temperature measurements were performed before using metal thermocouples [4]. This had the disadvantage that temperature could not be measured in or very near the plasma because the vicinity of metal would lead to arcing. In this paper, we present temperatures obtained using liquid crystal strips, which give more reliable and realistic results. The possibility of reduction of helium flow rate is investigated because a lower flow rate would be beneficial for application of plasma on vulnerable tissues and in small openings. Furthermore, reducing helium consumption will reduce costs.

The plasma that is generated in this way is small (below 1-mm diameter) and can thus be used with a high precision. This is different from other sources, for example, the centimeters-long plasma plume, recently presented by Laroussi and Lu [5].

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The plasma was applied successfully on several types of cultured cells [4], [6]. It appeared that irradiated cells detached from the surface they adhered to. This was the principal effect of plasma treatment, which was readily observed in all types of cells. It has not yet been decided what caused the detachment of cells because the concept of cell adhesion is complex [7], [8] and may be influenced by electrostatic charging by electrons, damage by radicals, or an activation of a certain mobilization process within the cell. In any case, the effect was of temporary nature, and the detached cells were able to reattach in a few hours [4]. However, long-term effects were not extensively studied until now. These long-term effects become especially important if the detached cells are further employed in tissue repair, e.g., transferred to a wound bed or to the surface of implants. Cell detachment was a very pronounced effect, which also overshadowed other possible cell reactions. Therefore, in this study, we shall attempt to separate plasma-induced detachment and to concentrate on issues related to cell activity and apoptosis.

Cell activity after plasma treatment was assessed by observing their rate of reattachment. For this purpose, cells suspended in medium after administration of trypsine were studied. Cell adhesion molecules were already destroyed by trypsine prior to plasma treatment, both in control and in plasma-exposed samples. These molecules had to be reconstructed, so that the cells could reattach. The "speed" of reattachment is the speed of damage repair; thus, it can be used as an indicator for cell activity. A remarkable increase of repair activity after plasma exposure was found.

Another potentially useful effect of plasma treatment is the induction of apoptosis. If cells die by apoptosis, the cell is divided in small membrane-bound apoptotic bodies. These can be ingested by neighboring cells, and inflammation reactions generally do not occur as opposed to cell death by necrosis (accidental cell death). It is known that reactive oxygen species (ROS) may induce this type of cell death [9]–[11]. Inasmuch as these particles are produced in the plasma, apoptosis after plasma treatment belongs to expected responses. In this work, apoptosis was induced in attached cell samples. At the same time, cell detachment occurred; detached cells were examined, and apoptosis was assayed visually by observing cell shapes.

II. Experimental

A. Plasma Needle

The plasma needle consisted of a sharpened metal pin (0.3-mm diameter) that was confined in a Perspex tube (Fig. 1).

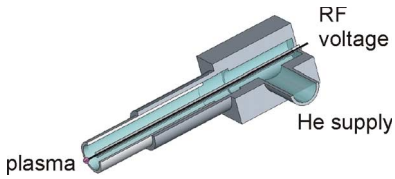

Fig. 1. Schematic drawing of the plasma needle.

Helium was supplied through the tube (2 L min^{-1}), which provided plasma operating in helium/air mixtures [2].

A radio-frequency (RF) signal (13.56 MHz) was generated by a waveform generator (Hewlett Packard 33120A) and amplified by an RF amplifier (Kalmus model 125C-CE). From the amplifier, the signal was directed to a home-built matching network. The resulting voltage at the tip of the needle was around 200 $\mathrm{V_{rms}}$.

An Amplifier Research PM 2002 power meter with a dual directional coupler was placed between source and matching network to measure forward and reflected power. Dissipated plasma power was determined using a subtraction method [12]: The difference in nominal power with and without plasma at the same voltage was used to estimate dissipated plasma power. The value used for treatment will be indicated in the text.

B. Temperature Measurements

Temperatures were estimated using liquid crystal temperature strips (VWR). At the specific temperature, phase transition in liquid crystal occurred, and the color of the strip turned black. The strips had a working range of 37 °C–65 °C in steps of 3 °C–5 °C. The needle was placed for 1 min above the strips with the tip of the needle at 1-mm distance from the strips.

Temperatures were measured both on dry strips and through a thin layer of liquid.

C. Cells

3T3 mouse fibroblast cells were transferred every 2–3 days to a new flask with fresh culture medium and incubated at 37 °C with 5% CO_2. The culture medium was composed of 45% Dulbecco's modified Eagle's medium (DMEM) with low glucose (1 g/l, BioWhittaker, Verviers, Belgium), 45% Ham's F12 (Bio Whittaker), 9% fetal bovine serum (FBS, Biochrom AG, Berlin), 0.9% penicillin/streptomycin (10 000 U/10 000 μg/ml, Biochrom AG), and 0.5% L-glutamine (200 mM in 0.85% NaCl solution, BioWhittaker). For sample preparation, cells were trypsinized (0.05% trypsine/0.02% ethylenediaminetetraacetic acid (EDTA) solution in phosphate-buffered saline (PBS), Biochrom AG) and transferred to sterile Petri dishes.

D. Attachment

To examine the condition of the cells after treatment, we monitored their reattachment. In the experiments described in this paper, we compared the adhesion of cells after treatment with trypsine and subsequent plasma treatment with the adhesion of cells treated only with trypsine (see Fig. 2). Trypsine is an enzyme that breaks down several types of proteins. When this enzyme is used on attached cultured cells, cell adhesion

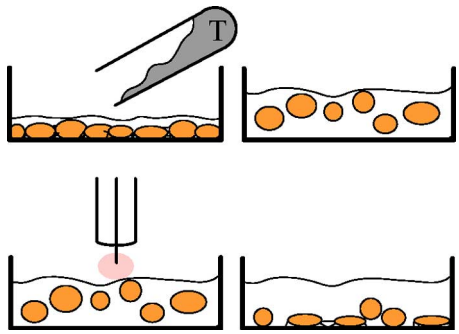

Fig. 2. Schematic drawing of attachment experiments. Cells are detached using trypsine (T), then treated by plasma. Afterward, the attachment is observed.

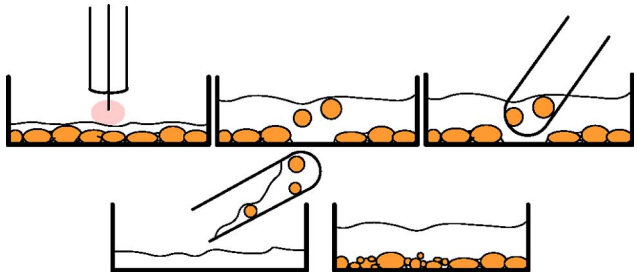

Fig. 3. Schematic drawing of apoptosis experiments. Cells are detached by plasma treatment. Afterward, detached cells are transferred to another Petri dish, and apoptosis is observed.

molecules are broken, and cells round up and become suspended in the medium. Using trypsine provides a good method to obtain detached cell samples because cellular damage is low. We used suspended cells because we intended to concentrate only on the reattachment processes. Plasma treatment also causes cleavage of cell adhesion molecules, but in this experiment, we wanted to discard the plasma-induced detachment effect.

This way, the influence of plasma treatment on attachment of loose cells was tested. Cells were suspended in 400-μL liquid using trypsine. They were treated for 30, 60, and 120 s using a 300-mW plasma at 1-mm distance from the liquid surface, and after plasma treatment, another 400 μL of medium was added. Cells were observed under a light microscope, and their attachment was compared with that of untreated cells. Both loose and settled cells were counted on five different samples, 1.5 h after plasma treatment. From this, the percentage of attached cells was determined.

The plasma power used in this experiment was relatively high. However, as cells were in suspension, the thermal capacity of the liquid prevented them from damage by heat. Furthermore, large amounts of liquid assured no desiccation.

E. Apoptosis

Nearly confluent samples of attached 3T3 cells were treated with plasma. During treatment, samples were covered with a PBS solution of 0.1-mm thickness. This caused detachment of the irradiated cells. These detached cells were then collected and transferred to a new well, where they were allowed to reattach and grow (see Fig. 3).

TABLE I
PLASMA TREATMENT PARAMETERS

Sample	Plasma power (mW)	Treatment time (s)
Control	0	0
1	129	40
2	129	70
3	129	120
4	223	40

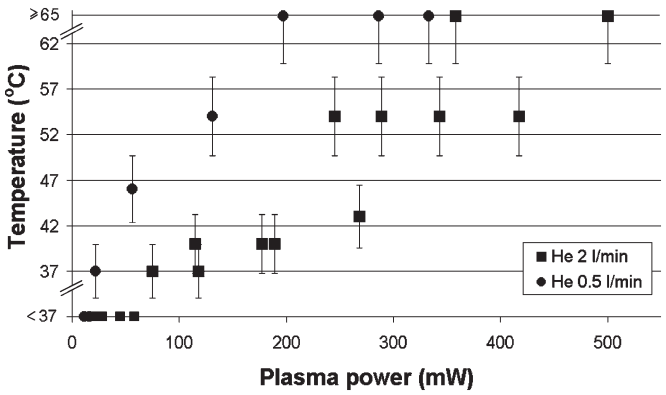

Fig. 4. Temperature in air at 1-mm distance from the needle using 2 and 0.5 L min^{-1} helium flow. Error bars indicate accuracy of the strips. The exact temperature of points below 37 °C and of points at or above 65 °C could not be measured.

Cell behavior was followed in time for two days, up to 52 h after plasma treatment. Their growth was compared with untreated cells that were transferred using trypsine (see methods described in Section II-D).

Several plasma powers and treatment times were tested (Table I).

Photographs of samples were collected using a 10× objective lens with a numerical aperture (NA) of 0.30.

III. RESULTS

A. Temperature

Fig. 4 displays temperatures measured at 1 mm from the needle tip after 1 min of treatment. We were not able to measure temperatures below 37 °C and above 65 °C. Thus, when 65 °C is indicated in the figure, this means that the temperature was at least 65 °C. Temperature was measured using both the standard 2.0 and 0.5 L min^{-1} helium flow. This is to investigate the possibility of reduction of helium usage. This would be economical, and in vulnerable tissue samples, it could possibly reduce damage due to dehydration.

Temperatures in the figure are valid for plasma treatment in dry environment. For a helium flow of 2 L min^{-1}, plasma powers up to 100 mW could be applied without risk of thermal damage as the temperature remained at 37 °C or below. When the flow rate was decreased to 0.5 L min^{-1}, temperature increased quickly with increasing power.

The temperatures displayed in Fig. 4 may be higher than the temperature of a cell sample at the end of plasma treatment. One reason is that before the start of the treatment, samples are at room temperature. Furthermore, the thermal capacity of the cells covered with liquid is larger than that of the strips. This means that more energy is required for an increase in temperature of the samples than for the same increase in temperature of the strips.

Furthermore, temperature was measured underneath a thin layer of water (about 0.5 mm) to simulate conditions relevant for cell treatment. In such a wet environment, temperature of 37 °C was not exceeded for plasma powers up to 700 mW. However, the temperature was strongly dependent on the thickness of the liquid layer, which could not be measured accurately.

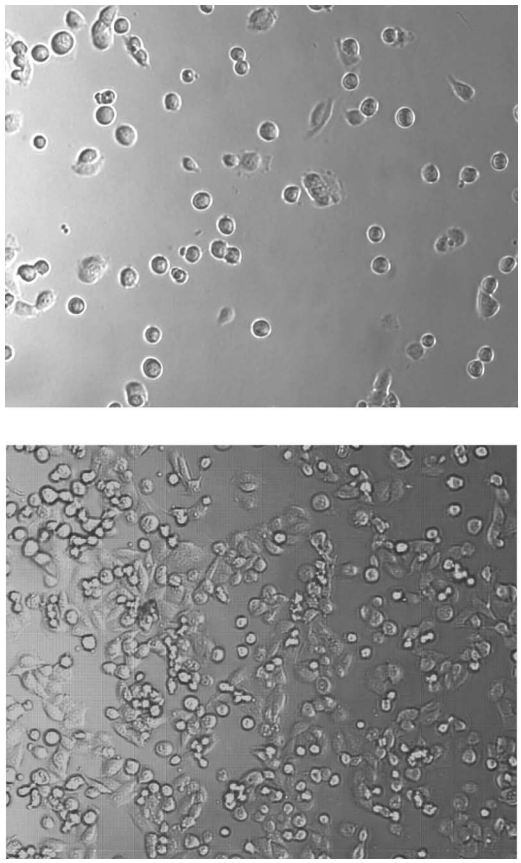

Fig. 5. Cell reattachment and growth after plasma treatment. Top, control sample; bottom, sample treated at 200 mW for 30 s and 1-mm needle-to-liquid distance. Observation was made 2 h after treatment.

B. Attachment

Fig. 5 shows photographs of a treated sample 2 h after plasma exposure and an untreated (control) sample. Quantitative data showing percentages of reattached and loose cells are given in Fig. 6. It is clear that plasma treatment enhanced cell reattachment of suspended cells. The effect of faster attachment was observed 1.5 h after treatment. On a longer timescale (more than 8 h), there was no difference in the appearance of treated and control samples: All cells were attached, and the samples were nearly confluent.

It is interesting to note that the enhanced attachment is most pronounced for short plasma treatment times (30 and 60 s). The effect fades away after longer plasma treatment times.

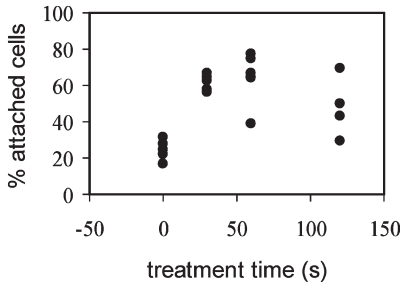

Fig. 6. Percentages of attached cells after plasma treatment (300 mW, 1-mm distance) with varying treatment times. Observation was made 1.5 h after treatment. Cells were counted under a microscope. The treatment time of 0 s corresponds to an untreated (control) sample.

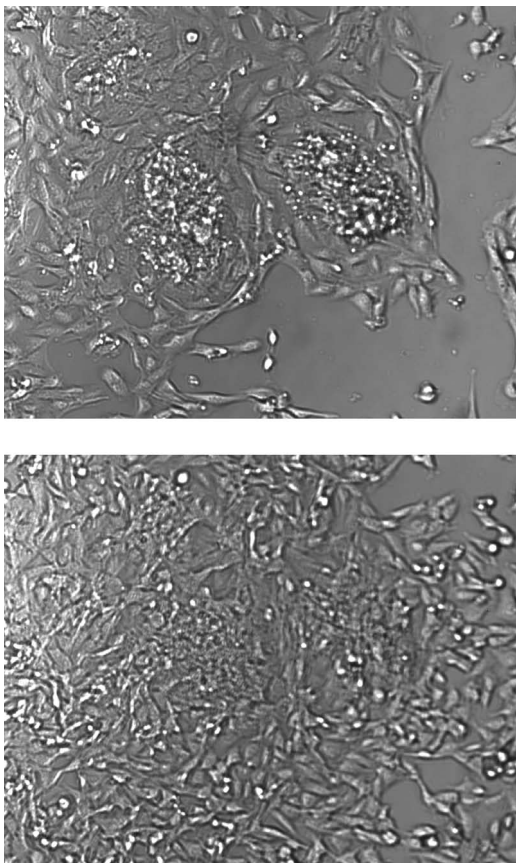

Fig. 7. Apoptosis induced by 129-mW plasma treatment for 70 s. Observation after 21 h (top) and 42 h (bottom) at the same position. Clusters of shapeless fragmented cells are visible; these cells are in the late stadium of apoptosis.

C. Apoptosis

After plasma treatment, all cells within the treated spot were detached. The typical diameter of the detached area was 5 mm. The detached cells were collected and transferred to new wells. Cells appeared to be viable and capable of reattachment. However, in the transferred samples of irradiated cells, apoptosis was detected. Areas were found where most likely several cells underwent apoptosis (see Fig. 7); this produced characteristic clusters of apoptotic bodies. Neighboring cells appeared healthy; they surrounded the apoptotic clusters, presumably to consume the apoptotic bodies. In the literature, apoptosis is normally observed from 4 h after the incentive. Here, it was still observed in samples after 16 h and even after 26 h. In total, the number of apoptotic cells was estimated to be between 20% and 40%. This is much higher than the percentage achieved in earlier experiments [13], where observations were made 6 h after treatment. The time window of high apoptosis rates is determined among others by the cell type and the apoptosis inducer [14]. Apparently, plasma induction of apoptosis is slow, and results may be expected after about 24 h instead of after 6 h.

Cells that were not apoptotic show regular cell growth. In time, the apoptotic bodies were overgrown by healthy cells.

No apoptosis was observed in control (untreated) samples prepared by using trypsine. There was no difference observed between apoptosis caused by plasma in samples treated for 40, 70, and 120 s nor between those treated with low (129 mW) or high (223 mW) plasma power. This is a typical saturation effect that was observed in other plasma–cell interactions, i.e., cell detachment and bacterial inactivation [6], [15]. Results were obtained above a certain threshold, and further increase of plasma dosage was of no avail. This is a positive feature because high apoptosis rates may be attained using short treatment times and low plasma powers. This is favorable from the point of view of patient safety. Optimization of apoptosis can be performed by changing the composition of the plasma, which would result in production of specific radicals (e.g., NO).

IV. Discussion

A low helium flow rate leads to a substantial increase in temperature. This shows the importance of convective cooling on thermal properties of the plasma needle. Another reason for high temperature at low flows is the admixture of air molecules in the helium, which results in a lower heat conductivity of the gas and in additional gas heating because of energy dissipation in rovibrational transitions of the molecules. Unfortunately, these findings imply that flow rates of more than 1 l/min must be maintained in the present configuration. Possibly, some modifications can be made to promote cooling, e.g., by reducing the diameter of gas outlet nozzle (increasing flow velocity) and/or pulsing the discharge. Another solution may be to change the design of the needle and its helium supply by using high flow rates and recycling of helium.

We hypothesize that there is a certain cell activation mechanism due to plasma treatment. In cell suspension, the dosage of plasma species is relatively low: The majority of chemical agents are short living and thus unable to survive in plenty of liquid. For example, a 1.5-mm layer of liquid filters out 90%–95% of energetic plasma particles [16]. Thus, we realize that only relatively long-living species have the largest chance to affect the cells. The role of ultraviolet (UV) photons was studied separately by using UV sources [17], but none of such effects were observed. From the long-living particles, singlet oxygen ($^1\Delta_g O_2$; a metastable state of molecular oxygen with energy of 0.98 eV above the ground state) is expected to be most abundant, in the range of 10% of the total oxygen content [18]. Its lifetime can be even in the millisecond range [19]. Singlet oxygen and other long-living species may induce oxidative stress in the cells. The latter will use their full resources for repair, and this may be expressed in activation

and faster reattachment. This effect can be expected when the damage is small to moderate, so that the cells are still functional. After higher plasma dosages (longer treatment times), the cellular damage is larger and repair is impossible. This explains why the effect of cell activation fades away at long irradiation times.

Another explanation of increased cell activity may be a certain chemical stimulus to the cells. For example, singlet oxygen carries a substantial energy, which can help in lowering activation thresholds of various reactions. As commonly known, ground-state oxygen is abundantly used in cellular respiratory chain—Combustion of 1 mol of glucose requires 6 mol of oxygen. In the aerobic breathing (citric acid cycle), oxygen reacts primarily by attaching an electron [20]. The attachment cross section for singlet oxygen is four times higher than that for ground-state oxygen [21]. Besides, the activation energy is lowered, which implies that attachment rate for low-energy electrons can be orders of magnitude higher. Involvement of energetic oxygen in the respiratory chain could thus speed up the energy production. The produced energy can be stored as ATP and employed in various metabolic processes. This would result in the observed "energy boost": the produced ATP would be used in reparation of adhesion molecules, and thus, cell reattachment would be enhanced. This mechanism can also explain the temporary nature of cell activation: The stored energy resources are limited, and thus, the effect vanishes after the energy has been consumed.

Apoptosis in plasma treatment is of a different nature than cell activation. Cells that underwent apoptosis received higher doses of plasma treatment because they were less protected by the liquid. Thus, more of the aggressive unstable species (ROS) could reach the cells. Apoptosis, or programmed cell death procedure, is started if the cell is damaged beyond repair and poses a threat to its surroundings. Apoptosis is a typical cell response to, e.g., mitochondrial damage, which can be induced by ROS. In fact, induction of apoptosis was initially the major motivation in the study of plasma-cell interactions: the effect was predicted beforehand, and the plasma needle was constructed to realize it. Inducing apoptosis is of major importance in "neat" surgery because it is a clean way of cell removal without inflammation reactions. First trials were disappointing [13]: apoptosis occurred in less than 5% of cell population, 6 h after treatment. However, the current study provides encouraging results for future medical treatment. Apparently, induction of apoptosis by plasma is a long-term effect because the top occurrence of this effect is at 24 h after treatment. There is no objection to delayed responses in medical treatment. In fact, slow reactions have the advantage that the organism will have enough time for tissue purification and repair.

V. Conclusion

Plasma treatment can be applied without causing thermal effects. With high helium flow rates, the gas temperature remains below physiological temperature. Biological samples usually remain cool because of short treatment times and sample coverage with liquid that has very high heat capacity. However, reduction of helium flow rate leads to an increase in temperature. In the conducted experiments, cell reactions are induced not by heat, but by chemical agents from the plasma.

It appears that plasma treatment can stimulate cultured cells and temporarily enhance their activity. Cell adhesion after plasma treatment is faster than attachment restoration after treatment with trypsine. We hypothesize that cells are slightly injured and therefore stimulated in the repair of both this injury and of the adhesion molecules. Alternatively, singlet oxygen from the plasma may provide extra energy that can be involved in the respiratory chain. Accelerated cell attachment may be used in, for example, wound healing.

Another important cell reaction after plasma treatment is the induction of apoptosis. Twenty-four hours after irradiation, apoptosis percentage is above 20%. In addition to earlier described cell detachment, apoptosis may provide an alternative method of "clean" cell removal.

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