Plasma Medicine, 3(1-2): 71–80 (2013)

Viability of Human Blood Leukocytes Compared with Their Respective Cell Lines after Plasma Treatment

Lena Bundscherer,[1,2,*] Sander Bekeschus,[1,2] Helena Tresp,[1,2] Sybille Hasse,[1,2] Stephan Reuter,[1,2] Klaus-Dieter Weltmann,[2] Ulrike Lindequist[3] & Kai Masur[1,2]

[1]Center for Innovation Competence *plasmatis*, Greifswald, Germany; [2]Leibniz Institute for Plasma Science and Technology, Greifswald, Germany; [3]Institute of Pharmacy, University of Greifswald, Greifswald, Germany

*Address all correspondence to: Lena Bundscherer, Leibniz Institute for Plasma Science and Technology, Felix-Hausdorff-Str. 2, 17489 Greifswald, Germany; lena.bundscherer@inp-greifswald.de.

ABSTRACT: Non-thermal plasma application has become a promising field of investigation in chronic wound healing research over the past few decades. In addition to its well-characterized antibacterial effects, plasma potentially promotes the growth of eukaryotic cells. To date, mainly epithelial skin cells have been examined regarding the impact of plasma treatment on chronic wound healing. However, immune cells also are involved in wound healing as well as the removal of pathogens. Therefore, we compared the survival behavior of 2 human leukocyte cell lines (a monocyte and a $CD4^+$ T helper cell line) and their respective human blood counterparts after exposure to plasma. Measurements of early and late apoptotic cells demonstrate that freshly isolated blood cells were more susceptible to apoptosis induction than the cell lines. Furthermore, blood and cell line monocytes tolerated longer plasma exposure compared with blood and cell line $CD4^+$ T helper cells.

KEY WORDS: apoptosis, $CD4^+$ T helper cells, Jurkat cells, monocytes, non-thermal plasma, pH effect, plasma medicine, THP-1 cells, wound healing

I. INTRODUCTION

Non-thermal plasma has drawn more and more attention worldwide in the field of medical applications over the past 2 decades.[1] Because of its complex composition (free radicals, excited and neutral species, ions, electrons, and ultraviolet, visible, and infrared radiation),[2,3] plasma can have lethal effects on bacteria and eukaryotic cells. In addition, it has potentially stimulating effects on mammalian cells.[4,5] Medical applications range from the removal of dental biofilms[6] and healing of infectious skin and nail diseases[7] to wound management.[8] In particular, chronic wounds pose one major target for plasma treatment.[9] However, future plasma applications require a thorough examination of plasma-cell interactions, ensuring the safety and reliability of devices in advance of their clinical use. So far, most studies concerning chronic wound healing have investigated the effect of plasma on skin cells. Nevertheless, immune cells play an important role in skin health, wound healing, and bacterial cleaning.[10,11] Epidermal T cells are known to

1947-5764/13/$35.00 © 2013 by Begell House, Inc.

be involved in wound regeneration. Among others, their ability to produce insulin-like growth factor 1 has been shown to be essential for a proper acute wound healing process, whereas this growth factor was absent in T cells isolated from chronic wounds.[10] Next to T cells, monocytes contribute to wound recovery. After monocytes are recruited to the wound, they are stimulated by various cytokines or bacterial products to differentiate into macrophages. These leukocytes are able to engulf senescent cells and debris as well as pathogens spreading the wound bed. Moreover, they exert functions that promote wound healing, such as stimulation of collagen production, angiogenesis, and re-epithelialization.[11] This study reveals for the first time the major differences regarding the survival of human blood $CD4^+$ T helper cells and monocytes compared with their homologous cell lines after plasma exposure.

II. MATERIALS AND METHODS

A. Cell Culture

The THP-1 human monocyte (Cell Lines Service GmbH, Eppelheim, Germany) and Jurkat $CD4^+$ T helper cell lines (DSMZ, Braunschweig, Germany) were cultured in RPMI 1640 (Lonza Group AG, Basel, Switzerland) containing 10% (THP-1) or 8% (Jurkat) fetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO), 2 mM L-glutamine, and 1% penicillin/streptomycin (both from Lonza Group AG).

Peripheral blood mononuclear cells were isolated from human blood from healthy donors by density gradient centrifugation (Histopaque-1077; Sigma-Aldrich). Monocytes were subsequently isolated by negative selection (Pan monocyte isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany); $CD4^+$ T lymphocytes were isolated by positive selection (human $CD4^+$ selection kit; Stem Cell Technology, Grenoble, France). The purity of T helper cells and monocytes was greater than 85%, as evaluated by staining with specific fluorochrome-conjugated antibodies and flow cytometry measurements (data not shown). Cell culture medium was used as described for THP-1 cells (supplemented with 10% FCS).

B. Plasma Treatment

For non-thermal plasma treatment, the plasma jet kinpen 09 (neoplas GmbH, Greifswald, Germany) was used based on the setup described by Weltmann *et al*.[12] It was operated with argon (99.999% purity) at a flow rate of 3 standard L/minute, and a radio frequency of around 1 MHz was applied. For assessment of apoptosis 1×10^6 cells were incubated with 5 mL of plasma-treated cell culture medium. Different plasma treatment times were chosen (5, 15, 60, 120, 180 seconds and an additional 360 seconds for monocytes and THP-1 cells) to distinguish different plasma sensitivities of the investigated cell types. Cells were left untreated (0 seconds) as a negative control or were treated with 100 μM hydrogen peroxide (H_2O_2; Sigma-Aldrich) as a positive control for apoptosis induced by reactive oxygen species. For the detection of plasma-mediated pH

variations, cell culture medium (8% FCS) was treated with plasma for 60, 180, and 300 seconds or left untreated (0 seconds) as a negative control.

C. Apoptosis Assay

Twelve hours after treatment, cells were harvested and 0.5×10^6 cells were stained with 1 µL (1 µg) of the apoptosis marker annexin V (conjugated with fluorescein isothiocyanate; Enzo Life Sciences, Lörrach, Germany) in binding buffer (10 mM HEPES/sodium hydroxide [pH 7.4], 140 mM sodium chloride, 2.5 mM calcium chloride) for 10 minutes in the dark. Cells were washed once and resuspended in 300 µL of binding buffer. Late apoptotic or necrotic cells were stained with 1 µL 7-aminoactinomycin D (7AAD; eBioscience, San Diego, CA) for 5 minutes. The percentage of early (annexin V positive/7AAD negative) and late (annexin V and 7AAD positive) apoptotic cells was subsequently measured using a Gallios flow cytometer and analyzed using Kaluza flow analysis software (both from Beckman Coulter, Brea, CA) and FlowJo 7.6.5 software (TreeStar Inc., Ashland, OR), respectively.

D. pH Measurement

The pH value of plasma-exposed cell culture medium was determined 1 minute after treatment. These measurements were performed with a pH meter (Sevenmulti M47; Mettler Toledo, Giessen, Germany).

E. Statistics

Two-way analyses of variance and Holm-Sidak's post hoc test (Prism 6.0; GraphPad Software, La Jolla, CA) were used to calculate the statistical significance of the cell culture experiments. The mean of 3 independent experiments per cell line was compared with samples from 3 individual donors. Bars and error bars represent the mean and range of duplicates per donor and triplicates of the cell lines, respectively. Statistics of pH liquid analyses were determined by one-way analysis of variance and Dunnett's multiple comparison test (Prism 6.0) referring to the negative untreated control. The means of 3 independent experiments were plotted as bar graphs with error bars that represent the maximum and minimum of the measured pH values. Statistical significance of all experiments is displayed in the following way: $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

III. RESULTS AND DISCUSSION

The gating strategy of the flow cytometric annexin V/7AAD apoptosis assay is depicted in Fig. 1, which allows both early and late apoptotic cells at one time point to be recorded. After gating all cells in a forward scatter (FSC)/side scatter (SSC) dot plot, they were separated by their annexin V and 7AAD staining properties. Cells that were negative for both annexin V and 7AAD (annexin V^-/$7AAD^-$) were regarded as living cells. Cells

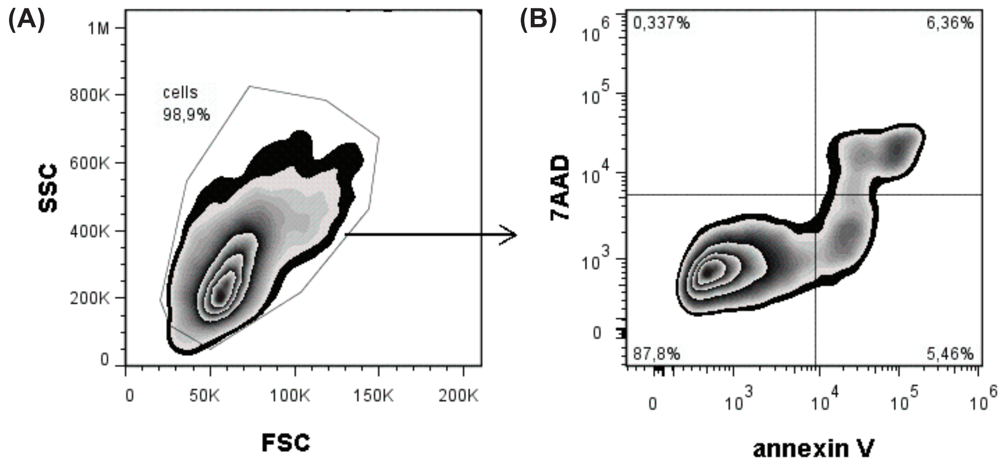

FIG. 1. Flow cytometric gating of early and late apoptotic cells. **A:** All cells (excluding cell debris) were first gated in a forward scatter (FSC)/side scatter (SSC) plot and subsequently categorized into subgroups regarding the staining ability of the apoptotic marker annexin V and the late apoptotic/necrotic dye 7-aminoactinomycin D (7AAD). **B:** Percentage of early (annexin $V^+/7AAD^-$; bottom right quadrant) and late (annexin $V^+/7AAD^+$; top right quadrant) cells were analyzed further. No necrotic cells could be detected.

that stained positive for annexin V but negative for 7AAD (annexin $V^+/7AAD^-$) were undergoing early apoptosis. Moreover, cells positive for both annexin V and 7AAD (annexin $V^+/7AAD^+$) were already in a late apoptotic stage. In contrast to apoptotic cells, necrotic cells can be stained with 7AAD but not with the marker annexin V (annexin $V^-/7AAD^+$). Preliminary time course experiments showed that plasma treatment with the kinpen 09 induced only apoptotic processes in all investigated cells, whereas almost no necrotic cells could be detected (data not shown), as previously described by Bekeschus *et al.*[13] for peripheral blood mononuclear cells. Thus, inflammatory reactions due to necrosis could be neglected.

Figure 2 represents the percentages of early (Fig. 2A) and late (Fig. 2B) apoptotic blood $CD4^+$ T helper cells compared with the Jurkat cell line after plasma treatment. Percentages of both early (annexin $V^+/$ $7AAD^-$) and late apoptotic (annexin $V^+/$ $7AAD^+$) $CD4^+$ T helper cells and Jurkat cells increased with plasma treatment time prolonged up to 120 seconds. Because of donor variations, $CD4^+$ T helper cells treated for 180 seconds behaved differently: While the percentage of early apoptotic cells of donor II further increased, those of donors I and III dropped below the value of the cell sample treated for 120 seconds. In contrast to the level of late apoptotic cells of donors I and III, which increased further, donor II reached a plateau. Apoptotic rates of primary $CD4^+$ T helper cells always exceeded those of the Jurkat cell line. Nevertheless, strong differences between the donors of the primary cells were detected. There was no significant increase of cell death after 5 seconds of plasma treatment for all investigated freshly isolated

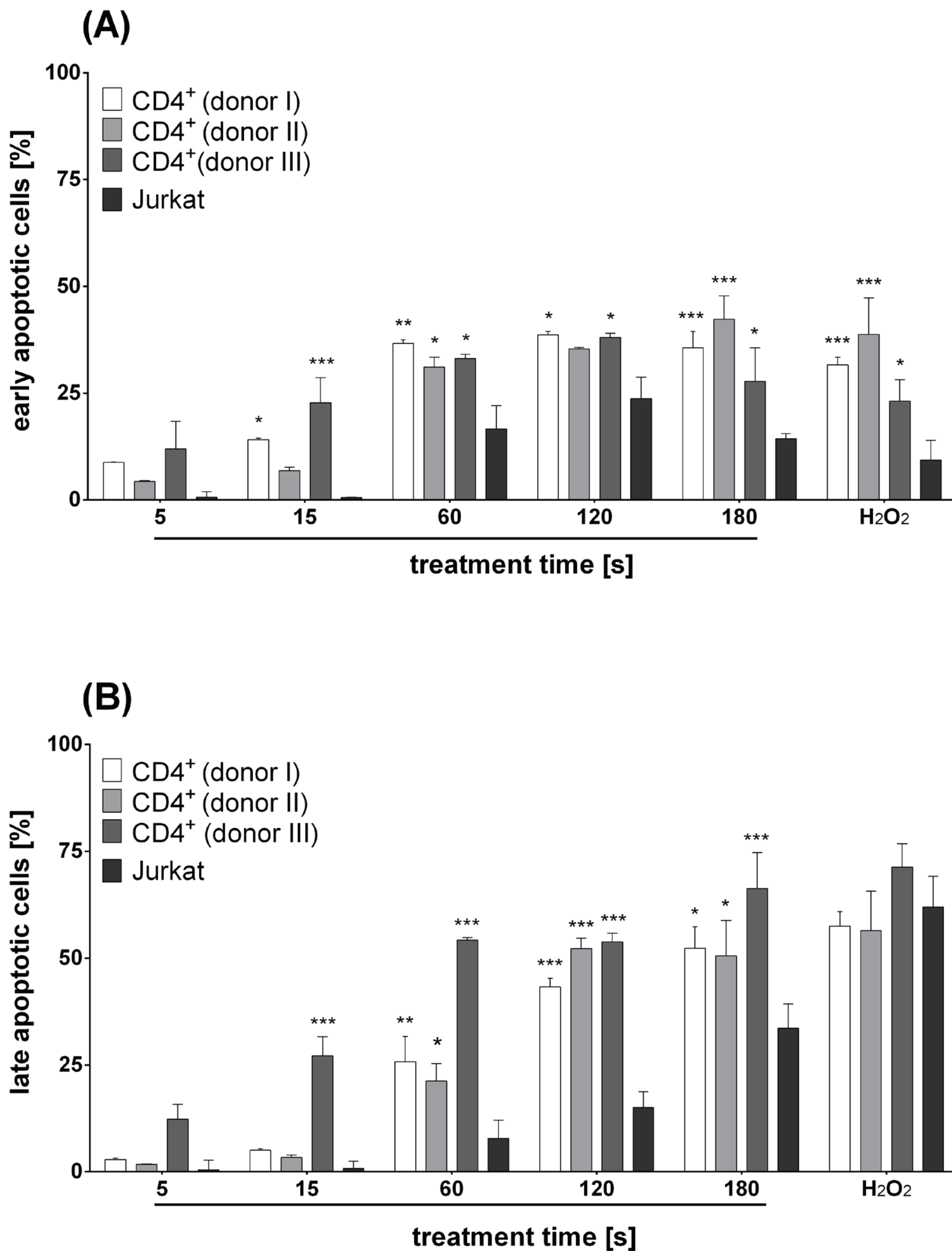

FIG. 2. Comparison of apoptotic rates of isolated CD4⁺ T helper cells versus a Jurkat cell line after plasma treatment. Percentages of early apoptotic cells annexin V⁺/7-aminoactinomycin D [7AAD⁻]) (**A**) and late (annexin V⁺/7AAD⁺) apoptotic cells (**B**) are displayed as bar diagrams, with the proportion of apoptotic untreated cells subtracted for each investigated cell type; 100 μM hydrogen peroxide was used to trigger apoptosis. Representative data from 3 independent experiments are shown.

$CD4^+$ T helper cells compared with Jurkat cells. While early apoptosis after 15 seconds of plasma treatment was significantly higher for cells of 2 blood donors, only one donor revealed a significant increase in late apoptotic $CD4^+$ T helper cells. For nearly all tested samples, plasma treatment for 60 seconds and longer resulted in a significant difference between primary $CD4^+$ T helper cells of these 3 donors and the Jurkat cell line (from $p < 0.05$ to <0.001). In comparison, treatment of primary $CD4^+$ T helper cells with H_2O_2 led to an increased number of early but not late apoptotic cells compared with Jurkat cells. Percentages of early (Fig. 3A) and late apoptotic cells (Fig. 3B) of freshly isolated monocytes were compared with the THP-1 cell line. Similar to the T helper cells, the percentage of apoptotic cells increased in a plasma treatment time–dependent manner in both investigated cell types. For the THP-1 cell line, only plasma treatment of 360 seconds resulted in increased early and late apoptosis. Furthermore, all examined cells displayed much lower percentages of early apoptotic cells than the amounts of corresponding late apoptotic cells. Primary monocytes showed significantly higher (from $p < 0.05$ to <0.001) early and late apoptosis rates compared with THP-1 cells for treatment times exceeding 60 and 15 seconds of plasma exposure, respectively. Thus, isolated monocytes were more susceptible to non-thermal plasma treatment than the THP-1 cell line. In addition, incubation with 100 μM of H_2O_2 significantly increased early and late apoptosis in primary monocytes compared with THP-1 cells.

Non-thermal physical plasma was able to induce apoptosis in all examined immune cell types. Both primary $CD4^+$ T lymphocytes and primary monocytes showed higher early and late apoptotic percentages than their cell line counterparts. However, both types of primary cells displayed a significantly higher percentage of apoptotic cells already in the untreated control than the cell lines (data not shown). For statistical reasons, baseline percentages of apoptosis in untreated controls were subtracted from the corresponding treated samples. The reason why the investigated cell lines tolerated higher plasma doses than the primary cells might be the fact that both cell lines descend from patients with leukemia.[14,15] Their immortalization and adaption to cell culture may have led to an increased capability to deal with oxidative stress and prevent apoptosis. Even though working with cell lines is more convenient and an effective tool for establishing experiments, it is necessary for future research to focus also on the impact of non-thermal plasma treatment on primary immune cells. These cells are closer to a real *in vivo* situation and may reveal differences between healthy donors and patients with chronic wounds.

Moreover, blood monocytes and the respective THP-1 cell line were less sensitive to the induction of early and late apoptosis than blood $CD4^+$ T helper cells and the Jurkat cell line in response to plasma and H_2O_2 treatment, indicating strong resistance of monocytes toward plasma chemistry and radicals. As previously described,[16] this might be because monocytes—but not $CD4^+$ T helper cells—are known to produce reactive oxygen species, including H_2O_2, themselves during respiratory bursts.[17] Treatment with 100 μM of H_2O_2 did not even induce apoptosis in THP-1 monocytes. In contrast to H_2O_2, it is already known that etoposide is able to provoke apoptosis in THP-1 cells via

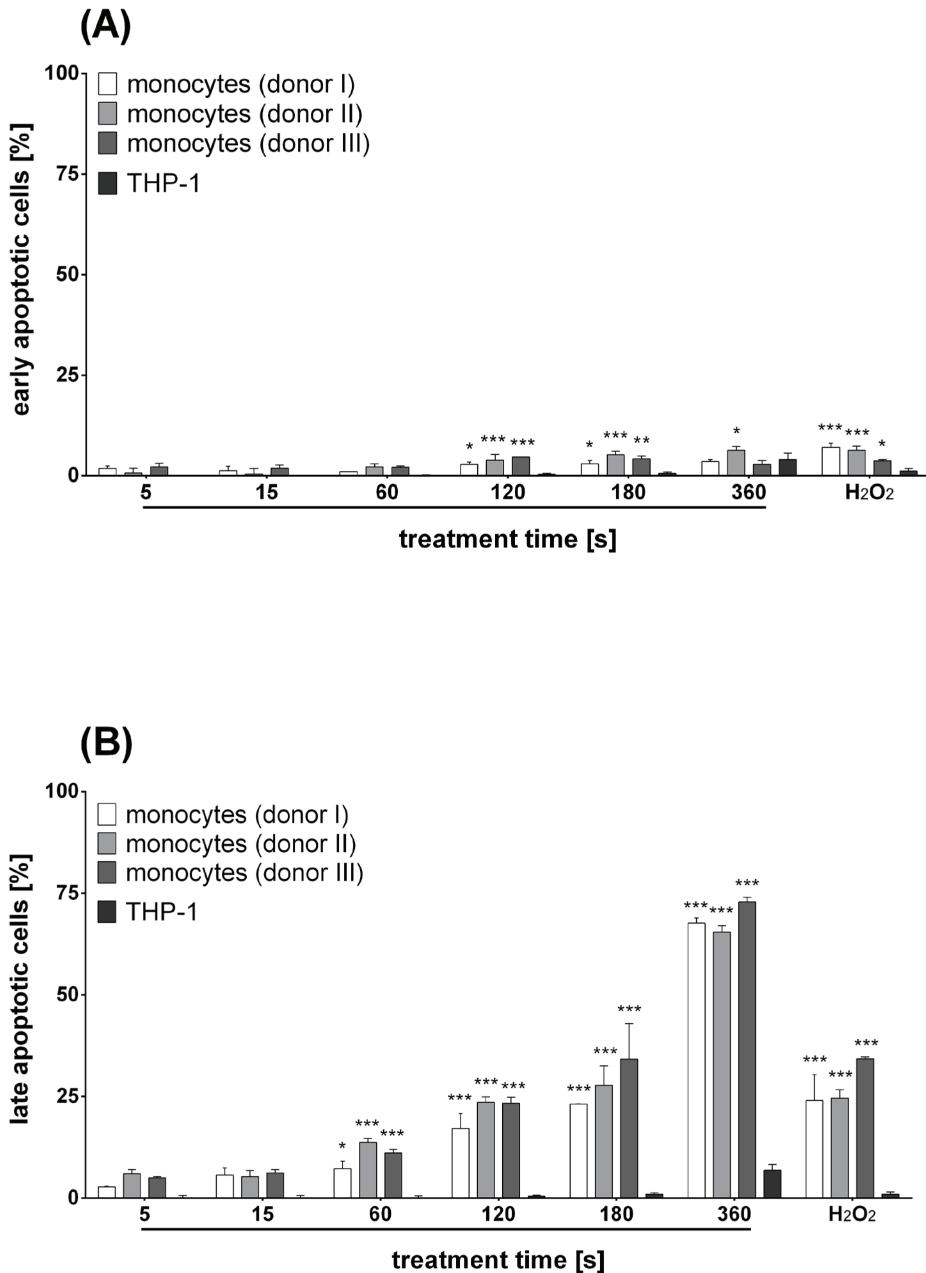

FIG. 3. Comparison of apoptotic rates of isolated monocytes versus a THP-1 cell line after plasma treatment. Percentages of early apoptotic cells (annexin V^+/7-aminoactinomycin D [$7AAD^-$]) (**A**) and late (annexin V^+/$7AAD^+$) apoptotic cells (**B**) are displayed as bar diagrams, with the proportion of apoptotic untreated cells subtracted for each investigated cell types; 100 µM hydrogen peroxide was used to trigger apoptosis. Representative data from 3 independent experiments are shown.

a different mode of action.[16] However, H_2O_2 is one major stable product of argon plasma treatment of the kinpen 09 in liquids.[18] Thus, it is not surprising that the same treatment doses of both plasma and H_2O_2 induced less apoptosis in monocytes than in $CD4^+$ T helper cells. Furthermore, incubation with 100 µM of H_2O_2 showed similar effects on the level of apoptosis activation as a treatment time of 180 seconds in all investigated cell types. Since comparable H_2O_2 levels were measured in cell culture medium treated with plasma for 180 seconds,[19] H_2O_2 seems to be one of the main plasma components to affect cell viability in susceptible cells.

Next to the formation of H_2O_2, plasma treatment may alter the pH of the treated cell culture medium, which can then affect cell viability. Therefore, we additionally investigated plasma-modulated pH changes, which are depicted in Fig. 4. A slight increase in the pH value could be measured with increasing treatment time. The longest plasma exposure of 300 seconds yielded a pH value of 8.3 in the medium compared to a pH value of 7.8 in the untreated medium.

This can be explained by a degassing effect of the carbonate buffer sodium bicarbonate, a main component of the RPMI medium buffer system. This loss of carbon dioxide was due to the argon flow of the plasma jet.[20] The elevated pH level of the culture medium may contribute to apoptosis induction since it is known that alkaline stress can cause apoptosis in human cells.[21]

Moreover, it is already known that reactive species such as superoxide anions and hydroxyl radicals are formed by plasma treatment of liquids.[22] Therefore, it has to be further investigated what effects these molecules and the pH have on cellular level.

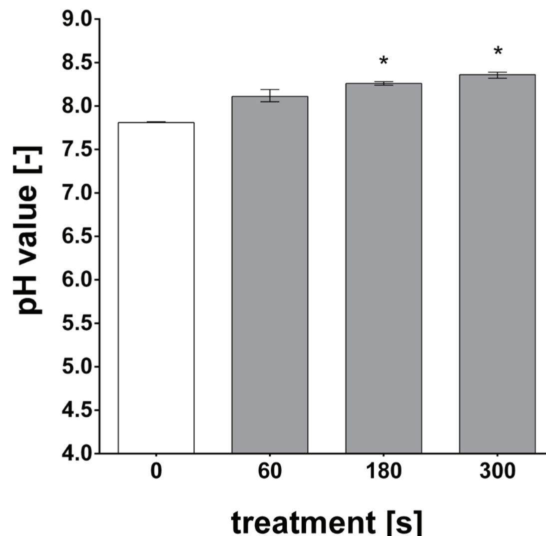

FIG. 4. pH measurements of the plasma-treated cell culture medium. One minute after plasma exposure, pH values of the treated medium were determined with a pH meter. Representative data from 3 independent experiments are shown.

IV. CONCLUSION

We showed that the percentage of early and late apoptotic leukocytes increased with plasma treatment time. However, the magnitude of apoptosis induction was strongly dependent on the cell type investigated. Monocytes were less sensitive than $CD4^+$ T helper cells, whereas the cell lines displayed higher survival rates compared with their human blood counterparts. We additionally demonstrated that prolonged plasma treatment led to an increased pH value of the cell culture medium, which might aggravate apoptosis induction. Taken together, we showed significant differences regarding apoptosis induction after plasma treatment between different immune cell types as well as between primary cells and cell lines. Future investigations of the molecular basis of these effects are necessary to figure out what mechanisms are involved in cell survival.

ACKNOWLEDGEMENTS

This work was supported by a grant funded by the German Ministry of Education and Research (BMBF grant nos. 03Z2DN11 and 03Z2DN12). We thank Dr. Andreas Greinacher (head of the Institute of Transfusion Medicine, University of Greifswald) for allocating human donor blood and Liane Kantz for her technical contributions.

REFERENCES

1. Heinlin J, Isbary G, Stolz W, Morfill G, Landthaler M, Shimizu T, Steffes B, Nosenko T, Zimmermann J, Karrer S. Plasma applications in medicine with a special focus on dermatology. J Eur Acad Dermatol Venereol. 2011;25(1):1–11.
2. Heinlin J, Morfill G, Landthaler M, Stolz W, Isbary G, Zimmermann JL, Shimizu T, Karrer S. Plasma medicine: possible applications in dermatology. J Dtsch Dermatol Ges. 2010;8(12):968–76.
3. Weltmann KD, Polak M, Masur K, von Woedtke T, Winter J, Reuter S. Plasma processes and plasma sources in medicine. Contrib Plasma Phys. 2012;52(7):644–54.
4. Kalghatgi S, Friedman G, Fridman A, Clyne AM. Endothelial cell proliferation is enhanced by low dose non-thermal plasma through fibroblast growth factor-2 release. Ann Biomed Eng. 2010;38(3):748–57.
5. Wende K, Landsberg K, Lindequist U, Weltmann KD, von Woedtke T. Distinctive activity of a nonthermal atmospheric-pressure plasma jet on eukaryotic and prokaryotic cells in a cocultivation approach of keratinocytes and microorganisms. IEEE Trans Plasma Sci. 2010;38(9):2479–85.
6. Koban I, Holtfreter B, Hubner NO, Matthes R, Sietmann R, Kindel E, Weltmann KD, Welk A, Kramer A, Kocher T. Antimicrobial efficacy of non-thermal plasma in comparison to chlorhexidine against dental biofilms on titanium discs *in vitro* - proof of principle experiment. J Clin Periodontol. 2011;38(10):956–65.
7. Daeschlein G, Scholz S, von Woedtke T, Niggemeier M, Kindel E, Brandenburg R, Weltmann KD, Junger M. *In vitro* killing of clinical fungal strains by low-temperature atmospheric-pressure plasma jet. IEEE Trans Plasma Sci. 2011;39(2):815–21.

8. Fridman G, Peddinghaus M, Balasubramanian M, Ayan H, Fridman A, Gutsol A, Brooks A. Blood coagulation and living tissue sterilization by floating-electrode dielectric barrier discharge in air. Plasma Chem Plasma Process. 2006;26(4):425–42.
9. Isbary G, Morfill G, Schmidt HU, Georgi M, Ramrath K, Heinlin J, Karrer S, Landthaler M, Shimizu T, Steffes B, Bunk W, Monetti R, Zimmermann JL, Pompl R, Stolz W. A first prospective randomized controlled trial to decrease bacterial load using cold atmospheric argon plasma on chronic wounds in patients. Br J Dermatol. 2010;163(1):78–82.
10. Toulon A, Breton L, Taylor KR, Tenenhaus M, Bhavsar D, Lanigan C, Rudolph R, Jameson J, Havran WL. A role for human skin-resident T cells in wound healing. J Exp Med. 2009;206(4):743–50.
11. Mahdavian Delavary B, van der Veer WM, van Egmond M, Niessen FB, Beelen RH. Macrophages in skin injury and repair. Immunobiology. 2011;216(7):753–62.
12. Weltmann KD, Kindel E, Brandenburg R, Meyer C, Bussiahn R, Wilke C, von Woedtke T. Atmospheric pressure plasma jet for medical therapy: plasma parameters and risk estimation. Contrib Plasma Phys. 2009;49(9):631–40.
13. Bekeschus S, Masur K, Kolata J, Wende K, Schmidt A, Bundscherer L, Barton A, Kramer A, Bröker B, Weltmann KD. Human mononuclear cell survival and proliferation is modulated by cold atmospheric plasma jet. Plasma Process Polym. 2013;10(8):706–13.
14. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer. 1980;26(2):171–6.
15. Schneider U, Schwenk HU, Bornkamm G. Characterization of EBV-genome negative “null” and “T” cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. Int J Cancer. 1977;19(5):621–6.
16. Bundscherer L, Wende K, Ottmüller K, Barton A, Schmidt A, Bekeschus S, Hasse S, Weltmann KD, Masur K, Lindequist U. Impact of non-thermal plasma treatment on MAPK signaling pathways of human immune cell lines. Immunobiology. 2013;218(10):1248–55.
17. Forman HJ, Torres M. Reactive oxygen species and cell signaling: respiratory burst in macrophage signaling. Am J Respir Crit Care Med. 2002;166(12 Pt 2):S4–8.
18. Winter J, Wende K, Masur K, Iseni S, Dünnbier M, Hammer MU, Tresp H, Weltmann KD, Reuter S. Feed gas humidity: a vital parameter affecting a cold atmospheric-pressure plasma jet and plasma-treated human skin cells. J Phys D Appl Phys 2013;46(29):1–11.
19. Tresp H, Hammer MU, Weltmann KD, Reuter S. Plasma generated reactive species in biologically relevant solutions. Plasma Med. In press 2014.
20. Buxton GV, Elliot AJ. Rate constant for reaction of hydroxyl radicals with bicarbonate ions. Int J Radiat Appl Instrum C Radiat Phys Chem. 1986;27(3):241–3.
21. Cutaia M, Black AD, Cohen I, Cassai ND, Sidhu GS. Alkaline stress-induced apoptosis in human pulmonary artery endothelial cells. Apoptosis. 2005;10(6):1457–67.
22. Tresp H, Hammer MU, Winter J, Weltmann KD, Reuter S. Quantitative detection of plasma-generated radicals in liquids by electron paramagnetic resonance spectroscopy. J Phys D Appl Phys 2013;46(43):435401.