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Nonthermal Plasma Increases Expression of Wound Healing Related Genes in a Keratinocyte Cell Line

Annemarie Barton,[1,2] Kristian Wende,[1,2] Lena Bundscherer,[1,2] Sybille Hasse,[1,2] Anke Schmidt,[1,2] Sander Bekeschus,[1,2] Klaus-Dieter Weltmann,[2] Ulrike Lindequist,[3] & Kai Masur[1,2,*]

[1]Centre for Innovation Competence *plasmatis*, Greifswald, Germany; [2]Leibniz Institute for Plasma Science and Technology (INP Greifswald), Greifswald, Germany; [3]Institute of Pharmacy, Ernst Moritz Arndt University of Greifswald, Felix-Hausdorff-Str. 2, 17489 Greifswald, Germany

[*]Address all correspondence to: K. Masur, Centre for Innovation Competence *plasmatis*, Greifswald, Germany; E-mail: kai.masur@inp-greifswald.de

ABSTRACT: The application of physical plasma in medicine has great potential in wound healing. Due to the generation of reactive oxygen and nitrogen species (ROS, RNS), emission of UV radiation and the generated electric fields can be used to stimulate epithelial and immune cells. To understand the processes on a molecular level the human keratinocyte cell line HaCaT was treated with a nonthermal atmospheric pressure argon plasma jet (kinpen). Subsequently, cellular RNA was isolated to conduct a quantitative polymerase chain reaction (qPCR) to monitor the magnitude of translation of genes related to wound healing. Plasma treatment induced an upregulation of vascular endothelial growth factor-a (VEGF-A), heparin-binding epidermal growth factor (EGF)-like growth factor (HBEGF), granulocyte macrophage colony-stimulating factor (GM-CSF), prostaglandin-endoperoxide synthase 2 (PTGS2) and interleukin-6 (IL-6) at the mRNA levels. This is a very promising result as the corresponding proteins are likely to be secreted and promote the wound healing process. Therefore plasma potentially induces the secretion of certain cytokines and growth factors, and hence, it could be the stimulus which is necessary to induce chronic wounds to heal.

KEY WORDS: nonthermal plasma; keratinocytes; wound healing; gene expression; HaCaT cells, plasma medicine

ABBREVIATIONS

CDH1: cadherin 1; **CXCL2:** chemokine (C-X-C motif) ligand 2; **EGF:** epidermal growth factor; **EGFR:** epidermal growth factor receptor; **FGF, -7, 10:** fibroblast growth factor, -7, -10; **G-CSF:** granulocyte colony-stimulating factor; **GM-CSF:** granulocyte macrophage colony-stimulating factor; **HBEGF:** heparin-binding epidermal growth factor-like growth factor; **IGF-1, -10:** insulin-like growth factor-1, -10; **IL-1, -6, -8:** interleukin-1, -6, -8; **IFNγ:** interferon γ; **KGF:** keratinocyte growth factor; **MMP9:** matrix metallopeptidase 9; **PTGS2:** prostaglandin-endoperoxide synthase 2; **TGFα:** transforming growth factor α; **TGFβ1:** transforming growth factor β1; **TNF:** tumor necrosis factor; **VEGF-A:** vascular endothelial growth factor-A

I. INTRODUCTION

With its tissue-tolerable temperature, nonthermal atmospheric pressure plasma is a very attractive tool for medical application.[1,2] It has been reported that plasma can have an-

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timicrobial effects on chronic wounds, whereas a negative impact on the tissue was not detectable.[3,4] Plasma generates ROS, RNS, UV-radiation, and an electric field. Especially the complex collection of reactive species including H_2O_2, O_3, O_2^*, NO, NO_2, N_2^*, and OH may enhance wound healing.[5] For ROS and RNS, electric fields, and UV radiation, generally it is known that they could speed up healing processes by stimulating cytokine secretion of different cell types.[6–8] Therefore, plasma medicine emerges as a promising research field, especially wound healing. Wound healing is a well-orchestrated and complex mechanism. Different cell types, e.g., leukocytes, lymphocytes, monocytes, neutrophils, fibroblasts, and keratinocytes are involved in this process. Inflammation, proliferation, and remodeling are the three phases of wound healing.[6,9,10] During these overlapping phases the cells secrete diverse cytokines such as growth factors or interleukins to stimulate the surrounding tissue and enhance the wound healing process.[10] During inflammation platelets become activated and subsequently release many anti-inflammatory mediators such as IL-8, CXCL2, TGFβ1, and IL-1 to recruit macrophages, neutrophils, or fibroblasts to the wound site.[8] Simultaneously γδ T-cells, immune cells, which are located in the tissue, release a wide range of growth factors such as FGF-7 and -10, IGF-10, and KGFs to induce the proliferation and differentiation of keratinocytes.[8] If this well-organized process is disturbed, i.e., in diabetic patients, it may result in a nonhealing chronic wound, which is associated with an additional colonization of bacteria. It is very difficult to cure chronic wounds and there is a worldwide need for new and more effective treatment possibilities. A new option can be the treatment of wounds with atmospheric pressure plasma. It has been shown in vitro that human skin and immune cells react in a dose-dependent manner after plasma treatment.[11–14] At long treatment times the cells induce apoptosis whereas short treatments promote normal proliferation. This effect is accompanied by a change in gene expression and protein activation or secretion.[7,15,16]

The aim of this study was to examine the influence of nonthermal atmospheric pressure argon plasma on keratinocytes' gene expression during wound healing. Therefore, the changes of mRNA transcription of the human keratinocyte cell line HaCaT were analyzed after plasma treatment. The studied genes belong to different subgroups like extracellular matrix, cell adhesion, growth factors, inflammatory cytokines and chemokines, and signal transduction which are very important for the different phases in wound healing. The keratinocytes play a crucial role in wound healing, especially in the remodeling phase where they migrate and proliferate. But also the cross talk of the keratinocytes with each other or with different cell types, by signaling molecules such as cytokines and growth factors, is very important.[10,17,18] To screen which genes could be triggered by plasma treatment the 84 most important genes for the following proteins were investigated: collagens, E-cadherin, integrins, angiopoietin 1, GM-CSF, G-CSF, EGF, FGFs, HBEGF, IGF1, TGFα, TGFβ1, TNF, VEGF-A, chemokine ligands, IFNγ, interleukins, Wnt-pathway related proteins, and EGFR. Changes in the respective gene expressions could indicate the ability of plasma to affect the wound healing processes and hence are a promising outcome of the present study.

II. MATERIAL AND METHODS

A. Cell Culture

This experiment was conducted with the human keratinocyte cell line HaCaT (DKFZ, Heidelberg, Germany), which was passaged every 3 to 4 days. Therefore, they were washed with PBS/EDTA (5 mM, Lonza Group AG, Basel, Switzerland) and subsequently incubated in PBS/EDTA for 10 min (37°C, 95% humidity, 5% CO_2). For detachment trypsin (170,000 $U \cdot L^{-1}$)/EDTA (200 $mg \cdot L^{-1}$) (Lonza Group AG, Basel, Switzerland) was added to the cells and incubated for 4 min. The detached cells were gathered with Roswell Park Memorial Institute (RPMI) 1640 medium (Lonza Group AG, Basel, Switzerland) supplemented with 8% fetal calf serum (FCS; Sigma-Aldrich, St. Louis, United States of America), 2 mM L-glutamine, 0.1 $mg \cdot L^{-1}$ streptomycin and 100 $U \cdot mL^{-1}$ penicillin (Lonza Group AG, Basel, Switzerland), which was the cell culture standard medium. Detached cells were centrifuged at 250 g for 3 min at room temperature. The supernatant was discarded and the pellet was resuspended in 10 mL of standard medium. The HaCaT cells were counted via a Buerker counting chamber and seeded with a density of 1.3×10^5 cells mL^{-1}.

B. Plasma Treatment

HaCaT cells were subcultivated and 2×10^5 cells mL^{-1} were seeded in a 60 mm petri dish, 24 h before plasma treatment. Plasma treatment was conducted with the nonthermal atmospheric pressure plasma jet kinpen09 (neoplas GmbH, Greifswald, Germany), which according to the manufacturer, operates at a voltage between 2 and 6 kV_{pp} at a frequency around 1 MHz. An argon gas flow rate of 3 standard liters per minute was used.[1] As a direct treatment induces additional mechanical stress to the cells due to the gas flow, which displaces the water layer in the petri dish, an indirect treatment was chosen. Therefore 5 mL standard medium in a 60 mm petri dish was treated for 120 or 180 s with the plasma jet in a meandering pattern. The cells were immediately exposed to the treated medium by replacing the cell culture medium with the treated one. The control cells obtained the same treatment, while the medium was not plasma treated. The medium from all samples was exchanged by untreated standard medium after the first hour post-treatment. All samples were incubated for 6 or 12 h after treatment in total. From previous studies it is known that plasma induced changes of the gene expression can be detected after 6 h incubation time. To probe the further temporal development of the signal, an incubation time of 12 h was also chosen.

Additionally, the impact of different plasma treatment times, ranging from 5 s to 5 min, on cell survival of the HaCaT cells had also been investigated before. It was shown that treatments up to 120 s did not influence cell survival and the treated HaCaT cells behaved similarly to untreated control cells. However, a plasma treatment of 180 s induced a slight increase in apoptosis but the majority was still viable (around 85%). Therefore, a short (120 s) and a long (180 s) plasma treatment were chosen for these experiments.

C. Sample Preparation

After incubation time the medium was removed and the keratinocytes were washed with 1 mL of ice-cold PBS (1×, w/o Ca^{2+}/Mg^{2+}, PAA Laboratories GmbH, Pasching, Austria). Subsequently, 1 mL ice-cold PBS was added to the cells and the cell layer was detached via cell scraper and transferred into a microvial. The cells were centrifuged at 250*g* at 4°C for 5 min. The supernatant was removed and the cell pellet deep-frozen at –80°C.

RNA of the samples was isolated according to the manufacturer's instruction (Bio&SELL e.K., Feucht, Germany) and the cDNA synthesis was operated with the RT^2 First Strand Kit (Qiagen, Hilden, Germany). Subsequently, qPCR was performed with the RT^2 Profiler PCR Array (Human Wound Healing PCR Array, Qiagen, Hilden, Germany) with the LightCycler 480 (Roche Diagnostics GmbH, Mannheim, Germany). The experiments were repeated three times and the gene expression was considered significantly changed if a twofold up- or downregulation compared to the untreated control was detected. After amplification of the target a melting cure was performed. With this step the specificity of the used primers was controlled. During the melting cure the temperature was continuously increased from 60 to 95°C. The analyzed genes are listed at the URL.[19]

III. RESULTS AND DISCUSSION

The human keratinocyte cell line HaCaT reacts with a change of gene expression due to plasma treatment. Table 1 displays the 21 genes, which are significantly up- or downregulated after plasma exposure. Among them are the genes, which encode the prominent proteins VEGF-A, HBEGF, GM-CSF, or PTGS2. Due to the high number of analyzed genes only the most significant are presented and discussed here. Some of the most prominently changed gene expressions encode for proteins involved in angiogenesis, the generation of new blood vessels, which is an important process during wound healing. The plasma treatment of the HaCaT cells enhanced the transcription rate of the angiogenesis factor VEGF-A within the first 6 h after treatment (Table 1). Both 120 and 180 s plasma treatment induced a fourfold upregulation of the gene expression. It is known that RNS such as peroxynitrite, which is formed by superoxide anion and nitric oxide, augment a VEGF-A release in fibroblasts.[20] Therefore, it could be that the same effect leads to the observed increase in HaCaT cells. A transient upregulation of VEGF-A could induce or speed up the angiogenesis in damaged tissues.[21,22] However, a permanent increase of VEGF-A, which was not shown in our results, could also have negative effects and is associated with diseases such as psoriasis.[23]

Another angiogenesis factor is angiopoietin 1, which rapidly declined within the first 6 h after plasma treatment (Table 1). It has been reported to be upregulated during wound healing [24,25] and other studies indicate that interleukin-6 can cause a downregulation of angiopoietin 1 and at the same time a VEGF upregulation,[26] which is in good agreement with the detected changes after plasma treatment in our experiments (Table 1).

TABLE 1. Genes which showed a significantly-changed-fold regulation after plasma treatment compared to the untreated sample. A positive-fold regulation describes an upregulation (red) and a negative a downregulation (blue) of the gene.

Short gene name	Gene name	Treatment and incubation time	Fold regulation
ACTA2	Actin, alpha 2	120 s, 6 h	4
		180 s, 6 h	8
ANGPT1	Angiopoietin 1	120 s, 6 h	–52
		180 s, 6 h	–45
B2M	Beta-2-microglobulin	180 s, 6 h	–10
CCL2	Chemokine (C-C motif) ligand 2	120 s, 6 h	–5
		180 s, 12 h	–5
CDH1	Cadherin 1, type 1, E-cadherin	180 s, 6 h	–13
		120 s, 12 h	5
COL5A2	Collagen, type V, alpha 2	180 s, 6 h	20
CSF2 (GM-CSF)	Colony-stimulating factor 2 (granulocyte-macrophage)	180 s, 6 h	13
		120 s, 12 h	6
		180 s, 12 h	7
CSF3	Colony-stimulating factor 3 (granulocyte)	180 s, 6 h	6
CXCL1	Chemokine (C-X-C motif) ligand 1	180 s, 12 h	–4
CXCL2	Chemokine (C-X-C motif) ligand 2	180 s, 6 h	12
FGF10	Fibroblast growth factor 10	180 s, 12 h	9
HBEGF	Heparin-binding EGF-like growth factor	120 s, 6 h	6
		180 s, 6 h	13
		180 s, 12 h	4
IL1B	Interleukin 1, beta	180 s, 12 h	5
IL6	Interleukin 6	120 s, 6 h	7
		180 s, 6 h	29
ITGA5	Integrin, alpha 5	180 s, 6 h	6
ITGB6	Integrin, beta 6	180 s, 6 h	5
MMP9	Matrix metallopeptidase 9	180 s, 6 h	5
PLAUR	Plasminogen activator, urokinase receptor	180 s, 6 h	5
PTGS2	Prostaglandin-endoperoxide synthase 2	180 s, 6 h	7
TAGLN	Transgelin	180 s, 6 h	4
VEGFA	Vascular endothelial growth factor A	120 s, 6 h	4
		180 s, 6 h	4

A further protein that influences angiogenesis is granulocyte macrophage colony-stimulating factor (GM-CSF). An overexpression of GM-CSF improves wound healing by recruitment of leukocytes, enhances keratinocyte proliferation, and increases angiogenesis via VEGF upregulation.[27–29] Keratinocytes are able to produce this stimulating factor immediately post– skin injury.[27,30] Table 1 visualizes the increased gene expression of GM-CSF in HaCaT cells after plasma treatment. Twelve hours post-treatment a sixfold upregulation after 120 s treatment and a sevenfold upregulation after 180 s was observed. For the short incubation time of 6 h a doubled upregulation was detectable for 180 s of treatment, whereas a treatment of 120 s has not shown any changes in gene expression of GM-CSF. Studies by Imokawa et al. and Fang et al. have shown a correlation between UV radiation and increase of GM-CSF and IL-6 production.[31,32] ROS, which were generated during UV radiation, are probably responsible for this cellular reaction. Accordingly, also the plasma generated ROS can have triggered the GM-CSF gene expression in the same way as the UV generated ROS in the studies by Imokawa et al. and Fang et al.

Angiogenesis could also be regulated via the protein prostaglandin-endoperoxide synthase 2, which is encoded by the gene PTGS2. Table 1 illustrates a PTGS2 regulation by plasma. An upregulation after a 180 s treatment and 6 h of incubation was observed, whereas for 120 s no significant changes were detected. After the longer incubation time (12 h) both samples (120 and 180 s) did not show any changes in regulation. It is known that the expression rate of PTGS2 can be increased via UV radiation, followed by ROS production.[33] Therefore, also the plasma generated ROS could impact the gene expression of PTGS2. The reason for activation only in the first 6 h could be that PTGS2 is an intermediate early gene in wound healing.[34] After 12 h the activation of PTGS2 was decreased and the expression was not significantly changed compared to control. This could be a hint that PTGS2 is only upregulated by higher amounts of generated ROS. It is also known that PTGS2 could be stimulated by cytokines and growth factors[35] and further investigations have to examine if some mediators, which were regulated after plasma treatment in the early phase, activated PTGS2 upregulation, e.g., IL-6. In addition to angiogenesis, PTGS2 can also promote cell proliferation[33] and therefore it is a very important agent during wound healing.

Another growth factor, which was regulated by plasma, is HBEGF. In human wounds HBEGF is an early gene, which is upregulated within the first hours.[36] Interestingly this behavior is shown in HaCaT cells, too. The heparin-binding EGF-like growth factor is well known to induce keratinocyte proliferation and regeneration during injury.[37,38] It also has mitogenic and cell survival promoting properties on fibroblasts and further multiple cell types.[24,33] After the first 6 h this gene showed a sixfold and twelvefold upregulation for 120 and 180 s of treatment, shown in Table 1. After 12 h incubation, this upregulation vanished and 180 s of treatment even led to a minor decrease of gene expression. The expression of HBEGF is described to be rapidly increased by oxidative stress, tissue damage, during wound healing, and regeneration.[38] After oxidative stress using hydrogen peroxide (H_2O_2) an increased mRNA level and elevated release of HBEGF into the medium had been shown.[36,38–40] Therefore, a post-plasma treatment increased gene expression

was expected. The differences between the 120 and 180 s treatment duration could be explained by H_2O_2 generated during plasma exposure. The longer the plasma treatment duration the higher the amount of produced ROS. An upregulation of HBEGF by plasma treatment would have positive effects for keratinocytes and other wound related cells and might deliver the necessary stimulus to initiate wound closure.

Besides the release of growth factors and angiogenesis promoting mediators, another important mechanism during wound healing is the migration of keratinocytes. One example is interleukin-6 whose stimulation by plasma is demonstrated in Table 1. The gene, which encodes IL-6, was upregulated in both samples at the incubation time of 6 h, and for 180 s the gene expression was exceedingly increased (29-fold). It is known that both ROS[41–43] and RNS[44] can increase the level of IL-6 in a dose-dependent manner, which could explain these results. Moreover, the upregulation of IL-6 is also correlated with an increase of further stimulating factors such as GM-CSF, which was found to be upregulated in our experiments, too.[31,32] Interleukin-6 is an anti-inflammatory cytokine, which enhances wound healing.[30]

For a successful cell migration of the wound-edged keratinocytes the ECM has to be degraded. Therefore, MMPs, like MMP-9, are synthesized by the keratinocytes to remove and reorder the provisional matrices.[10,45] A plasma treatment for 180 s led to a fivefold upregulation of MMP-9 6 h later which could initiate the degradation of the ECM in the wound bed and increase the migration of wound edged keratinocytes. However, 12 h post–plasma exposure MMP-9 was not significantly regulated by plasma. It is known that the expression of MMP-9 can be repressed by TAGLN, the gene which encodes the actin-binding protein transgelin, which was also upregulated after plasma treatment.[46]

Next to IL-6 and MMP-9 also the fibroblast growth factor-10 (FGF-10) enhances proliferation and migration of keratinocytes, which is very important for the re-epithelization in wound healing. Furthermore, this mitogenic growth factor can reduce the ROS-induced apoptosis in keratinocytes by the transcription of ROS detoxifying factors.[47,48] In order to react to the plasma generated ROS the FGF-10 was upregulated (ninefold) 12 h after long-term plasma treatment (180 s).

Other important cytokines and growth factors inducing keratinocyte migration are epidermal growth factor (EGF), interleukin-1α (IL-1α), and transforming growth factor-β1 (TGFβ1).[49] However, these three candidates were not found to be significantly regulated by plasma in the investigated keratinocyte cell line.

During migration cellular adhesion plays a very important role and it is known that after ROS or plasma exposure the adhesion molecule E-cadherin was less detectable.[50,51] Therefore it was to be expected that the gene CDH1, which encodes E-cadherin was downregulated post–plasma exposure. However, the results showed both a down- and an upregulation (Table 1) of CDH1. One hypothesis could be that the cells decreased CDH1 to induce proliferation and subsequently it was upregulated to affect adhesion again. It was also shown that abundance of the epidermal growth factor receptor (EGFR) can be decreased post–plasma treatment,[51] but in our experiments the amount of EGFR gene expression was not significantly changed compared to untreated cells. This could

be caused by the different experimental setups, e.g., the incubation time (6 and 12 h vs. 24 h). Additionally, in our study the mRNA level and not the proteins were analyzed. One hypothesis could be that the receptor at the cell membrane was modified by plasma generated ROS and due to these morphological changes the used antibody was not able to bind. It also could be that the receptor was internalized and was therefore not detected.

Two further studies investigated the influence of plasma on the adhesion molecules α_2-integrin and β_1-integrin of human keratinocytes. Interestingly, two different plasma sources were used: on the one hand a dielectric barrier discharge, and on the other hand the kinpen plasma jet, which was used for these experiments, too. It was shown that the effects on integrins could vary by different plasma sources and experimental designs.[14,51] Additionally, during the work of Haertel et al. the incubation time was extended up to 24 h. These changes at the experimental design could be the reason why α_2-integrin and β_1-integrin were changed in their experiments, but not in ours, after plasma exposure.

IV. CONCLUSION

Our work shows that plasma can have modulating effects on the transcriptome of the human keratinocyte cell line HaCaT. The regulation of wound healing related genes, which encode for, e.g., growth factors and cytokines, could be a necessary stimulus to trigger the healing of chronic wounds. Many research groups study both acute as well as chronic wounds in order to develop new wound healing therapies. One new approach is the addition of exogenous growth factors such as HBEGF to the wound to stimulate a quicker re-epithelialization and wound closure.[37] But it is known that a combination of various growth factors has better synergistic effects than the addition of a growth factor alone.[52] Therefore, if wounds were treated with plasma many important wound healing mediators could be expressed and subsequently released by the different cell types, which could stimulate the healing process. We showed that keratinocytes in vitro increased the gene expression of numerous wound healing related factors such as VEGF, GM-CSF, FGF-10, HBEGF, IL-1β and -6, integrin-α5 and -β6, MMP9, and transgelin. Most of the plasma regulated genes encode proteins, which are responsible for angiogenesis or proliferation, but also recruitment or cell adhesion, and interestingly only a few for extracellular matrix. The reasons for the changed gene expression following plasma exposure have to be clarified. Some changes can result from the reactive nitrogen species or reactive oxygen species, generated by plasma. It is also known that some genes were regulated due to other mediators, which were activated by plasma earlier. Further investigations have to analyze if the up- or downregulation of these genes is associated with a changed translation and, e.g., growth factors and cytokines are secreted into the cellular environment.

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