31st Annual International Conference of the IEEE EMBS
Minneapolis, Minnesota, USA, September 2-6, 2009

Cell proliferation following non-thermal plasma is related to reactive oxygen species induced fibroblast growth factor-2 release.

Sameer U. Kalghatgi, Member IEEE, Alexander Fridman, Gary Friedman, and Alisa Morss Clyne

Abstract— **Non-thermal dielectric barrier discharge plasma is currently being developed for a wide range of medical applications, including blood coagulation, malignant cell apoptosis, and wound healing. However, the effect of non-thermal plasma on the vasculature is unclear. Blood vessels are affected during plasma treatment of many tissues, and vessels themselves may be an important clinical plasma therapy target. We investigated the effect of non-thermal plasma treatment on endothelial cells, which line the inner surface of blood vessels. Non-thermal plasma treatment at short exposures (up to 30 seconds; 4 J/cm^2) was relatively non-toxic to endothelial cells. Endothelial cells treated with plasma for 30 seconds demonstrated twice as much proliferation as untreated cells five days after plasma treatment. Proliferation was abrogated by a fibroblast growth factor-2 neutralizing antibody and reactive oxygen species inhibitors. This suggests that plasma-induced endothelial cell proliferation is caused by growth factor release following reactive oxygen species cell membrane damage. These data suggest that low power non-thermal plasma treatment is a potential novel therapy for promotion of endothelial cell mediated angiogenesis.**

I. Introduction

Non-thermal plasma has recently emerged as a novel tool for medical applications, including blood coagulation, malignant cell apoptosis, and wound healing [1–3]. Dielectric Barrier Discharge (DBD) plasma occurs at atmospheric pressure in air or other gases when high voltage of sinusoidal waveform or short duration pulses is applied between two electrodes, with at least one electrode being insulated [4]. The insulator prevents current build-up between the electrodes, creating electrically safe plasma without substantial gas heating. This approach allows direct treatment of biological systems without the thermal damage observed in more conventional thermal plasma [5].

Plasma interaction with the vasculature is important to understand prior to treating any vascularized tissue. Endothelial cells, which line all blood contacting surfaces in the body, control many aspects of the vasculature ranging from vascular tone to coagulation to inflammation. Endothelial cells also play a guiding role in angiogenesis, the growth of new blood vessels from existing vessels [6]. Endothelial cells produce and secrete angiogenic growth factors such as fibroblast growth factor-2 (FGF2), which in conjunction with many other signals induces endothelial cells to invade the surrounding tissue, proliferate, and develop into new blood vessels [7].

Plasma-induced angiogenesis is of particular interest for wound healing. Wound healing follows a tightly regulated timeline, proceeding from the inflammatory phase to the proliferative phase to the remodeling phase. In chronic wounds, this timeline may be altered, with varying phases overlapping and inhibiting effective healing. Plasma devices have shown remarkable effects in promoting wound healing [8]. We believe that plasma properties can be tuned to further enhance healing through increased angiogenesis.

We have previously shown that low dose non-thermal plasma treatment is relatively non-toxic to endothelial cells [9]. In this study, we used an *in vitro* model to investigate the effect of non-thermal plasma on endothelial cell proliferation.

II. Materials and Methods

A. Cell culture

Porcine aortic endothelial cells (PAEC) were maintained in low glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. For plasma treatment, cells were washed with phosphate buffered saline, detached with 0.1% trypsin and seeded near confluence on 18 mm diameter glass cover slips in 12-well plates. Cells were cultured for 24 hours prior to plasma treatment in 1.5 ml supplemented medium in a 37°C, 5% CO_2 incubator to allow full attachment and spreading. Recombinant human FGF2 was from Peprotech, and the neutralizing FGF2 antibody was from Upstate Biotechnology. *N*-Acetyl-L-cysteine, an intracellular reactive oxygen species (ROS) scavenger, and sodium pyruvate, an extracellular ROS scavenger were from Sigma.

B. Non-thermal plasma treatment

Non-thermal atmospheric pressure dielectric barrier discharge plasma was produced using an experimental setup similar to one previously described and illustrated in Fig. 1 [2]. Plasma was generated by applying alternating polarity pulsed (500 Hz – 1.5 kHz) voltage of ~20 kV magnitude (peak to peak) between the insulated high voltage electrode and the sample undergoing treatment using a variable voltage and variable frequency power supply. Discharge power density was measured to be 0.13 Watts/cm^2 (at

Manuscript received April 7 2009.

Sameer Kalghatgi and Gary Friedman are in the Department of Electrical and Computer Engineering, Drexel University, Philadelphia, PA 19104, USA (phone: 215-895-2909; e-mail: sameer.kalghatgi@drexel.edu, gary@cbis.ece.drexel.edu).

Alexander Fridman and Alisa Morss Clyne are in the Department of Mechanical Engineering and Mechanics, Drexel University, Philadelphia, PA 19104, USA (e-mail: af55@drexel.edu, asm67@drexel.edu)

500Hz) and 0.31 Watts/cm^2 (at 1.5 kHz).

PAEC on glass cover slips were exposed to low power plasma for 0 to 60 seconds. 50 µl of serum free medium was added to the glass cover slip before plasma treatment to prevent sample drying. Following plasma treatment, cover slips were immediately placed in a new 12-well plate, 1.5 ml of supplemented medium was added to each well, and the samples were returned to the incubator.

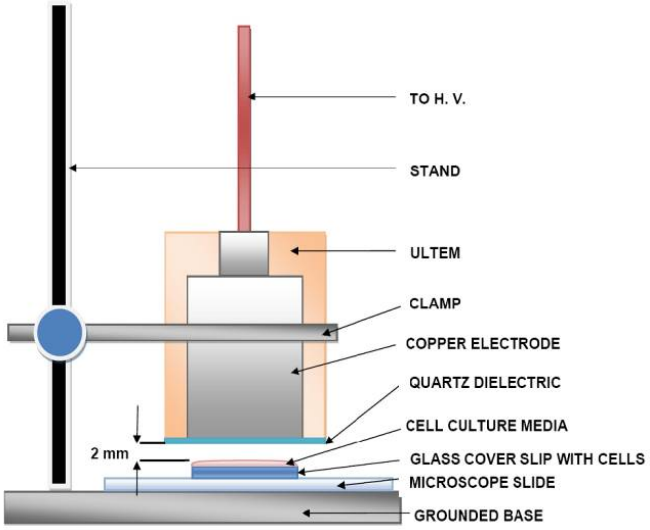

Fig. 1 DBD plasma device schematic showing the high voltage electrode and the same holder.

C. Endothelial cell proliferation

Endothelial cell number was determined by counting trypsin-detached cells in a Coulter counter. Counted cells were confirmed as viable since they are attached to the tissue culture plate, and by trypan blue. Endothelial cell proliferation was measured through cell counts either on directly treated cells or through a conditioned medium assay. Conditioned medium was prepared by incubating control or plasma-treated cells in serum free medium for 24 hours. For cell counts, 10,000 PAEC were seeded on 18 mm diameter cover slips in 12-well plates. Cells were plasma treated as described and incubated for an additional 7 days with a medium change on days 2, 4 and 6. Cell number was quantified on days 1, 3, 5 and 7 by counting trypsin-detached cells using a Coulter counter. For directly treated cells, fold proliferation was determined by taking the ratio of cell number on day five to day one.

D. Fibroblast growth factor-2 release

FGF2 release from plasma-treated cells was measured by enzyme linked immunosorbent assay (ELISA) using an FGF Elisa Kit (R&D Systems) as per manufacturer instructions. FGF2 effects were blocked using an FGF2 neutralizing antibody (10 µg/ml), which was pre-incubated for 30 min with the conditioned medium prior to adding it to cells.

E. Statistical analysis

Data are mean±SD. Statistical significance was evaluated using Student's t test (2 groups) and ANOVA (>2 groups).

III. Results

A. Endothelial cell proliferation

Endothelial cell proliferation was enhanced by low dose non-thermal plasma treatment (Fig. 2). Cells treated with plasma showed greater cell number than control with up to 30 seconds of plasma treatment. Counted cell viability was confirmed by trypan blue. With the 30 second treatment, endothelial cells demonstrated twice as many viable cells on day 5 as untreated controls. Cell proliferation was confirmed by measuring increased DNA synthesis via a BrdU assay.

All plasma treatment times resulted in some cell death. Death occurred within 24 hours of plasma treatment, and no further cell death was observed at later time. Dead cells were removed during medium changes prior to day 5. Plasma-induced cell death likely occurs due to apoptosis, which was confirmed via an Annexin V assay. At high plasma treatment times (60 seconds), early cell death resulted in a lower overall cell number at day 5.

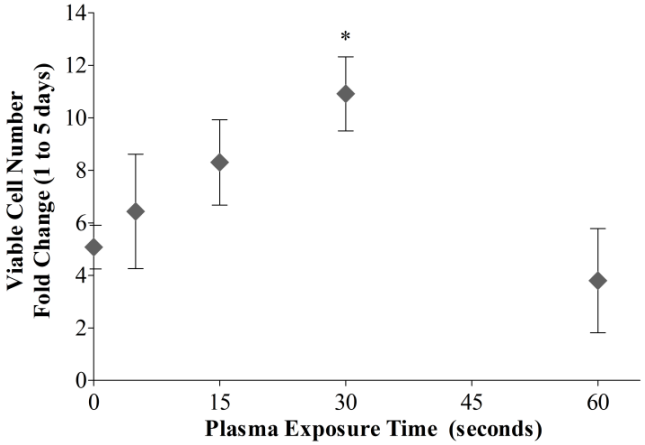

Fig. 2 Endothelial cell proliferation is enhanced 5 days after plasma treatment, as measured by viable cell counts.*p < 0.01

When conditioned medium from 30 second plasma-treated cells was added to untreated cells, a significant cell number increase was observed at 3 days (Fig. 3). By seven days, cells incubated with plasma-treated cell conditioned medium had twice as many cells as untreated control.

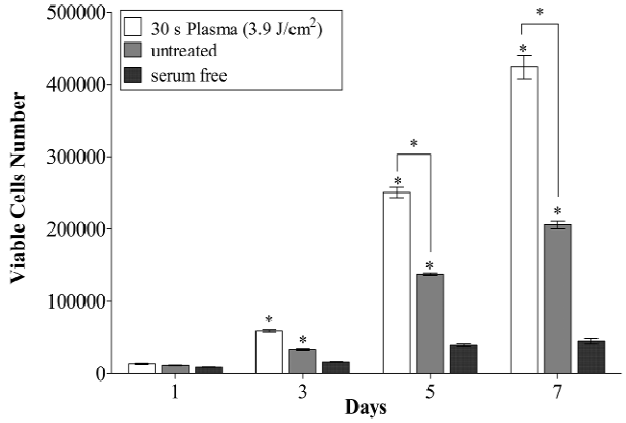

Fig. 3 Endothelial cell proliferation is enhanced in cells incubated in non-thermal plasma treated conditioned medium.*p < 0.01

B. Fibroblast growth factor-2 release

To determine if enhanced proliferation related to growth factor release, we measured FGF2 in cell conditioned medium following 30 second plasma treatment (Fig. 4). The medium FGF2 level increased up to 3 hours after plasma treatment and then rapidly decreased up to 24 hours after plasma treatment. In contrast, FGF2 medium levels for cells treated with 10 ng/ml TNF-α as a positive control rose more slowly but continued to rise up to 24 hours.

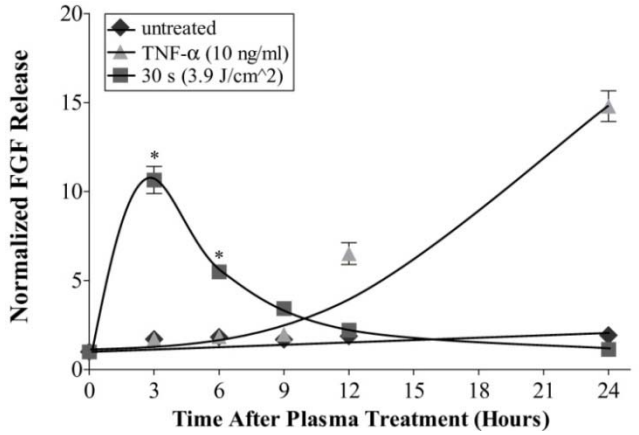

Fig. 4 Non-thermal plasma induces FGF-2 release from endothelial cells. Release peaks 3 hours after plasma treatment and subsequently decreases up to 24 hours after plasma treatment.*p<0.01

C. FGF-2 related cell proliferation

The role of FGF2 release in enhanced endothelial cell proliferation by plasma treatment was investigated using a neutralizing antibody to block FGF2 (Fig. 5). The FGF2 antibody binds to cell-released FGF2 and prevents it from activating cell surface receptors. The FGF2 neutralizing antibody had no effect on cells exposed to untreated cell conditioned medium. However, the FGF2 antibody significantly suppressed proliferation in endothelial cells exposed to plasma-treated cell conditioned medium. Viable cell number for samples incubated in plasma-treated cell conditioned medium with a neutralizing antibody was similar to samples incubated in untreated cell conditioned medium. The cell proliferation decrease following FGF2 neutralization was confirmed by BrdU DNA synthesis assay.

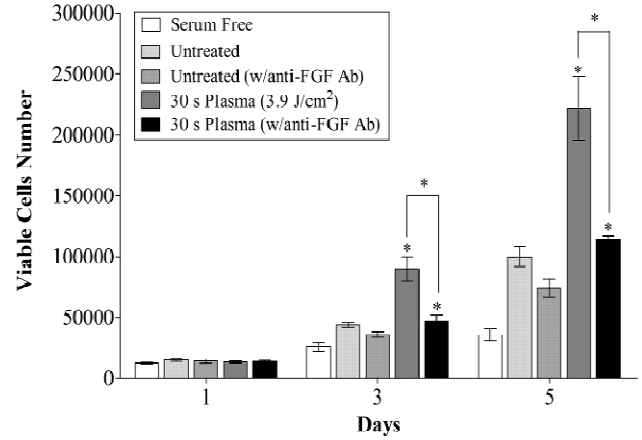

Fig. 5 FGF-2 blockade using an FGF-neutralizing antibody inhibits proliferation of non-thermal plasma treated endothelial cells.*p < 0.01

D. Reactive oxygen species

Non-thermal plasma produces large amounts of ROS. These ROS may interact with the endothelial cell membrane, leading to damage that allows FGF2 release from plasma-treated cells. To test the role of ROS in the plasma-induced cell FGF2 release, endothelial cells were pre-incubated in 4 mM *N*-acetyl cysteine (NAC) to scavenge intracellular ROS and then plasma-treated in supplemented medium with or without 50 mM sodium pyruvate to scavenge extracellular ROS. Both NAC and sodium pyruvate significantly suppressed FGF2 release from plasma treated cells (Fig. 6), suggesting that intracellular and extracellular ROS may mediate the plasma-induced endothelial cell FGF2 release.

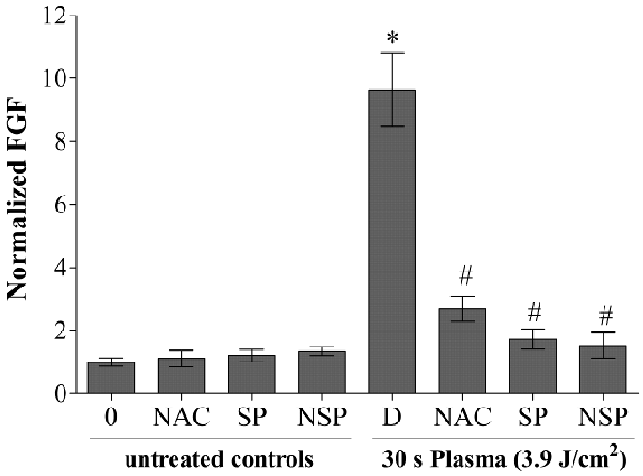

Fig. 6 ROS scavengers block FGF2 release from endothelial cells post plasma treatment..NAC: N-acetyl cysteine (4 mM), SP: sodium pyruvate (50 mM), NSP: NAC and SP. *p < 0.01, #p<0.05 as compared to control.

IV. Discussion

We now show that low dose plasma exposure enhances endothelial cell proliferation through reactive oxygen species-induced FGF2 release. These data suggest that plasma devices may be able to promote wound healing by promoting angiogenesis in a dose dependent manner.

Endothelial cells produce angiogenic growth factors, such as FGF2, which in conjunction with other cell signaling pathways induce endothelial cell proliferation, migration, and tube formation. Since FGF2 has no signal sequence for secretion, it is only known to be released with sub-lethal cell membrane damage or cell death [7]. Other stimuli that induce sub-lethal cell membrane damage also lead to FGF2 release, including mechanical forces, ionizing radiation, and inductively coupled pulsed electromagnetic fields [10-12]. Non-thermal plasma differs from irradiation and electromagnetic fields in that the latter are either penetrating and therefore injure surrounding tissue, or they need extensive and expensive setup to be generated safely and applied to human tissue. Non-thermal plasma provides a novel and safer means to induce FGF2 release and angiogenesis since it is non-penetrating and therefore provides precise control of treatment area and depth. Non-

thermal plasma devices are also small and relatively simple to produce.

Plasma-induced FGF2 release is likely related to neutral ROS. Non-thermal plasmas produce long lived (O_3, NO, HO_2, H_2O_2) and short lived (OH, O, electronically excited O (1D), O_2 (1Δg)) neutral particles and charged particles (ions and electrons). Both charged and neutral particles can lead to additional ROS production in the treated fluid. When endothelial cells were treated directly or indirectly (using a grounded mesh to exclude charged particles, data not shown), endothelial cell FGF2 release was not significantly altered. Non-thermal plasma produces a large concentration of reactive oxygen species in the extracellular environment (cell medium) covering the cells during treatment. The endothelial cell membrane damage, and the subsequent release of FGF2, appear to be related to intracellular and extracellular reactive oxygen species produced by active short living and long living neutral plasma components.

When endothelial cells are exposed to plasma, the conditioned medium FGF2 level peaks three hours after plasma treatment and then declines up to 24 hours. This could allow tight control of FGF2 signaling in wound healing. In contrast, cells treated with TNF-α show a gradual increase in medium FGF2 up to 24 hours. Thus plasma FGF2 release kinetics are essentially different from those of TNF-α. Possible explanations include that TNF-α remains in the medium continuously for 24 hours whereas plasma treatment occurs over a short, finite time period, or that while both plasma and TNF-α likely release FGF2 related to ROS effects, TNF-α takes longer to produce ROS due to signaling requirements [13].

We believe that non-thermal plasma could be used *in vitro* and *in vivo* to stimulate angiogenesis. Potential applications of plasma treatment include tissue engineering structure vascularization, enhanced transplanted tissue incorporation, and accelerated wound healing. Our two-dimensional treatment model, which consists of an endothelial cell monolayer on a glass substrate covered with a thin medium film (~ 100-200 microns), is likely to be more severe than what would be experienced by cells either *in vivo* or as part of three-dimensional *in vitro* models. This suggests that even lower toxicity may actually be observed.

V. Conclusions and Future Work

Low dose, non-thermal atmospheric pressure dielectric barrier discharge plasma enhances endothelial cell proliferation via FGF2 release. These studies suggest that modulation of plasma treatment dose may be able to progress blood vessel growth. Future work includes cell treatment within a three-dimensional model.

Acknowledgments

We would like to thank Gregory Fridman for building the plasma device.

References

[1] G. Fridman, M. Peddinghaus, H. Ayan, M. Balasubramanian, A. Gutsol, A. Brooks, A. Fridman, and G. Friedman. "Blood Coagulation and Living Tissue Sterilization by Floating-Electrode Dielectric Barrier Discharge in Air," *Plasma Chem. and Plasma Proc.* 26(4): 425-442, 2006.

[2] G. Fridman, A. Shereshevsky, M.M. Jost, A.D. Brooks, A. Fridman, A. Gutsol, V. Vasilets, and G. Friedman. "Floating Electrode Dielectric Barrier Discharge Plasma in Air Promoting Apoptotic Behavior in Melanoma Skin Cancer Cell Lines," *Plasma Chemistry and Plasma Processing,* 27(2): 163-176, 2007.

[3] V. Gostev and D. Dobrynin. "Medical microplasmatron." In 3rd International Workshop on Microplasmas (IWM-2006). Greifswald, Germany 2006.

[4] W. Egli, U. Kogelschatz, and B. Elliason. "Modeling of Dielectric Barrier Discharge Chemistry," *Pure and Appl. Chem.*, 66(6):1275-1286, 1994.

[5] J.J. Vargo. "Clinical Applications of Argon Plasma Coagulator," *Gastrointestinal Endoscopy*, 59(1): 81-88, 2004.

[6] J. Folkman. "Angiogenesis," *Annu. Rev. Med.*, 57:1-18, 2006.

[7] M. Nugent and R. Iozzo. "Fibroblast Growth Factor-2," *Int. J. Biochem. Cell Bio.* 32:115-120, 2000.

[8] A.B. Shekhter, V. Serezhenkovb, T. Rudenkoa, A. Pekshevc and A. Vanin. "Beneficial effect of gaseous nitric oxide on the healing of skin wounds," *Nitric Oxide-Biology and Chemistry*, 12(4): 210-219, 2005.

[9] S. Kalghatgi, G. Fridman, A. Fridman, G. Friedman, and A. Morss Clyne, "Non-thermal dielectric barrier discharge plasma treatment of endothelial cells," IEEE EMBS 2008.

[10] L. Muthukrishnan, E. Warder, and P. McNeil, "Basic fibroblast growth factor is efficiently released from a cytolsolic storage site through plasma membrane disruptions of endothelial cells," *J. Cell Physiol.* 148(1):1-16, 1991.

[11] Z. Fuks, R. S. Persaud, A. Alfieri, M. McLoughlin, D. Ehleiter, J. L. Schwartz, A. P. Seddon, C. Cordon-Cardo, and A. Haimovitz-Friedman. "Basic fibroblast growth factor protects endothelial cells against radiation-induced programmed cell death in vitro and in vivo," *Cancer Res*. 54: 2582–2590, 1994.

[12] M.J. Callaghan, L. Chang, N. Sieser, S. Aarabi, S. Ghali, E. Kinnucan, B. Simon, B., and C.J. Gutner, "Pulsed electromagnetic fields accelerate normal and diabetic wound healing by increasing endogenous FGF-2 release," *Plast Reconstr Surg.* 121(1):130-41, 2006.

[13] W. Fiers, R. Beyaert, W. Declercq and P. Vandenabeele. "More than one way to die: apoptosis, necrosis and reactive oxygen damage," *Oncogene,* 18: 7719-7730, 1999.