medical sciences forum

MDPI

Proceeding Paper

Neuroimmunomodulation in Chronic Wound Healing after Treatment with Photodynamic Therapy: The Role of iNOs †

Lorenzo Notari [1], Patrizia Nardini [1], Vieri Grandi [2], Alessandro Corsi [3], Nicola Pimpinelli [2] and Stefano Bacci [4,*]

[1] Research Unit of Histology and Embriology, Department of Clinical and Experimental Medicine, University of Florence, 50121 Firenze, Italy; lorenzo.notari@stud.unifi.it (L.N.); patrizia.nardini@unifi.it (P.N.)
[2] Department of Health Sciences, Division of Dermatology, University of Florence, 50121 Firenze, Italy; vieri.grandi@unifi.it (V.G.); pimpi@unifi.it (N.P.)
[3] S. Raffaele Hospital, 20158 Milan, Italy; corsi.alessandro@hsr.it
[4] Research Unit of Histology and Embriology, Department of Biology, University of Florence, 50121 Firenze, Italy
* Correspondence: stefano.bacci@unifi.it
† Presented at the 2nd International Electronic Conference on Biomedicines, 1–31 March 2023; Available online: https://ecb2023.sciforum.net/.

Abstract: The term neuroimmunomodulation defines the modulating role of the nervous system in relation to immune functions. This phenomenon is possible due to the existence of receptors for neurotransmitters in immune cells. The neuronal mediators are also able to direct and modulate many of the events in the wound-healing process. Among these substances, nitric oxide (NO) is a neuromodulator involved in the control of vascular tone and blood pressure, which has a vasodilator and antimicrobial effect. Photodynamic treatments in venous leg ulcers have shown how this therapy stimulates the activity of immune cells involved in healing, which have, among their functions, the function of releasing NO into the extracellular space. The experimental results showed an increase in the expression of iNOs (the enzyme involved in the secretion of NO) in PDT-treated lesions, underlining its central role in improving the clinical condition of the wound. In light of such evidence, the versatility of this protein would therefore assume a key role in the definition of new clinical therapies, as well as in the study of the process of wound healing itself.

Keywords: chronic wounds; iNOs; photodynamic therapy

Citation: Notari, L.; Nardini, P.; Grandi, V.; Corsi, A.; Pimpinelli, N.; Bacci, S. Neuroimmunomodulation in Chronic Wound Healing after Treatment with Photodynamic Therapy: The Role of iNOs. *Med. Sci. Forum* **2023**, *21*, 44. https://doi.org/10.3390/ECB2023-14135

Academic Editor: Allan Stensballe

Published: 8 March 2023

1. Introduction

Wounds that do not heal within 6/8 weeks are considered chronic. The difficulties or even the impossibility of treating these types of wounds entail high costs in terms of care for the various communities [1]. In general, the processes involved in chronic wound healing are like those in acute wound healing, but dysregulation of MMP secretion is strongly associated with chronic wounds [2]. In addition, prolonged inflammation in chronic wounds is mainly mediated by various cell types present in the cell infiltrate [1]. Photodynamic therapy (PDT) is a treatment based on the interaction between a photosensitizing agent, such as 5-aminolevulinic acid (ALA), and molecular oxygen. ALA-PDT has been proposed as an adjuvant tool for the treatment of chronic wounds, especially in long-lasting wounds with multi-resistant bacteria colonization and infection [3]. Among the multiple properties of PDT, there is evidence of a strong immune-modulating activity [1]. However, the activity of the immune system can be modulated by the nervous system [4]; this close correlation is also documented in healing wounds. Recently it was described that in chronic wounds, after PDT treatment, the expression of some neuronal mediators involved in wound healing was increased in neurons, while mast cells (MCs) other than express VIP and NGF increased their degranulation index [5]. Nitric oxide (NO) is an extracellular

molecular messenger capable of freely crossing membranes, thanks to its gaseous nature, and it is characterized by a rather short half-life [6]. In mammals, NO is synthesized from the enzyme complex nitric oxide synthase (NOs), of which there are three different homodimeric isoforms: (1) neuronal isoform, nNOs (or NOs I); (2) inducible isoform, iNOs (or NOs II); and (3) endothelial isoform, eNOs (or NOs III) [6]. All NOs isoforms use L-arginine as a substrate and molecular oxygen and NADPH as co-substrates. As regards the functions of the different isoforms, the nNOs isoform is expressed in specific neurons of the central nervous system, and is implicated in synaptic plasticity and blood pressure regulation. The eNOs isoform is mainly expressed in endothelial cells, where it helps to keep blood vessels dilated and control blood pressure, as well as having vasoprotective and anti-atherosclerotic effects. The inducible iNOs isoform is up-regulated under stress conditions; in fact, the expression of the enzyme increases in the presence of inflammatory cytokines, apoptotic bodies, or bacterial antigens [6]. The involvement of iNOs has also been hypothesized in the inflammatory phase of wound repair, during which it favors vasodilation and antibacterial activity and affects the function of fibroblasts [1,7].

In this preliminary study in untreated or ALA-PDT-treated chronic wounds, we investigated the expression of iNOs and its content in some cellular types of cellular infiltrate involved during wound healing processes.

2. Material and Methods

2.1. Study Population, Clinical Study Details, and Photodynamic Therapy

As for the clinical outcomes of this study, the model, and the use of the instrument, see [5] for details.

2.2. Immunohistochemical Analysis

Cryostatic sections were labeled with primary antibodies against the following antigen: iNOs (Abcam, Milan, Italy). The following labels were combined with iNOs: Avidin (for MCs; Sigma, Milan, Italy), NSE (for neurons, Abcam), and SPM250 (for granulocytes, Genetex, Irwine, CA, USA). Photomicrographs were taken with an Axiophot microscope (Zeiss, Berlin, Germany) equipped with epifluorescence and a digital photo camera (Zeiss) connected to a personal computer hosting the software Axiovision 4 (Zeiss) or Leica TCS SP5 confocal microscope.

2.3. Morphometry

For the evaluation of the degranulation index of MCs and iNOs expression, see [5,8] for details.

2.4. Statistical Analysis

For every parameter, the average value of each skin sample was taken as a sample unit. All differences were subjected to a one-way analysis of variance among all experimental groups (including Bonferroni-corrected t-tests or Tukey HSD tests). When significant results were obtained, the values for each time frame were compared to controls by Student's *t*-test for unpaired values with two tails. $p < 0.05$ was assumed as significant. The results of this analysis are given as median values and standard error (SE) of the median. For values concerning percentages, the chi-square test was applied.

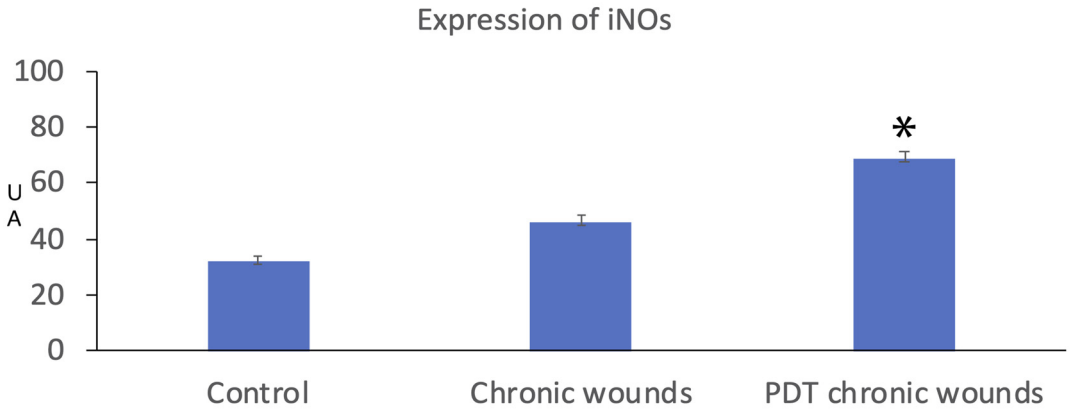
3. Results

(a) The morphometric evaluation of iNOs shows an increase in average surface area of about 50% in the wound and about double in the treated one, underlining the ability of PDT to influence the activity of iNOs itself (Figure 1A);

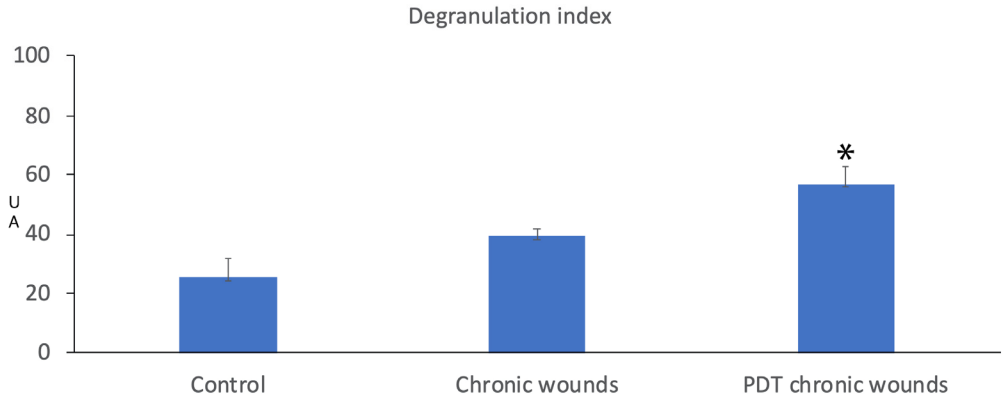
(b) Avidin-positive cells, i.e., MCs, were located around vessels and nerve fibers. They increase in density as well as their degranulation index upon photodynamic therapy (Figure 1B, see page 4);

(c) The percentage of MCs containing iNOs decreases significantly upon PDT (Figure 1C). Simultaneously, the % of cells containing iNOs increases significantly;

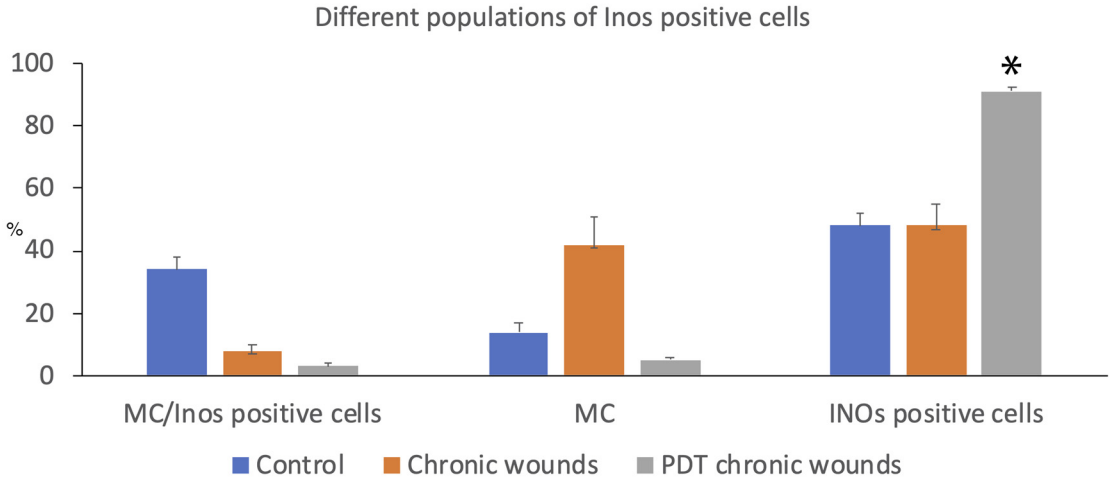
(d) How the percentage of iNOs increases after treatment with PDTI is observed in the neuronal population. Surprisingly, the proportion of other cell types containing iNOS after treatment with photodynamic therapy decreases significantly compared to the control (Figure 1D, see page 5);
(e) The granulocytes expressed iNOs in chronic wounds and also in those treated with PDT (Figure 1E).



(**A**)

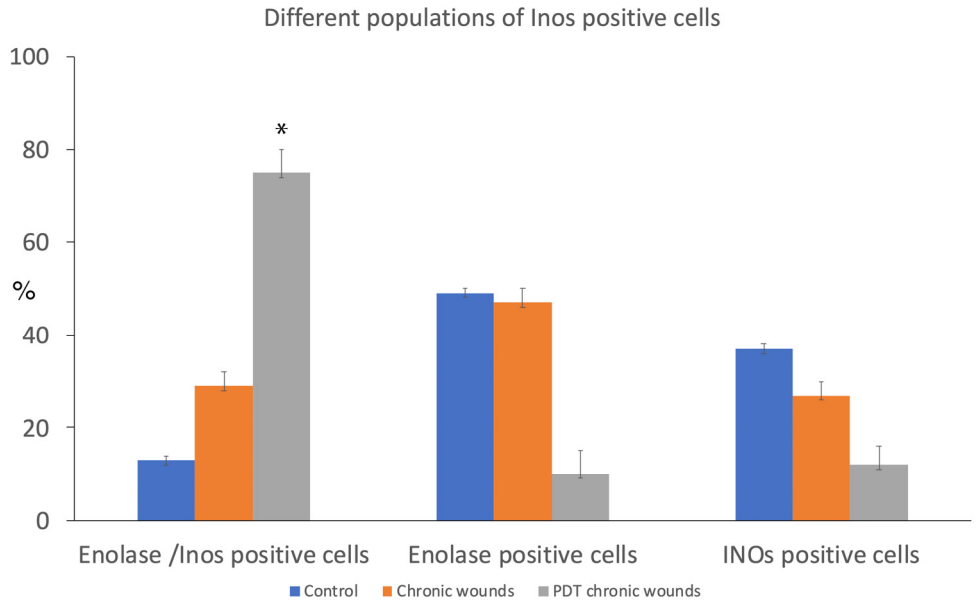


(**B**)

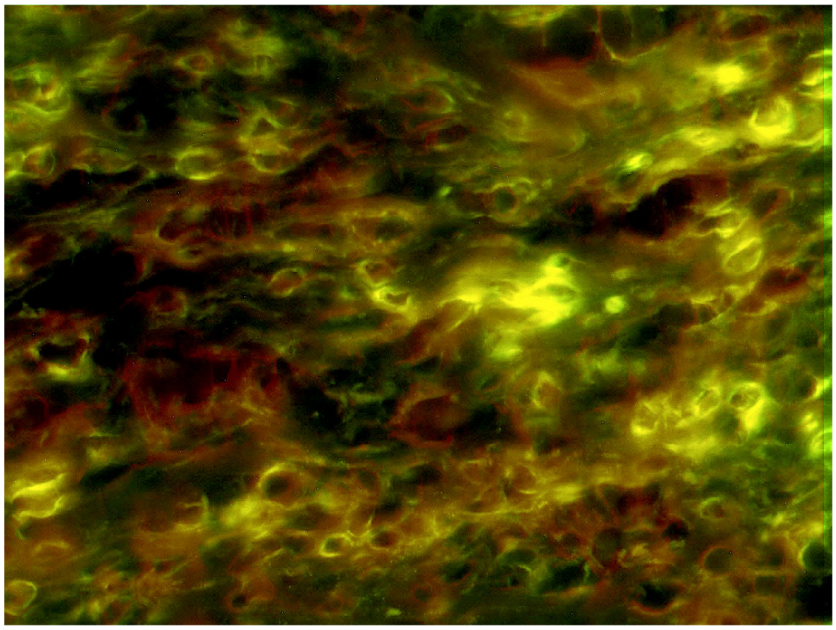


(**C**)

Figure 1. *Cont.*



(**D**)



(**E**)

Figure 1. (**A**) Different expression of iNOs, in Control, Chronic wounds and PDT treated wounds (**B**) Degranulation index by MCs in the same conditions as described in A (**C**) Different expression of iNOs by MCs, in the same conditions as described in A (**D**) Different expression of iNOs by neurons, in the same conditions as described in A (**E**) Co-expression of iNOs (in green) in granulocytes marked with Spm 250 (in red), fluorescence microscopy, ×40. Data are expressed as mean ± SE: * $p < 0.05$ vs. control.

4. Conclusions

Previously, we compared the distribution and content of SP, NKA, CGRP, NPY, VIP, NGF, and PGP 9.5 in neuronal cells before and after a single exposure to ALA-PDT; the choice of these mediators was strictly related to their involvement in different phases of wound healing [5]. This time, we have considered iNOs because of the great importance of this substance during wound healing [1,7]. The preliminary results of the experiments demonstrate that the expression of iNOs increases in chronic wounds treated with PDT,

and that neurons are the predominant cells that contain this mediator. The degranulation index of MCs also increases, and they contain iNOs as well as granulocytes. As far as the neuronal population is concerned, it is observed that the majority of neurons contain iNOs as opposed to other cell types present. However, the response of other cells involved in iNOs secretion and in wound healing upon PDT is currently underway in the laboratory.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ECB2023-14135/s1.

Author Contributions: Conceptualization, S.B.; methodology, L.N. and P.N.; validation, S.B.; formal analysis, S.B.; investigation, V.G., A.C., and S.B.; resources, S.B.; data curation, S.B.; writing—original draft preparation, S.B.; writing—review and editing, S.B.; supervision, N.P. and S.B.; funding acquisition, S.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of Azienda Sanitaria di Firenze (281/2008).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Grandi, V.; Corsi, A.; Pimpinelli, N.; Bacci, S. Cellular Mechanisms in Acute and Chronic Wounds after PDT Therapy: An Update. *Biomedicines* **2022**, *10*, 1624. [CrossRef]
2. Tottoli, E.M.; Dorati, R.; Genta, I.; Chiesa, E.; Pisani, S.; Conti, B. Skin Wound Healing Process and New Emerging Technologies for Skin Wound Care and Regeneration. *Pharmaceutics* **2020**, *12*, 735. [CrossRef]
3. Grandi, V.; Sessa, M.; Pisano, L.; Rossi, R.; Galvan, A.; Gattai, R.; Mori, M.; Tiradritti, L.; Bacci, S.; Zuccati, G.; et al. Photodynamic therapy with topical photosensitizers in mucosal and semimucosal areas: Review from a dermatologic perspective. *Photodiaggnosis Photodyn. Ther.* **2018**, *23*, 119–131. [CrossRef]
4. Steinman, L. Elaborate interactions between the immune and nervous systems. *Nature Immunol.* **2004**, *5*, 575–581. [CrossRef]
5. Grandi, V.; Paroli, G.; Puliti, E.; Bacci, S.; Pimpinelli, N. Single ALA-PDT irradiation induces increase in mast cells degranulation and neuropeptide acute response in chronic venous ulcers: A pilot study. *Photodiagnosis Photodyn Ther.* **2021**, *34*, 102222. [CrossRef]
6. Lee, M.; Rey, K.; Besler, K.; Wang, C.; Choy, J. Immunobiology of Nitric Oxide and Regulation of Inducible Nitric Oxide Synthase. *Results Probl. Cell Diff.* **2017**, *62*, 181–207. [CrossRef]
7. Shi, H.P.; Most, D.; Efron, D.T.; Tantry, U.; Fischel, M.H.; Barbul, A. The role of iNOs in wound healing. *Surgery* **2001**, *130*, 225–229. [CrossRef]
8. Bacci, S.; Pieri, L.; Buccoliero, A.M.; Bonelli, A.; Taddei, G.; Romagnoli, P. Smooth muscle cells, dendritic cells and mast cells are sources of TNFalpha and nitric oxide in human carotid artery atherosclerosis. *Thrombosis Res.* **2008**, *122*, 657–667. [CrossRef]