Nicotine effect on inflammatory and growth factor responses in murine cutaneous wound healing

Citation for published version (APA):

Xanthouleas, S., Deliaert, A., Romano, A., Rensen, S. S., Buurman, W. A., & van der Hulst, R. R. (2013). Nicotine effect on inflammatory and growth factor responses in murine cutaneous wound healing. *International Immunopharmacology*, 17(4), 1155-1164. <https://doi.org/10.1016/j.intimp.2013.10.022>

Document status and date:

Published: 01/12/2013

DOI:

[10.1016/j.intimp.2013.10.022](https://doi.org/10.1016/j.intimp.2013.10.022)

Document Version:

Publisher's PDF, also known as Version of record

Document license:

Taverne

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

Download date: 26 Apr. 2023

Nicotine effect on inflammatory and growth factor responses in murine cutaneous wound healing

Sofia Xanthoulea^{a,c,*}, An Deliaert^a, Andrea Romano^b, Sander S. Rensen^c,
Wim A. Buurman^c, Rene' RWJ van der Hulst^a

^a Department of Plastic Surgery, Maastricht University Medical Center, P.O. Box 5800, 6202 AZ, Maastricht, The Netherlands

^b GROW, School for Oncology and Developmental Biology, Maastricht University Medical Centre, Maastricht, The Netherlands

^c Department of Surgery, NUTRIM School for Nutrition, Toxicology & Metabolism, Maastricht University Medical Centre, Maastricht, The Netherlands

ARTICLE INFO

Article history:

Received 14 June 2013

Received in revised form 3 September 2013

Accepted 15 October 2013

Available online 6 November 2013

Keywords:

Cytokines
Growth factors
Inflammation
Macrophages
Nicotine
Wound repair

ABSTRACT

The aim of the current study was to investigate the effect of nicotine in an experimental mouse model of cutaneous injury and healing responses, during the inflammatory phase of repair. Nicotine injection in full-thickness excisional skin wounds minimally affected inflammatory mediators like TNF, IL-6 and IL-12 while it induced a down-regulation in the expression of growth factors like VEGF, PDGF, TGF- β 1 and TGF- β 2, and the anti-inflammatory cytokine IL-10. Analysis of wound closure rate indicated no significant differences between nicotine and saline injected controls. *In-vitro* studies using bone marrow derived macrophages, resident peritoneal macrophages and RAW 264.7 macrophages, indicated that nicotine down-regulates TNF production. Moreover, nicotine was shown to down-regulate VEGF, PDGF and TGF- β 1 in both bone marrow derived macrophages and RAW 264.7 cells. Using an NF- κ B luciferase reporter RAW 264.7 cell line, we show that nicotine effects are minimally dependent on NF- κ B inhibition. Moreover, nicotinic acetylcholine receptor (nAChR) subunit expression analyses indicated that while β 2 nAChR subunit is expressed in mouse macrophages, α 7 nAChR is not. In conclusion, while skin inflammatory parameters were not significantly affected by nicotine, a down-regulation of growth factor expression in both mouse skin and macrophages was observed. Reduced growth factor expression by nicotine might contribute, at least in part, to the overall detrimental effects of tobacco use in wound healing and skin diseases.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Cutaneous wound healing after an injury is a complex and highly dynamic process that involves interaction of different players like resident cells of the skin, inflammatory leukocytes, extracellular matrix components and soluble mediators. The healing process can be divided in three consecutive and partially overlapping phases i.e. inflammatory phase, proliferative phase and remodelling phase. The repair process finally results in the formation of a mass of fibrotic tissue known as scar [1,2].

Numerous experimental and clinical studies have determined that inflammation plays a crucial although still not completely clear role during cutaneous wound healing and influences the quality of the resulting scar. Inflammatory cells and particularly macrophages appear to be essential for proper healing by, among other mechanisms, stimulating

growth factor and anti-inflammatory cytokine production that are necessary for repair [3]. Moreover, pathological functioning of macrophages and excessive inflammation in the wound healing process can result in derailed healing, like the formation of ulcers, chronic wounds, hypertrophic scars and keloids [4].

Nicotine, a major constituent of tobacco smoke, has been shown to exert anti-inflammatory effects on different cell types and to be beneficial in disorders where inflammation-related mechanisms are involved like in ulcerative colitis and obesity [5]. Nicotine actions are mediated through binding to cholinergic receptors termed nicotinic acetylcholine receptors (nAChR) that are expressed in many different tissues and cells in the body, including immune cells. In both human and mouse macrophages, nicotine was shown to inhibit the release of pro-inflammatory cytokines through a specific “nicotinic anti-inflammatory pathway” that involves signalling through the α 7 nAChR and prevents activation of the NF- κ B pathway [6–9]. In human microvascular endothelial cells, nicotine was found to inhibit TNF-induced NF- κ B activation, to suppress adhesion molecule and chemokine expression and to reduce adhesion of leukocytes to activated endothelium and consequent inflammation [10]. Moreover, nicotine was shown to have angiogenic effects and to increase endothelial cell proliferation and vascular growth in different *in-vitro* and *in-vivo* models [11,12].

Abbreviations: TNF, tumor necrosis factor; IL-6, interleukin 6; IL-12, interleukin 12; VEGF, vascular endothelial growth factor; PDGF, platelet derived growth factor; TGF- β 1, transforming growth factor beta 1; TGF- β 2, transforming growth factor beta 2.

* Corresponding author at: Maastricht University Medical Centre, Universiteitssingel 50, Maastricht 6229 ER, The Netherlands. Tel.: +31 433882128; fax: +31 433884154.

E-mail address: sofia.xanthoulea@maastrichtuniversity.nl (S. Xanthoulea).

In the skin, effects of nicotine are ambivalent and often unclear, but they generally appear more negative than positive. Although cigarette smoking has been reported to negatively affect cutaneous wound healing [13], the angiogenic action of nicotine prompted for studies for a potential beneficial role of nicotine in this process. It was reported that in either normal C57BL/6 [14] or genetically diabetic mice [15], nicotine promotes wound healing due to increased angiogenesis. Yet the effect of nicotine administration on wound inflammatory responses was not evaluated in these studies. Moreover, we have observed that scar formation, a process largely dependent on the extent of the preceding inflammatory process [16], appears to be improved in smokers, which tend to have faster and less erythematous scar healing compared to non-smokers [17].

In the present study, we aimed to evaluate the effect of nicotine during the inflammatory phase of cutaneous wound healing responses in mice. Quantification of wound closure rate indicated that nicotine did not significantly affect the wound healing process. However, nicotine administration in wounds was found to negatively regulate the production of growth factors like VEGF, PDGF, TGF- β 1 and TGF- β 2 but to only minimally affect wound inflammatory parameters. *In-vitro*, in both primary mouse macrophages and in the macrophage cell line RAW 264.7, nicotine was also found to induce a down-regulation of growth factor expression and to decrease TNF production. Nicotine mediated effects were found to be independent of NF- κ B inhibition and the expression of the α 7 nAChR.

2. Materials and methods

2.1. Animals and wound model

Ten to twelve weeks old C57BL/6 female mice ($n = 3$ –4 mice/group) were used for experiments. To generate wounds, mice were anesthetized by isoflurane inhalation and the dorsal surface was shaved and cleaned with 70% ethanol. Full-thickness excisional skin wounds were generated on either side of the dorsal midline using a 3 mm biopsy punch (Kai medical). Mice were housed individually in special paper bedding material (7089 Harlan Teklad Diamond Soft Bedding), to avoid bedding particles interfering with the healing wounds. Two wounds were generated on the same animal and mice were divided in groups. Mice in each group received only one treatment in both wounds (i.e. saline or nicotine solutions). Solutions were injected with 3 injections of 20 μ l (total 60 μ l) around the wounds once daily for 3 days. Wounds were photographed daily and wound area was quantified using Image J. The percentage of wound closure was calculated using the following formula: Wound closure (%) = [(wound area on day 0 – wound area on indicated day)/wound area on day 0] $\times$ 100. At sacrifice, wounds were excised and snap frozen in liquid N₂.

Experiments were performed twice. Mice were maintained under standard pathogen-free conditions and all experiments were approved by the Committee for Animal Welfare of Maastricht University. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Reagents

Nicotine (N3876) and LPS (L2630) were from Sigma–Aldrich Chemie BV (Zwijndrecht, The Netherlands). Nicotine solutions were prepared in culture medium or PBS and were made fresh prior to each experiment. ELISA kits for murine IL-6, IL-10 and IL-12 were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA, USA) and performed according to manufacturer's protocol. For murine TNF ELISA a hamster anti-murine TNF monoclonal (TN3) was used as capture antibody and a polyclonal rabbit anti-murine TNF (Genzyme Corporation, Cambridge, MA, USA) was used as a second antibody. The ELISA had a lower detection limit of 50 pg/ml. Quantikine TGF- β 1 and PDGF-AB ELISA kits (R&D systems) and VEGF ELISA kit (Life Technologies) were performed according to manufacturer's protocol.

2.3. Primary cell isolation and cell lines

For generation of bone marrow derived macrophages (BMDM), bone marrow was isolated from femur and tibia bones of C57BL/6 mice and cultured in 15-cm bacteriologic plastic petri dishes in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 10 mM Hepes and 15% L-929 cell conditioned medium (LCM) for 8 days. Resident peritoneal macrophages (RPM) were collected from C57BL/6 mice by flushing the peritoneal cavity with 5 ml of ice cold medium. Peritoneal cells were plated in RPMI medium supplemented with antibiotics, L-glutamine and 10% FCS and macrophages were left to adhere for 4 h. Cells were washed and the remaining macrophages were cultured overnight and stimulated the next day with nicotine and/or LPS for the indicated times. RAW 264.7 cells (American Type Culture Collection, number TIB-71) were cultured in RPMI medium supplemented with antibiotics, L-glutamine and 10% FCS. 3 T3-L1 murine fibroblasts (American Type Culture Collection, number CL-173) were cultured in DMEM supplemented with antibiotics, L-glutamine and 10% FCS and SVEC4-10 small-vessel murine endothelial cells (ATCC number CRL-2181) were cultured in F12 medium supplemented with antibiotics and 10% FCS. RAW 264.7 cells stably transfected with the 3 $\times$ - κ B-luc plasmid [18] were a generous gift from Dr. M. de Winther (AMC, Amsterdam).

Table 1
Primer sequences used in this study.

Primer name	Forward sequence	Reverse sequence
CD-68	5'-TGACCTGCTCTCTCTAAGGCTACA-3'	5'-TCACGGTTGCAAGAGAAACATG-3'
TNF	5'-CATCTTCTCAAATTCGAGTGACAA-3'	5'-TGGGAGTAGACAAGGTACAAACCC-3'
IL-6	5'-TTCAACCAAGAGGTAAGATTACATAA-3'	5'-CACTCTCTCTGTGACTCCAGCTT-3'
IL-12	5'-TGAGAACTACAGCACCAGCTTCTT-3'	5'-CTTCAAAGGCTTCATCTGCAAGT-3'
IL-10	5'-GCTCTTACTGACTGGCATGAG-3'	5'-CGCAGCTCTAGGAGCATGTG-3'
VEGF	5'-GCTTTACTGCTGTACCTCCACCA-3'	5'-GGGACTTCTGCTCTCTCTCTGTC-3'
PDGF	5'-CGCCTGCAAGTGTGAGACAG-3'	5'-GAATGGTCACCCGAGCTTGA-3'
TGF- β 1	5'-GCCCTTCTGCTCTCTCATG-3'	5'-CCGCACACAGCAGTTCCTCTC-3'
TGF- β 2	5'-GACTTAACATCTCCACCCA-3'	5'-CCATCAATACCTGCAATCTCG-3'
α 7 nAChR-1	5'-CACATTCCACACCAACGCTCTT-3'	5'-AAAAGGGAACACAGCTACATC-3'
α 7 nAChR-2	5'-TGCTGGTATTCTTGTGCTGTC-3'	5'-GGTGTGGCGAAGTACTGTGCTAT-3'
α 7 nAChR-3	5'-GCCTAAGTGACACAGGATCA-3'	5'-CTCGGAAGCCAATGTAGAGC-3'
β 2 nAChR	5'-GGGCAGGCACACTATTCTTC-3'	5'-TCCAATCTCTCTCACACTC-3'
cyclophilin	5'-TTCCTCTTTACAGAAATTATCCA-3'	5'-CCGCCAGTGCATTATGG-3'
GAPDH	5'-TTCACCACCATGGAGAAGGC-3'	5'-GGCATGGACTGTGGTCATGA-3'
β -actin	5'-GACAGGATGAGAGGAGATTACTG-3'	5'-CCACCGATCCACAGAGTACTT-3'

Fig. 1. Nicotine administration in wounds. (A) Macroscopic appearance of representative saline, 10⁻⁸ M and 10⁻⁴ M nicotine injected wounds. Day 0 pictures were taken immediately after wounding. (B) Quantification of wound closure rate. At the indicated days, wound areas were determined using image analysis and expressed as percentage of wound area immediately post-injury as described in methods ($n = 6-8$ wounds/group). (C) mRNA expression analysis by real-time PCR of CD-68, IL-12, TNF, IL-6, IL-10 and (D) VEGF, PDGF, TGF-β1, TGF-β2 in either unwounded (NMS, normal mouse skin) or wounded skin, after three days from wounding. Unwounded skin expression levels for each gene were set to one and for simplicity only one control is shown. $n = 3$ mice/group. (E) mRNA expression analysis by real-time PCR of CD-68, IL-12, TNF, IL-6, IL-10 and (F) VEGF, PDGF, TGF-β1, TGF-β2 in wounded skin after three days from wounding, injected with either saline or nicotine solutions (10⁻⁴ M or 10⁻⁸ M). Expression levels for each gene in unwounded (NMS, normal mouse skin) were set to one. $n = 3-4$ mice/group. Statistical significance was evaluated for each nicotine concentration compared to saline control or between wounded and non wounded skin, by t -test. * $p < 0.05$, ** $p < 0.01$.

2.4. RNA isolation and quantitative gene expression

RNA was isolated either with the RNeasy Fibrous Tissue kit (Qiagen GmbH, Hilden, Germany) for mouse skin tissue or with Trizol (Sigma-Aldrich Chemie BV) for cell monolayers. Residual DNA was digested with the RQ1 RNase-free DNase (Promega GmbH, Mannheim, Germany) and cDNA synthesis was performed using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Quantitative PCR was performed in 10 ng of cDNA, with 1 × Absolute qPCR SYBR Green Fluorescein Mix (Westburg, Leusden, The Netherlands) and 150 nM of gene specific forward and reverse primers. Cyclophilin A and β -actin were used as housekeeping genes. Primer sequences are indicated in Table 1.

2.5. MTT assay, luciferase activity and ELISA

For MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich Chemie BV) assay, 10^5 cells/well of BMDM, RAW 264.7, PEC or 5×10^4 cells/well of SVEC4-10 or 10^4 cells/well of 3 T3-L1 cells were plated in 96-well plates overnight and stimulated the following day with nicotine and/or LPS (100 ng/ml) for 24 h. Nicotine was added to cell cultures 30 min before LPS. MTT assay was performed after 24 h by addition of MTT solution to a final concentration of 0.5 mg/ml, for 2 h. Dye was solubilized with DMSO (Sigma-Aldrich Chemie BV) and absorbance was measured at 570 nm. Data represent mean $\pm$ SEM of 4 independent experiments performed in triplicate.

For luciferase activity, RAW 264.7 cells stably transfected with the 3 $\times$ - κ B-luc plasmid were plated at a density of 10^5 cells/well in 96 well plates and stimulated with the indicated compounds for the indicated

times. Cells were lysed in lysis buffer (Promega GmbH) for 20 min and 10 μ l lysate was added to 50 μ l luciferin (Steady-Glo Luciferase assay system, Promega GmbH). Luciferase activity was measured with a Lumac Biocounter M1500 luminometer (Promega GmbH). Data represent mean $\pm$ SEM of 2 independent experiments performed in quadruplicate.

For ELISA, 2×10^5 3 T3-L1 fibroblast cells or 5×10^5 macrophages or SVEC4-10 endothelial cells were plated in 500 μ l medium in triplicate/condition in 24-well plates and stimulated for 6 or 24 h with nicotine and/or LPS. Supernatants were analyzed by ELISA. Since significant levels of growth factors are present in bovine serum used in tissue culture medium, for quantification of VEGF, PDGF-AB and TGF- β 1 in macrophage supernatants, medium was changed to Optimem-1 (Gibco-BRL) overnight and cells were stimulated the following morning with nicotine for 6 or 24 h. Experiments were performed in triplicate and data represent mean $\pm$ SEM of 3 independent experiments.

2.6. Statistical analysis

Statistical analyses were performed using Graphpad Prism (Graphpad Software) or SigmaPlot statistical tests. Data are expressed as means $\pm$ SEM. A $p < 0.05$ is considered statistically significant.

3. Results

3.1. Nicotine down-regulates growth factor expression in skin wounds

The initial response upon a cutaneous injury is characterized by a strong inflammatory reaction with induction of different inflammatory

Fig. 2. Nicotine down-regulates TNF production by mouse macrophages. ELISA for (A) TNF, (B) IL-6, (C) IL-10 and (D) IL-12 in culture supernatants of either mouse bone marrow derived macrophages (BMDM), resident peritoneal macrophages (RPM) or RAW 264.7 macrophages stimulated with 100 ng/ml LPS in the presence or absence of different doses of nicotine (10^{-4} M, 10^{-6} M, 10^{-8} M), after 24 h. Left Y axis shows protein levels for BMDM and either RPM or RAW 264.7 cells. Right Y axis shows protein levels for RAW 264.7 or RPM cells. * $p < 0.05$, ** $p < 0.01$. Statistical significance was evaluated for each nicotine concentration compared to no nicotine control by t-test.

mediators. Since nicotine was previously shown to negatively regulate inflammation [6–9], and to promote wound healing in normal [14] and genetically diabetic mice [15], we aimed to examine the effect of nicotine administration during the inflammatory phase of cutaneous wound healing in mice. Full thickness excisional wounds were generated on the dorsum of C57BL/6 mice and mice were divided in groups that received either saline or nicotine solutions. Saline or nicotine solutions at two different concentrations (10^{-4} M or 10^{-8} M) were injected around the wounds daily for three days, similar to other studies [14] and as described in the methods section. Quantification of wound area indicated a not significant delay in wound closure in the nicotine injected wounds compared to saline controls (Fig. 1 A and B). After three days, real time PCR analysis of RNA isolated from either wounded mice or non-wounded controls (normal mouse skin, NMS) was performed. As shown in Fig. 1 C, wounded skin exhibits a strong inflammatory response which is absent in non-wounded controls. CD-68 was over five-fold increased indicating extensive macrophage infiltration and expression of different inflammatory mediators (TNF, IL-6, IL-10) was also found several fold increased (Fig. 1C). A milder effect of wounding on expression of growth factors was observed (Fig. 1D).

Nicotine injections resulted in significant mild down-regulation in the expression of IL-10 in the wounds of 10^{-8} M nicotine injected wounds compared to saline controls (Fig. 1E). Interestingly, nicotine administration indicated a clear down-regulation in the mRNA expression of growth factors, with PDGF, TGF- β 1 and TGF- β 2 showing significant differences while VEGF expression had a similar trend but differences were borderline not significant (Fig. 1F).

3.2. Nicotine down-regulates TNF and growth factor expression in mouse macrophages

Macrophages play a crucial role during cutaneous wound healing and represent a major source of cytokines and growth factors in the wound [3]. Previous studies have shown that nicotine attenuates macrophage activation and inhibits the production of a number of pro-inflammatory cytokines [7,9]. To evaluate the effect of nicotine in our setting, we used primary mouse macrophages like bone marrow derived (BMDM) and resident peritoneal macrophages (RPM) and the mouse macrophage cell line RAW 264.7. Cells were stimulated with LPS in order to induce cytokine production, and with or without addition of nicotine in the culture medium (nicotine added 30 min. before LPS) for either 6 or 24 h. Nicotine concentrations used were comparable to those in similar studies [7,9] and in the range of nicotine concentrations in blood during smoking [19]. No major effect of nicotine on the production of TNF, IL-6, IL-12 and IL-10 was observed after 6 h, as measured by ELISA in culture supernatants (data not shown). However after 24 h, we could confirm previous studies showing that nicotine induces a significant dose-dependent down-regulation in the production of TNF in all macrophage cells tested (Fig. 2A). No significant effect of nicotine on the production of IL-6, IL-10 or IL-12 was observed (Fig. 2B, C, D).

Since macrophages are major sources of growth factors and nicotine administration in wounds indicated a down-regulation in growth factor expression, we analyzed macrophage growth factor gene expression in the presence of nicotine. Similar to the *in-vivo* findings, nicotine was

Fig. 3. Nicotine inhibits growth factor expression in mouse macrophages. mRNA expression analysis by real-time PCR of VEGF, PDGF, TGF- β 1 and TGF- β 2 in either (A) bone marrow derived macrophages or (B) RAW 264.7 macrophages stimulated with nicotine (10^{-4} M, 10^{-6} M, 10^{-8} M), after 24 h. Expression levels in cells not stimulated with nicotine (no nic.) for each gene were set to one and for simplicity only one control is shown. (C) ELISA for VEGF, PDGF and TGF- β 1 in culture supernatants of mouse bone marrow derived macrophages not stimulated or stimulated with nicotine (10^{-4} M, 10^{-8} M) for 24 h. (D) MTT assay performed in bone marrow derived macrophages (BMDM), resident peritoneal macrophages (RPM) or RAW 264.7 macrophages in the presence of different doses of nicotine (10^{-4} M, 10^{-6} M, 10^{-8} M), after 24 h. * $p < 0.05$, ** $p < 0.01$. Statistical significance was evaluated for each nicotine concentration compared to no nicotine control by *t*-test.

found to down-regulate mRNA levels of VEGF, PDGF and TGF- β 1 in BMDM and RAW 264.7 macrophages (Fig. 3A and B respectively) (TGF- β 2 levels were undetectable). A comparable down-regulation in the protein levels of VEGF, PDGF and TGF- β 1 was measured in BMDM culture supernatants after 24 h of incubation with nicotine (Fig. 3C). Expression levels were below detection limit in supernatants of RAW 264.7 cells. Cell viability measurement by tetrazolium salt MTT assay showed that concentrations of nicotine used did not result in significant differences in cell survival and therefore the observed effects were not due to cytotoxicity (Fig. 3D).

3.3. Nicotine does not significantly affect NF- κ B transcriptional activity in mouse macrophages

Nicotine has been proposed to inhibit pro-inflammatory gene expression through a nicotinic anti-inflammatory pathway which results in the inhibition of NF- κ B [8,20]. Moreover, NF- κ B regulates the expression of a number of growth factors as VEGF and PDGF [21]. To determine the effect of nicotine on NF- κ B transcriptional activity we used a macrophage RAW 264.7 cell line stably transfected with an NF- κ B luciferase reporter and that expresses luciferase under the control of NF- κ B. This cell line maintains the inflammatory characteristics of the parental line used in the previous experiments [18]. As shown in Fig. 4A, nicotine

alone (without LPS induction), does not activate NF- κ B in any of the concentrations or time points examined (1, 6 or 24 h; for simplicity only the one hour time point is shown). Activation of RAW 264.7 macrophages with LPS induces NF- κ B activation already after one hour, as measured by luciferase activity. NF- κ B transcriptional activity peaked at six hours and returned to baseline after 24 h. However, nicotine showed only a very mild and statistically non-significant reduction in NF- κ B activity at one and six hours indicating that NF- κ B inhibition by nicotine is only minimal.

Since nicotine dependent NF- κ B inhibition in human macrophages was reported to be mediated by the α 7 nicotinic acetylcholine receptor (α 7 nAChR) [8,20], we evaluated the expression of this receptor in the different macrophage cell types used in our study. RNA was isolated from BMDM, RPM and RAW 264.7 cells and PCR was performed on cDNA for the identification of nAChR transcripts. As shown by previous studies [22], while expression of another nicotinic receptor like β 2 nAChR was detected in mouse macrophages, α 7 nAChR transcript was not detected in any of cell types analyzed (Fig. 4B). Use of two additional primer pairs for the α 7 nAChR failed as well to detect transcripts for this receptor (data not shown; primer sequences listed in Table 1). Similar analysis of RNA isolated from either unwounded or wounded mouse skin confirmed the presence of β 2 nAChR and the absence of α 7 nAChR transcripts in mouse skin.

Fig. 4. Minimal inhibition of NF- κ B transcriptional activity by nicotine. (A) Luciferase assay performed in RAW 264.7 macrophages stably transfected with an NF- κ B luciferase reporter, stimulated for 1, 6 or 24 h with 100 ng/ml LPS in the presence or absence of different doses of nicotine (10^{-4} M, 10^{-6} M, 10^{-8} M). In the absence of LPS, no luciferase activity was detected (for simplicity only the 1 h time point is shown). Statistical significance was evaluated for each nicotine concentration compared to no nicotine control by *t*-test. (B) RT-PCR showing transcript expression of nAChR α 7 and β 2 subunits in RNA derived from mouse brain, bone marrow derived macrophages (BMDM), resident peritoneal macrophages (RPM) or RAW 264.7 macrophages not stimulated or stimulated for 24 h with 100 ng/ml LPS, and in unwounded or day 3 wounded mouse skin. RNA derived from mouse brain was used as a positive control for nicotinic acetylcholine receptor expression while GAPDH was used as loading control.

3.4. Nicotine effect on endothelial and fibroblast cells

In addition to macrophages, fibroblasts and endothelial cells are abundantly present in the skin and have pivotal functions in the process of cutaneous wound healing. To evaluate the effect of nicotine on these cell types, we analyzed cytokine responses in the murine endothelial cell-line SVEC4-10 and in 3 T3-L1 murine fibroblasts after LPS activation in the presence or absence of nicotine (nicotine added to the medium 30 min before LPS). In both cell types no detectable amounts of TNF, IL-12 or IL-10 were measured by ELISA after 24 h of either LPS or LPS and nicotine incubation (not shown). On the contrary, IL-6 was measured in the culture supernatants of both SVEC4-10 cells (Fig. 5A) and 3 T3-L1 fibroblasts (Fig. 6A), but no differences were seen in the presence of different nicotine concentrations. MTT toxicity assay showed that the concentrations of nicotine used (10^{-4} M, 10^{-6} M, 10^{-8} M) were not toxic to either cell line since toxicity effects were only seen from concentrations higher than 10^{-3} M (Figs. 5B and 6B). Contrarily, MTT assay showed that nicotine actually promoted proliferation of 3 T3-L1 fibroblasts. As shown in Fig. 6B, in the absence of LPS stimulation, nicotine induces an approximately 10–20% increase in cell proliferation compared to non-treated cells. Addition of LPS to 3 T3-L1 cells induces a similar increase in cell proliferation but there is no further increase if nicotine is added in the presence of LPS.

To evaluate $\alpha 7$ nAChR expression in 3 T3-L1 and SVEC4-10 cells, RNA was isolated from either non-stimulated cells or cells stimulated with LPS for 24 h and cDNA was synthesized. Neither cell type was found to express $\alpha 7$ nAChR mRNA (Figs. 5C and 6C). SVEC4-10 cells expressed the $\beta 2$ nAChR transcripts while this nicotinic receptor subunit was absent from 3 T3-L1 fibroblasts. Moreover, real-time PCR analysis of RNA isolated from nicotine stimulated SVEC4-10 or 3 T3-L1

cells indicated that contrary to macrophages, nicotine does not induce a significant down-regulation of growth factors in these cell-types and actually shows a trend towards increased PDGF expression in 3 T3-L1 cells (Figs. 5D and 6D respectively).

4. Discussion

The discovery approximately a decade ago of a nicotinic anti-inflammatory pathway mediated by the $\alpha 7$ nAChR has raised interest in the use of nicotine or nicotinic agonists as potential therapeutic agents in different chronic inflammatory conditions. The therapeutic use of nicotine has been suggested for the treatment of inflammatory disorders such as Crohn's disease, ulcerative colitis and obesity [5,23]. However, the potential of a therapeutic role of nicotine in the skin is unclear and controversies exist as to whether the overall effect must be regarded as positive or negative [13].

4.1. Effect of nicotine on wound healing

Previous studies regarding the effect of nicotine in cutaneous wound healing responses in mice have shown contradictory results. In one study, day eight wounds treated with 10^{-3} M nicotine solution resulted in delayed wound healing with a larger wound area compared to saline controls, while wounds treated with a nicotine concentration of 10^{-4} M showed improved healing and a smaller wound area. Treatment with nicotine concentrations of 10^{-7} M or 10^{-10} M showed no significant differences [14]. In a different study, nicotine concentrations of 10^{-8} M and 10^{-9} M accelerated wound healing only in genetically diabetic (Lepr^{db}) mice but not in control mice (heterozygous for the diabetes allele) compared to saline treated controls [15]. In both studies,

Fig. 5. Analysis of nicotine action on SVEC4-10 murine endothelial cells. (A) ELISA for IL-6 in supernatants of SVEC4-10 cells stimulated with 100 ng/ml LPS in the presence or not of different doses of nicotine (10^{-4} M, 10^{-6} M, 10^{-8} M), after 24 h. (B) MTT assay performed in SVEC4-10 cells in the presence or absence of different doses of nicotine (10^{-4} M, 10^{-6} M, 10^{-8} M) and not stimulated or stimulated with 100 ng/ml LPS, after 24 h. (C) RT-PCR showing transcript expression of nAChR $\alpha 7$ and $\beta 2$ subunits in RNA derived from mouse brain and SVEC4-10 cells stimulated or not for 24 h with 100 ng/ml LPS. (D) mRNA expression analysis by real-time PCR of VEGF, PDGF, TGF- $\beta 1$ and TGF- $\beta 2$ in SVEC4-10 cells in the presence of different doses of nicotine (10^{-4} M, 10^{-6} M, 10^{-8} M), after 24 h. Statistical significance was evaluated for each nicotine concentration compared to no nicotine control by *t*-test.

improved healing was associated with improved wound angiogenesis. In our wound healing experiment we could not observe an improvement in wound healing induced by nicotine treatment in wild type mice compared to controls. Differences with previous studies regard both the background of the mice but also the time-point in which wound area is measured (the first three days in our setting versus days eight or fourteen in other studies). Although no significant dysregulation in wound inflammatory parameters like TNF, IL-6 and IL-12 was detected, nicotine induced a down-regulation in the expression of anti-inflammatory IL-10 and growth factors like VEGF, PDGF, TGF- β 1 and TGF- β 2. This latter effect was also confirmed *in-vitro* in both primary mouse bone marrow derived macrophages and RAW 264.7 cells, at both the RNA and protein levels, while a similar down-regulation was not observed in either fibroblasts or endothelial cells. Therefore, it is likely that the inhibition of growth factor production by wound macrophages accounts for the down-regulation of growth factors observed in nicotine-injected wounds.

4.2. Nicotine action on growth factor expression

Previous studies have suggested that the effect of nicotine on growth factor expression is likely to be cell-type specific. Chronic exposure to nicotine was found to reduce plasma VEGF levels in mice and to impair cholinergic angiogenesis [24]. In other studies, while nicotine was shown to induce VEGF and FGF in vascular smooth muscle cells and endothelial cells [25–27], it inhibited VEGF, PDGF and TGF- β 1 in rabbit osteoblasts [28] and VEGF in porcine retinal pigment epithelium [29]. Moreover, nicotine was shown to upregulate the expression of VEGF but suppress the expression of PDGF in nasopharyngeal carcinoma (NPC) cells [30]. Our data show that in macrophages, a major source

of growth factors like VEGF and TGF- β 1 in wounds [31], the effect of nicotine is inhibitory. In support of a cell-type specific nicotine action, we observed a trend towards increased PDGF production by nicotine in 3 T3-L1 fibroblasts.

4.3. Nicotine action on monocytes/macrophages

Nicotine was found to inhibit the production of pro-inflammatory cytokines in human and mouse monocytes or macrophages through activation of a Jak2-STAT3 pathway and inhibition of the NF- κ B transcriptional activity [6–9,20]. However, discrepancies from these findings in studies where nicotine was shown to augment TNF and pro-inflammatory synthesis in macrophages exist [32]. Using either primary mouse cells like bone marrow derived macrophages and resident peritoneal macrophages, or the mouse macrophage cell line RAW 264.7, we could confirm previous findings of an inhibitory action of nicotine on TNF production. Using an NF- κ B luciferase reporter system, we also show that, although nicotine induces down-regulation of TNF expression in RAW 264.7 macrophages, this is only minimally dependent on inhibition of NF- κ B transcriptional activity. Discrepancies from other studies [8,20] that show inhibition by nicotine of NF- κ B activity in luciferase assays may be explained by the differences in transiently transfected cells used in those studies compared to the stable transfected line we used in our experiments. Therefore, additional mechanisms to NF- κ B suppression seem to be responsible for the nicotine induced down-regulation of TNF expression in macrophages. In line with this, a different mechanism of nicotine mediated suppression of TNF release in human macrophages was recently reported, through induction of tristetrapirolin (TTP), an ARE-binding protein that binds and promotes degradation of the TNF transcript [33].

Fig. 6. Analysis of nicotine action on 3 T3-L1 murine fibroblasts. (A) ELISA for IL-6 in supernatants of 3 T3-L1 cells stimulated with 100 ng/ml LPS in the presence or absence of different doses of nicotine (10^{-4} M, 10^{-6} M, 10^{-8} M), after 24 h. (B) MTT assay performed in 3 T3-L1 cells in the presence or absence of different doses of nicotine (10^{-4} M, 10^{-6} M, 10^{-8} M) and stimulated or not with 100 ng/ml LPS, after 24 hours. (C) RT-PCR showing transcript expression of nAChR α 7 and β 2 subunits in RNA derived from mouse brain and 3 T3-L1 cells stimulated or not for 24 h with 100 ng/ml LPS. (D) mRNA expression analysis by real-time PCR of VEGF, PDGF, TGF- β 1 and TGF- β 2 in 3 T3-L1 cells in the presence of different doses of nicotine (10^{-4} M, 10^{-6} M, 10^{-8} M), after 24 h. * $p < 0.05$. Statistical significance was evaluated for each nicotine concentration compared to no nicotine control by *t*-test.

4.4. Nicotinic acetylcholine receptor expression

The anti-inflammatory effect of nicotine is considered to be mediated by the $\alpha 7$ nAChR expressed by many different cell types ranging from neurons to immune cells. Regarding the skin, the $\alpha 7$ nAChR has been detected in the upper spinous and granular layers of human scalp epidermis [34] and in skin of BALB/c mice, where $\alpha 7$ nAChR positive staining was observed in epidermis, hair follicles, sebaceous glands, endothelial cells, resident dermal fibroblasts, but also in inflammatory cells like macrophages and PMNs during skin wound healing [35]. However, concerns have been raised regarding the specificity of anti- $\alpha 7$ nAChR antibodies due to the discrepancies in the results between immunodetection data and mRNA or genotyping results in $\alpha 7$ nAChR deficient mice [22,36,37]. Using three different primer pairs, we were unable to detect mRNA of $\alpha 7$ nAChR in either unwounded or wounded skin in C57BL/6 mice or in primary mouse cells or cell-lines. Our results are supported by similar studies that show presence of other nAChR transcripts like the $\beta 2$ nAChR but absence of the $\alpha 7$ nAChR mRNA in mouse alveolar [38,39], intestinal, splenic or peritoneal macrophages [22]. Since $\alpha 7$ nAChR has been detected in human monocytes, macrophages, endothelial cells [6,10,12] but not in the corresponding mouse cells, it is likely that expression patterns of nicotinic acetylcholine receptors may differ between human and mouse tissues or between different mouse strains, and comparison of different studies or translation of mouse studies to humans should be done with caution.

Finally, to evaluate the effect of nicotine on additional cell types present in the skin, we have used the mouse vascular endothelial cell line SVEC4-10 and the 3 T3-L1 murine fibroblast cell line. In both cell types nicotine was found to have no effect on IL-6 secretion, the only cytokine among TNF, IL-12 and IL-10 that was produced after LPS stimulation. However, we observed that nicotine induced a mild increase in cell proliferation in 3 T3-L1 fibroblasts under non-LPS stimulated conditions. This effect is potentially mediated by nicotinic acetylcholine receptor subunits expressed by fibroblasts that are different from $\alpha 7$ nAChR or $\beta 2$ nAChR since expression analysis indicated absence of these subunits in mouse 3 T3-L1 cells. Similar nicotine-induced stimulation in cell proliferation was noted by others in endothelial cells [40,41], bone cells [42], epithelial cells [43], and chondrocytes [44].

Taken together, our results show that during the inflammatory phase of murine cutaneous wound healing, the main effect of nicotine administration was a negative regulation of growth factor expression, an effect which is likely to be due to reduced growth factor expression by wound macrophages. The inhibitory effect of nicotine on growth factor production may reflect, to a certain degree, the damaging effects of smoking on the skin vasculature and oxygenation and may provide critical insight into the overall detrimental effects of tobacco use in wound healing and general skin diseases.

Funding

This study was supported by internal funds of the Maastricht University Medical Centre.

Acknowledgements

We wish to thank Dr. M.P.J. de Winther (AMC, Amsterdam University) for providing us with the RAW 264.7 NF- κ B luciferase reporter line and Dr. P.J. Lindsey (MUMC, Maastricht University) for help with statistical analysis.

References

- [1] Singer AJ, Clark RA. Cutaneous wound healing. *N Engl J Med* 1999;341:738–46.
- [2] Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. *Nature* 2008;453:314–21.
- [3] Eming SA, Krieg T, Davidson JM. Inflammation in wound repair: molecular and cellular mechanisms. *J Invest Dermatol* 2007;127:514–25.
- [4] Mahdavian Delavary B, van der Veer WM, van Egmond M, Niessen FB, Beelen RH. Macrophages in skin injury and repair. *Immunobiology* 2011;216:753–62.
- [5] Lakhani SE, Kirchgessner A. Anti-inflammatory effects of nicotine in obesity and ulcerative colitis. *J Transl Med* 2011;9:129.
- [6] Wang H, Yu M, Ouchani M, Amella CA, Tanovic M, Susarla S, et al. Nicotinic acetylcholine receptor $\alpha 7$ subunit is an essential regulator of inflammation. *Nature* 2003;421:384–8.
- [7] Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, et al. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature* 2000;405:458–62.
- [8] Wang H, Liao H, Ouchani M, Justiniani M, Lin X, Yang L, et al. Cholinergic agonists inhibit HMGB1 release and improve survival in experimental sepsis. *Nat Med* 2004;10:1216–21.
- [9] de Jonge WJ, van der Zanden EP, The FO, Bijlsma MF, van Westerloo DJ, Bannink RJ, et al. Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. *Nat Immunol* 2005;6:844–51.
- [10] Saeed RW, Varma S, Peng-Nemeroff T, Sherry B, Balakhaneh D, Huston J, et al. Cholinergic stimulation blocks endothelial cell activation and leukocyte recruitment during inflammation. *J Exp Med* 2005;201:1113–23.
- [11] Heeschen C, Jang JJ, Weis M, Pathak A, Kaji S, Hu RS, et al. Nicotine stimulates angiogenesis and promotes tumor growth and atherosclerosis. *Nat Med* 2001;7:833–9.
- [12] Heeschen C, Weis M, Aicher A, Dimmeler S, Cooke JP. A novel angiogenic pathway mediated by non-neuronal nicotinic acetylcholine receptors. *J Clin Invest* 2002;110:527–36.
- [13] Misery L. Nicotine effects on skin: are they positive or negative? *Exp Dermatol* 2004;13:665–70.
- [14] Morimoto N, Takemoto S, Kawazoe T, Suzuki S. Nicotine at a low concentration promotes wound healing. *J Surg Res* 2008;145:199–204.
- [15] Jacobi J, Jang JJ, Sundram U, Dayoub H, Fajardo LF, Cooke JP. Nicotine accelerates angiogenesis and wound healing in genetically diabetic mice. *Am J Pathol* 2002;161:97–104.
- [16] Larson BJ, Longaker MT, Lorenz HP. Scarless fetal wound healing: a basic science review. *Plast Reconstr Surg* 2010;126:1172–80.
- [17] Deliaert AE, Van den Kerckhove E, Tuinder S, Noordzij SM, Dormaar TS, van der Hulst RR. Smoking and its effect on scar healing. *Eur J Plast Surg* 2012;35:421–4.
- [18] Carlsen H, Moskaug JO, Fromm SH, Blomhoff R. In vivo imaging of NF- κ B activity. *J Immunol* 2002;168:1441–6.
- [19] Henningfield JE, Stapleton JM, Benowitz NL, Grayson RF, London ED. Higher levels of nicotine in arterial than in venous blood after cigarette smoking. *Drug Alcohol Depend* 1993;33:23–9.
- [20] Yoshikawa H, Kurokawa M, Ozaki N, Nara K, Atou K, Takada E, et al. Nicotine inhibits the production of proinflammatory mediators in human monocytes by suppression of I- κ B phosphorylation and nuclear factor- κ B transcriptional activity through nicotinic acetylcholine receptor $\alpha 7$. *Clin Exp Immunol* 2006;146:116–23.
- [21] Gilmore L. NF- κ B Target Genes. Available: <http://www.bu.edu/nf-kb/gene-resources/target-genes/>.
- [22] van der Zanden EP, Snoek SA, Heinsbroek SE, Stanisor OI, Verseijden C, Boeckxstaens GE, et al. Vagus nerve activity augments intestinal macrophage phagocytosis via nicotinic acetylcholine receptor $\alpha 4\beta 2$. *Gastroenterology* 2009;137:1029–39 (39 e1–4).
- [23] Ulloa L. The vagus nerve and the nicotinic anti-inflammatory pathway. *Nat Rev Drug Discov* 2005;4:673–84.
- [24] Konishi H, Wu J, Cooke JP. Chronic exposure to nicotine impairs cholinergic angiogenesis. *Vasc Med* 2010;15:47–54.
- [25] Conklin BS, Zhao W, Zhong DS, Chen C. Nicotine and cotinine up-regulate vascular endothelial growth factor expression in endothelial cells. *Am J Pathol* 2002;160:413–8.
- [26] Zhen Y, Ruixing Y, Qi B, Jinzhen W. Nicotine potentiates vascular endothelial growth factor expression in balloon-injured rabbit aortas. *Growth Factors* 2008;26:284–92.
- [27] Kanda Y, Watanabe Y. Nicotine-induced vascular endothelial growth factor release via the EGFR-ERK pathway in rat vascular smooth muscle cells. *Life Sci* 2007;80:1409–14.
- [28] Ma L, Zwahlen RA, Zheng LW, Sham MH. Influence of nicotine on the biological activity of rabbit osteoblasts. *Clin Oral Implants Res* 2011;22:338–42.
- [29] Klettner AK, Doths J, Roider J. Nicotine reduces VEGF-secretion and phagocytotic activity in porcine RPE. *Graefes Arch Clin Exp Ophthalmol* 2011;250:33–8.
- [30] Shi D, Guo W, Chen W, Fu L, Wang J, Tian Y, et al. Nicotine promotes proliferation of human nasopharyngeal carcinoma cells by regulating $\alpha 7$ AChR, ERK, HIF-1 α and VEGF/PEDF signaling. *PLoS One* 2012;7:e43898.
- [31] Lucas T, Waisman A, Ranjan R, Roes J, Krieg T, Muller W, et al. Differential roles of macrophages in diverse phases of skin repair. *J Immunol* 2010;184:3964–77.
- [32] Lau PP, Li L, Merched AJ, Zhang AL, Ko KW, Chan L. Nicotine induces proinflammatory responses in macrophages and the aorta leading to acceleration of atherosclerosis in low-density lipoprotein receptor (–/–) mice. *Arterioscler Thromb Vasc Biol* 2006;26:143–9.
- [33] Joe Y, Kim HJ, Kim S, Chung J, Ko MS, Lee WH, et al. Tristetraprolin mediates anti-inflammatory effects of nicotine in lipopolysaccharide-stimulated macrophages. *J Biol Chem* 2011;286:24735–42.
- [34] Kurzen H, Berger H, Jager C, Hartschuh W, Naher H, Gratchev A, et al. Phenotypical and molecular profiling of the extraneuronal cholinergic system of the skin. *J Invest Dermatol* 2004;123:937–49.
- [35] Fan YY, Yu TS, Wang T, Liu WW, Zhao R, Zhang ST, et al. Nicotinic acetylcholine receptor $\alpha 7$ subunit is time-dependently expressed in distinct cell types during skin wound healing in mice. *Histochem Cell Biol* 2011;135:375–87.

- [36] Moser N, Mechawar N, Jones I, Gochberg-Sarver A, Orr-Urtreger A, Plomann M, et al. Evaluating the suitability of nicotinic acetylcholine receptor antibodies for standard immunodetection procedures. *J Neurochem* 2007;102:479–92.
- [37] Herber DL, Severance EG, Cuevas J, Morgan D, Gordon MN. Biochemical and histochemical evidence of nonspecific binding of alpha7nAChR antibodies to mouse brain tissue. *J Histochem Cytochem* 2004;52:1367–76.
- [38] Matsunaga K, Klein TW, Friedman H, Yamamoto Y. Involvement of nicotinic acetylcholine receptors in suppression of antimicrobial activity and cytokine responses of alveolar macrophages to *Legionella pneumophila* infection by nicotine. *J Immunol* 2001;167:6518–24.
- [39] Galvis G, Lips KS, Kummer W. Expression of nicotinic acetylcholine receptors on murine alveolar macrophages. *J Mol Neurosci* 2006;30:107–8.
- [40] Zimmerman M, McGeachie J. The effect of nicotine on aortic endothelial cell turnover. An autoradiographic study. *Atherosclerosis* 1985;58:39–47.
- [41] Villablanca AC. Nicotine stimulates DNA synthesis and proliferation in vascular endothelial cells in vitro. *J Appl Physiol* 1998;84:2089–98.
- [42] Ramp WK, Lenz LG, Galvin RJ. Nicotine inhibits collagen synthesis and alkaline phosphatase activity, but stimulates DNA synthesis in osteoblast-like cells. *Proc Soc Exp Biol Med* 1991;197:36–43.
- [43] Mazhari NJ, Mandal AK, Thusoo TK. Carcinogenic effect of nicotine on normal mammary ductal epithelial cells and the protective role of beta-carotene. *Indian J Pathol Microbiol* 2003;46:24–7.
- [44] Ying X, Cheng S, Shen Y, Cheng X, An Rompis F, Wang W, et al. Nicotine promotes proliferation and collagen synthesis of chondrocytes isolated from normal human and osteoarthritis patients. *Mol Cell Biochem* 2012;359:263–9.