



Published in final edited form as:

Biomaterials. 2011 June ; 32(18): 4238–4242. doi:10.1016/j.biomaterials.2011.02.043.

The acceleration of Wound Healing in Primates by the Local Administration of Immunostimulatory CpG Oligonucleotides

Masaki Yamamoto^{1,4}, Takashi Sato^{1,4}, Joel Beren², Daniela Verthelyi³, and Dennis M. Klinman¹

¹ Cancer and Inflammation Program, National Cancer Institute, Frederick, MD 21702

² Center for Biologics Evaluation and Research, FDA, Bethesda, MD 20894

³ Center for Drug Evaluation and Research, FDA, Bethesda, MD 20894

Abstract

The process of wound healing involves complex interactions between circulating immune cells and local epithelial and endothelial cells. Studies in murine models indicate that cells of the innate immune system activated via their Toll-like receptors (TLR) can accelerate wound healing. This work examines whether immunostimulatory CpG oligodeoxynucleotides (ODN) designed to trigger human immune cells via TLR9 can promote the healing of excisional skin biopsies in rhesus macaques. Results indicate that 'K' type CpG ODN significantly accelerate wound closure in non-human primates ($p < 0.05$). Contributing to this outcome was a CpG-dependent increase in both the production of basic fibroblast growth factor and in keratinocyte migration. Of interest, IL-1 α and TGF α normally present at sites of skin injury facilitated these effects. Current findings support the conclusion that the local administration of CpG ODN may provide an effective strategy for accelerating wound healing in humans.

Keywords

CpG oligonucleotide; primate; TLR9; wound healing

Introduction

The skin is a physical barrier that serves many functions, including protecting the host from environmental pathogens. Wounding breaches this barrier and increases host susceptibility to infection, fluid loss, and other adverse consequences [1]. Methods that accelerate wound healing are thus of considerable therapeutic interest.

Wound repair is a multi-step process that culminates in the regeneration of normal dermal and epidermal tissue [2]. The first stage is marked by an inflammatory response characterized by the infiltration of neutrophils and macrophages [3]. This is followed by a proliferative phase marked by the accumulation and proliferation of fibroblasts and the

Corresponding author: Dennis Klinman, Bldg 567 Rm 205 NCI in Frederick, Frederick, MD 21702, (W) 301 228 4265, (F) 301 228 4281, klinmand@mail.nih.gov.

⁴Current Address: Department of Internal Medicine and Clinical Immunology, Yokohama City University Graduate School of Medicine, Yokohama, Japan

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subsequent formation of granulation tissue [2]. These events are influenced by cytokines, chemokines and growth factors (including basic fibroblast growth factor) secreted by inflammatory and epithelial cells at the wound margins [4-8].

Recent studies suggest that the innate immune system can contribute to wound healing by facilitating the elimination of invading microbes and promoting the production of factors that accelerate tissue repair. For example, local administration of the TLR4 ligands LPS and HMGB1 accelerate wound healing [9;10]. Conversely, healing is delayed in mice with a defect in the TLR adapter molecule MyD88 [11]. TLRs recognize common determinants expressed by a wide variety of infectious microorganisms. TLR9, for example, is triggered by the unmethylated CpG motifs present at high frequency in bacterial DNA [12;13]. Synthetic oligonucleotides (ODN) expressing CpG motifs mimic the ability of bacterial DNA to stimulate cells expressing TLR9 [14-19].

We recently examined the ability of CpG ODN to affect wound repair in mice, demonstrating that CpG ODN accelerated healing by >40% [20]. This effect was mediated via TLR9, as CpG ODN treatment did not accelerate wound repair in TLR9 KO mice (a strain unresponsive to CpG stimulation) [17].

It is perilous to extrapolate results from murine studies of CpG ODN to humans because i) the cell types expressing TLR9 differ between these species [21], ii) murine and human TLR9 differ by 24% at the AA level [17], iii) the CpG flanking regions optimally recognized by human TLR9 differs from those optimally recognized by murine TLR9 [22;23] and iv) humans but not mice respond to several structurally and functionally distinct classes of CpG ODN [23;24]. 'D' type ODN in particular (also referred to as 'A' type) stimulate human pDC to secrete IFN α but have little activity in mice [23;24]. 'K' type ODN (also referred to as 'B' type) stimulate both human and murine B cells to proliferate and secrete Ig while triggering pDC to differentiate and produce TNF α [23-25]. Fortunately, TLR9 expression, function, and sequence recognition has been highly conserved in primates, such that the response of rhesus macaques to CpG ODN is highly predictive of their activity in Man [26;27]. This work employs a rhesus macaque wound healing model to examine the therapeutic utility of 'K' and 'D' type ODN designed for human use.

Materials and Methods

Animals and cells

Healthy 4 - 6 Kg rhesus macaques (*Macaca mulata*) were obtained from the FDA colony in South Carolina. Specific pathogen free female BALB/c mice were housed in a barrier environment and studied at 8-12 wk of age. All experiments were approved by the appropriate Animal Care and Use Committee and were conducted in AAALAC accredited facilities. Animals were monitored daily by veterinarians.

Adult human dermal fibroblasts were purchased from Lifeline Cell Technology (Walkersville, MD). Cells were maintained in Fibrolife basal medium supplemented with Fibrolife serum-free life factors (Lifeline Cell Technology, Walkersville, MD). For studies of bFGF production, cells were maintained in medium without rh FGF basic. The human HaCaT keratinocyte cell line was purchased from the German Cancer Research Center (Heidelberg, Germany). Cells were maintained in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 ug/ml streptomycin, 25 mM HEPES, 1 mM sodium pyruvate, 0.1 mM non essential amino acids.

Oligonucleotides and reagents

An equimolar mixture of three 'K' type phosphorothioate ODN (TCGTTTCGTTCTC, ATCGACTCTCGAGCGTTCTC and TCGAGCGTTCTC), and three 'D' type ODN (GGTgcatcgcagcaggGGGG, GGTgcaccggtgcaggGGGG and GGTgcatcgcaggggGG (upper case nucleotides were phosphorothioate and lower case were phosphodiester backbone), were used in all primate studies. These ODN mixtures were previously shown to be highly stimulatory in both rhesus macaques and human PBMC [27]. Phosphorothioate CpG ODN 1555 (GCTAGACGTTAGCGT) was used in murine studies. All ODNs were synthesized at the CBER Core Facility and were free of endotoxin and protein contamination. Growth factor reduced basement membrane extract (BME) without phenol red was purchased from Trevigen (Gaithersburg, MD) and used as a carrier of these ODN in animal studies.

Macaque in vivo wound repair model

A modification of the wound repair model of Devalaraja et al was used to analyze the effects of CpG ODN in vivo [28]. Animals were anesthetized by intramuscular injection of ketamine (10-12 mg/kg) plus xylazine (2 mg/kg) on the day of surgery [28]. The skin on the back was cleaned, shaved, and sterilized with betadine followed by 2% chlorhexidine in 70% isopropyl alcohol. 6 mm full thickness excisional punch biopsies (Acu Punch, Fort Lauderdale, FL) were taken from the right and left paravertebral region of each animal [29]. Individual Bx sites were coated with BME + 50 ug of 'K' or 'D' ODN (an amount selected on the basis of preliminary dose-ranging studies) on days 0, 2 and 4. Each site was covered with an Opsite FlexGrid dressing (Smith&Nephew, London, UK) secured in place with adhesive tape and then covered with a cotton bandage and Vetrap (3M, St. Paul, MN). The animals were placed in a jacket (Lomir Biomedical, Inc., Quebec, Canada) that did not restrict mobility but prevented them from scratching the biopsy site. Animals were treated with buprenorphine (0.02 mg/kg) twice daily to prevent pain. Biopsy sites were digitally photographed and Image J software (Ver 1.37) used to calculate changes in wound area over time. Repair of the wound area was calculated as $[1 - (\text{wound area}) / (\text{original wound area})] \times 100\%$. No systemic or local adverse reactions to treatment were observed.

Murine in vivo wound repair model

Mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) plus xylazine (10 mg/kg). As previously described, skin on the back was cleaned, shaved, and sterilized with a betadine solution followed by 70% ethanol [20]. A 6 mm full thickness (including the Pannilulus carnosus) excisional punch biopsies (Acu Punch, Fort Lauderdale, FL) were taken from the right and left upper paravertebral region of each animal [29]. Individual Bx sites were coated with BME mixed + 50 ug of ODN and the bx sites covered with non-adhesive sterile gauze. Mice were wrapped with a form fitting bandage to further protect the bx sites. Changes in wound size were monitored as described for rhesus macaques.

Quantitative RT-PCR

Tissue from the biopsy site was excised, homogenized in Trizol, and extracted with chloroform. Total RNA was isolated from the aqueous phase and passed through an RNeasy column as per manufacturer's suggestion. Five ug of total RNA was reverse-transcribed using the Quantitect Reverse Transcription Kit (Qiagen). Gene expression levels were analyzed using the StepOnePlus RT-PCR system (Applied Biosystems, Foster City, CA). All reagents and probes used in this system were purchased from Applied Biosystems.

In vitro bFGF assay

Human adult dermal fibroblasts were seeded at 10^5 cells/well in 96 well microtiter plates. After overnight incubation (to allow the formation of an adherent monolayer) cells were

stimulated with human rIL-1 γ (R&D systems, Minneapolis, MN) and/or 1 μ M of ODN for 48 h. FGF basic levels in the culture supernatants were measured using a commercial ELISA kit (RayBiotech Inc., Norcross, GA) as recommended by the manufacturer.

In vitro scratch assay

HaCaT cells were seeded at 10^5 cells/well in 24 well Linbro plates. After the cells formed an adherent monolayer, a linear scratch was created using a sterile P 200 pipette tip as previously described [9;30;31]. The monolayer was washed twice to remove cellular debris and incubated in DMEM containing 2% FCS plus 5 ng/ml of TGF β and/or 1 μ M of ODN. "Healing" was quantified by analysis of photomicrographs taken with an IX50 inverted microscope (Olympus, Tokyo, Japan). Changes in migration area over time were calculated using NIH Image J software. Results were derived from at least 4 independent experiments.

Statistical analysis

Statistical analysis was performed using SigmaStat, version 3.11 (Systat Software, Inc., Chicago, IL). Differences in the rate of healing of Bx sites were assessed using one way repeated measures analysis of variance (ANOVA). Area under the curve was calculated for overall changes in wound area using serial measurement. All values are expressed as means \pm SE unless otherwise noted.

Results

Effect of CpG ODN on wound healing in rhesus macaques

We previously demonstrated that CpG ODN could be formulated in liquid basement membrane extract (BME) and that the combination gelled when applied to wounded skin to create a protective layer from which ODN was released in bioactive form over 2 – 3 days [20]. To evaluate whether CpG ODN designed for human could accelerate wound healing in primates, multiple 6 mm full-thickness excisional skin biopsies were taken from the dorsal lumbar area of individual rhesus macaques. Different biopsies from the same animal were treated on days 0, 2 and 4 with 'K' or 'D' type CpG ODN formulated in BME. Sequential photographs provided a permanent record of the behavior of each biopsy site on each animal (Fig 1).

Those sites treated with 'K' ODN healed significantly more rapidly than those treated with 'D' ODN or BME alone ($p < 0.05$; two way repeated measures ANOVA). A 50% reduction in wound area was achieved after 5.9 ± 0.7 days at sites treated with 'K' ODN vs 10.3 ± 1.9 days for sites treated with 'D' ODN or BME alone ($p < 0.05$ for both comparisons, Fig 1). These findings indicate that 'K' (but not 'D') type CpG ODN significantly accelerate wound healing, confirming and extending previous results obtained using ODN optimized for use in mice [20].

Effect of CpG ODN on fibroblast production of bFGF

Murine studies indicated that CpG ODN treatment induced the production of vascular endothelial growth factor (VEGF) by cells at the wound site. VEGF accelerates blood vessel formation and thus promotes wound healing [20]. Consistent with that finding, VEGF production was significantly increased when human PBMC treated with CpG ODN ($p < .05$, supplementary Fig 1). However, this effect was mediated by both 'K' and 'D' ODN. Thus, while increased levels of VEGF might contribute to the repair of wounds treated with CpG DNA, production of VEGF could not explain the difference in activity between 'K' vs 'D' ODNs.

Fibroblasts contribute to wound repair, as they facilitate the formation of granulation tissue and differentiate into myofibroblasts that provide the contractile force needed for wound closure. This cell type is also a source of basic fibroblast growth factor (bFGF) [2], which is known to promote fibroblast proliferation and migration during wound healing [5]. Animal Care and Use Guidelines prohibit multiple surgical interventions in primates, preventing us from monitoring bFGF production at wound sites in rhesus macaques. The mechanism by which 'K' type ODN accelerated wound repair was therefore investigated in vivo in mice. mRNA was prepared from biopsy sites of mice treated with BME + 'K' ODN (mice do not respond to 'D' ODN). bFGF mRNA levels were significantly increased in wounds treated with 'K' ODN vs BME alone during the acute phase of wound healing (Fig 2a, $p < 0.05$).

Further evidence that CpG ODN could boost bFGF production was obtained during in vitro studies of human skin fibroblasts. Whereas CpG ODN alone had no effect on bFGF production, human fibroblasts treated with 'K' ODN plus IL-1 \forall significantly increased bFGF secretion (Fig 2b). 'D' ODN (alone or in combination with IL-1 \forall) had no effect on bFGF levels. IL-1 \forall is normally present at high concentrations at sites of tissue injury during the acute phase of wound repair, and PCR analysis of tissue obtained from murine biopsy sites ($N = 7$) showed that IL-1 \forall mRNA levels were elevated 12.2 ± 3.2 fold in wounded vs normal skin ($p < .01$). These findings suggest that the accelerated wound repair mediated by 'K' ODN involved increased production of bFGF, supported by IL-1 \forall present at the wound site.

Effect of CpG ODN on re-epithelialization and keratinocyte migration

Histologic evaluation of Bx sites in mice confirmed earlier results that CpG ODN Rx significantly accelerated re-epithelialization (Fig 3, $p < 0.01$). A key determinant of the rate of re-epithelialization is how effectively keratinocytes migrate into the wound [32]. To explore the effect of CpG ODN on primate keratinocyte migration, an in vitro wound repair model utilizing the human keratinocyte HaCaT cell line was employed. PCR studies confirmed that HaCaT cells, like primary murine and human keratinocytes [33], express TLR9 (data not shown).

In a pattern reminiscent of its effect on bFGF production, 'K' ODN alone had little effect on HaCaT cells (data not shown) but significantly accelerated cell migration when administered in combination with TGF \forall (which is present at high concentration at sites of skin injury [34]) (Fig 4). This effect was attributable to increased keratinocyte migration, as there was no difference in cell proliferation in cultures treated with TGF \forall + CpG ODN. 'D' ODN had no effect on human keratinocyte migration, indicating that 'K' ODN accelerate wound re-epithelialization in part by stimulating keratinocyte migration.

Discussion

This work is the first to show that 'K' type CpG ODN optimized for human use significantly accelerate wound healing in primates. The ability of TLR ligands to impact wound repair was initially established in studies of the TLR4 agonists LPS and HMGB1. The former promoted the migration of human bronchial cells in vitro [9] while the latter accelerated wound repair in diabetic mice in vivo [10]. Similarly, MyD88 KO mice show delayed healing (MyD88 is a TLR adapter molecule through which both TLR4 and TLR9 signal) [35]. Pursuing those observations, we recently demonstrated that CpG ODN significantly accelerated wound healing in mice [20]. That activity was TLR9 dependent, since CpG treatment had no effect on TLR9 KO mice [20].

Unfortunately, mice and humans differ in their recognition of and response to CpG DNA. Mice fail to recognize 'D' type ODN, and respond poorly to 'K' ODN optimized for human

use. In contrast, rhesus macaques mirror the human response to these structurally distinct classes of CpG ODN [23;24]. Using ODNs optimized to stimulate human PBMC, the healing of full-thickness biopsies in macaques treated with ‘K’ vs ‘D’ ODN was monitored. Results showed that 50% wound closure was achieved nearly twice as rapidly when biopsies on the same animal were treated with ‘K’ ODN vs ‘D’ ODN or BME alone (Fig 1). Previous studies in mice suggested that this acceleration in wound closure was attributable to multiple factors, including CpG-induced VEGF production [20]. While ‘K’ ODN stimulated a significant increase in the production of VEGF, so did ‘D’ ODN, indicating that the distinctive ability of ‘K’ ODN to accelerate wound repair must have some additional basis.

Our studies of macaques and mice indicate that CpG treatment is most effective when administered immediately after wounding (Fig 1 and ref 20). Focusing on that early period, we found that CpG ODN boosted the expression of bFGF at wound sites in mice within 24 hr (Fig 2). This finding was of interest because bFGF contributes to wound healing by inducing fibroblast proliferation [36;37]. Yet when human skin fibroblasts were cultured with ODN alone, no bFGF production was observed. Pursuing this matter further, ODN was co-administered with IL-1 \forall . IL-1 \forall was selected for study because i) it is known to stimulate growth factor production by fibroblasts [38;39] and ii) it is secreted at high levels by keratinocytes during the early inflammatory stage of wound healing when the effect of “K” type CpG was maximal [40]. Consistent with the latter finding, bFGF mRNA levels were increased 4-fold at biopsy sites. As seen in Fig. 2b, the combination of IL-1 \forall plus ‘K’ (but not ‘D’) ODN induced significant bFGF production by human skin fibroblasts. This set of observations led us to postulate that CpG ODN may not accelerate wound repair autonomously but rather in combination with other factors (such as IL-1 \forall) present at the injury site.

This interpretation was buttressed by studies examining the effect of CpG ODN on HaCaT human keratinocytes. HaCaT cells express TLR9 and form a monolayer when cultured in vitro. When “wounded” by scratching, changes in keratinocyte migration are readily visualized. Results showed that CpG ODN alone had no effect in this model, yet when co-administered with TGF \forall , ‘K’ (but not ‘D’) ODN significantly accelerated keratinocyte migration. Of note, TGF \forall up-regulates TLR9 expression by human keratinocytes [41] and is present at high levels at sites of skin injury [34]. This set of findings is consistent with the observation that the rate of wound re-epithelialization was significantly accelerated by CpG ODN in vivo (Fig 3).

CpG ODN are easy to manufacture, inexpensive, and stable. They have an excellent safety profile [42-45], and no adverse reactions were observed when these ODN were administered cutaneously to rhesus macaques or mice. Current results confirm and extend findings in mice by showing that CpG ODN designed for human use accelerate wound healing in primates. This was not accomplished simply by activating cells that express TLR9, but rather involved synergy with additional factors normally present at wound sites (such as TGF \forall and IL-1 \forall). Given the complexity of the wound healing process, additional factors and activities may also play primary or supportive roles in CpG-accelerated repair. As previous studies established that the response of rhesus macaques to CpG ODN closely mimics that of Man, current findings support the initiation of human clinical studies to assess the therapeutic utility of ‘K’ ODN on wound healing.

Conclusions

This work establishes that the topical administration of “K” type CpG ODN designed for clinical use significantly accelerates wound healing in rhesus macaques (a species that accurately models the response of humans). This effect is mediated by CpG-dependent

activation of TLR9 expressing cells but is critically dependent on other factors present at the wound site.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the Intramural Research Program of the National Cancer Institute of the National Institutes of Health.

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Abbreviations

BME	basement membrane extract
ODN	phosphorothioate oligodeoxynucleotide
LPS	lipopolysaccharide
bFGF	basic fibroblast growth factor

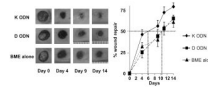


Figure 1.

Effect of CpG ODN on wound healing in rhesus macaques

Multiple independent 6 mm excisional biopsies, spaced 4 cm apart, were taken from the dorsum of rhesus macaques. BME combined with 50 ug of ‘K’ or ‘D’ ODN (or BME alone as negative control) was administered to each Bx site (N = 6/treatment) on days 0, 2 and 4. Wound size recorded by digital photography. The best fit first-order regression curve was calculated for each group, and the time needed to achieve a 50% reduction in the original area determined (dotted lines).

*, $p < .05$ for the integrated area under the curve of BME + ‘K’ ODN vs BME alone or BME + ‘D’ ODN by two way repeated measures ANOVA.

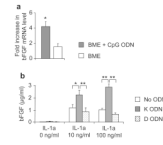


Figure 2.

Effect of CpG ODN on bFGF production.

A) Six mm excisional biopsies were taken from the right and left dorsum of individual BALB/c mice. One site (selected at random) was treated with BME alone while the other was treated with BME formulated with 50 :g of 'K' ODN. Total RNA was extracted from the Bx site at 24 hr. Quantitative RT-PCR was performed to detect bFGF expression. Results represent the mean \pm SE of at least 4 independent samples/treatment group. B) The concentration of bFGF in supernatants of cultured human skin fibroblasts was measured by ELISA. Results represent the mean \pm SD of 3 independent experiments performed in triplicate.

*; $p < .05$, **; $p < .01$.

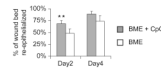


Figure 3.

Effect of CpG ODN on wound re-epithelialization.

a) Six mm excisional biopsies were taken from BALB/c mice as described in Fig 2. On each animal, one site was treated with BME alone while the other was treated with BME formulated with 50 :g of CpG ODN. Mean + SE of the total re-epithelialized area was measured on days 2 and 4. Results reflect the mean + SE of 6 independent samples/group. **, $p < .01$ for % re-epithelialized area of the entire wound bed of CpG + BME vs BME alone.

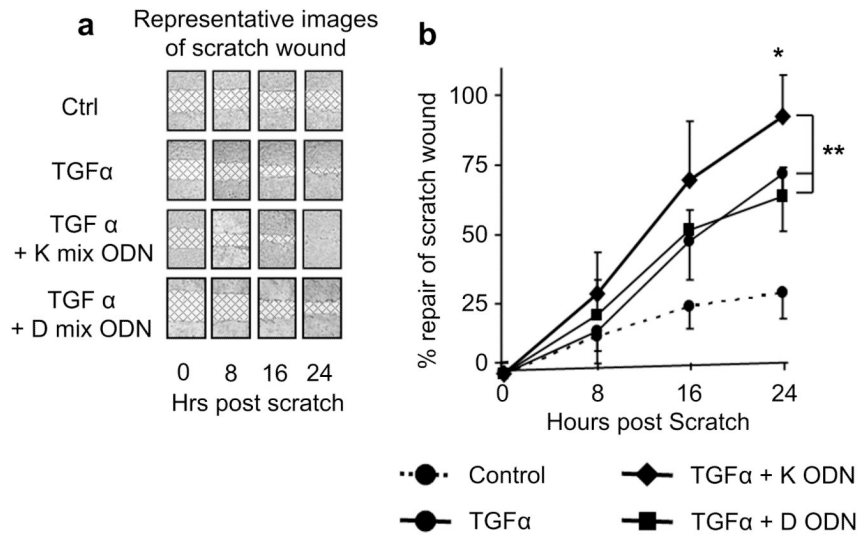


Figure 4. Effect of ‘K’ CpG ODN on keratinocyte migration.
 a) A linear “scratch wound” was made on a confluent monolayer of HaCaT cells. Digital photographs of representative wounds are shown, with the wound area cross hatched. b) The rate of repair of these scratch wounds was determined for each treatment over time. Results represent the mean \pm SD of 4 independent studies.
 **, $p < .01$ for the overall speed of wound healing of TGF α + ‘K’ ODN vs TGF α alone or TGF + ‘D’ ODN (Two way repeated measures ANOVA)