Gallium Nitrate Accelerates Partial Thickness Wound Repair and Alters Keratinocyte Integrin Expression to Favor a Motile Phenotype

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The nitrate form of the Group III transitional element gallium (GN) increases expression of specific structural components of the provisional wound matrix (i.e., collagen type I, fibronectin) in human dermal fibroblasts. To evaluate the potential of GN as a therapeutic option in management of cutaneous trauma, GN-treated partial thickness porcine wounds and experimentally “injured” human keratinocyte (NHK) monolayer cultures were compared with mirror image control (i.e., saline-treated) sites. GN suppressed cell proliferation in both models, as determined by reduced Ki-67 reactivity and significant lengthening of keratinocyte cell cycle transit times, while effectively promoting reepithelialization. The primary effect of GN was apparently to promote cell migration, as neither epidermal thickness nor epidermal differentiation was altered as a result of GN exposure in vivo or in vitro. Significantly enhanced epidermal reepithelialization was associated with alterations in expression of several keratinocyte integrin subunits. GN induced a significant increase in α5 expression. α5β1 switching is a characteristic of the motile phenotype in the setting of cutaneous injury. Concomitantly, GN treatment also induced a dramatic (70%) decrease in the expression of the α3 subunit; α3β1 binds laminin 5 and is associated with hemidesmosome formation and re-establishment of a nonmotile phenotype. Taken together, the GN-induced changes in integrin expression favor acellular migration. While the molecular mechanism of GN action on resident cells of the skin remains to be defined, these data suggest that GN administration which represses MMP activity in the wound and increases matrix synthesis also accelerates NHK motility and, thereby, may be a useful therapeutic agent for wound repair.

INTRODUCTION

Gallium, a Group IIIa transitional element, in nitrate form (GN) is an anti-resorptive agent and an effective U.S.-FDA-approved therapeutic option for the treatment of life-threatening cancer-related hypercalcemia [reviewed in 1–3]. GN actions on bone resorption are multifaceted, in part due to its direct effects on osteoclasts and several effects on osteoblast function including the regulation of osteocalcin gene expression [4–7]. GN may augment bone mass not only by inhibiting osteoblast activity but also by modulating synthesis and turnover of structural components of the extracellular matrix (ECM) [8–10]. GN increases bone calcium and crystallite perfection of hydroxyapatite [11]. In this regard, GN positively influences ECM deposition and accumulation through at least two distinct mechanisms. GN increases fibronectin and type I procollagen mRNA transcripts in primary rat calvarial osteoblasts as well as in normal human dermal fibroblasts (HDFs) [8]. Recent studies have also suggested that GN inhibits the activity of the matrix metalloproteinase (MMP) family of ECM-degrading enzymes [12]. The potential of GN to induce ECM synthesis while inhibiting MMP activity may contribute to an overall increase in ECM accumulation.

The ECM plays an important regulatory role in injury resolution [13]. Early after cutaneous trauma, a fibrin/fibronectin-rich matrix is deposited within the wound bed. The normally quiescent epidermal keratinocytes (NHKs) and HDFs are subsequently “activated” in response to cutaneous injury, a condition that in-
volves integrin switching to facilitate cellular migration through the provisional wound matrix [14, 15]. Once wound closure is complete, the provisional matrix undergoes a regulated and protracted period of remodeling largely under the control of MMPs and the plasmin-based pericellular proteolytic cascades. An imbalance in the rate of synthesis and degradation of the ECM may lead to specific healing deficits resulting in creation of a chronic wound [16–23]. Indeed, genetic analysis of the tissue response to injury has provided insights as to fundamental mechanisms involved in such complex processes as ECM remodeling. Recent findings suggest, for example, that efficient wound healing requires a functional overlap between the plasmin-based pericellular proteolytic cascade and members of the metalloproteinase family [24]. MMP inhibitors and plasminogen deficiency (Plg 

inhibits the healing process [25]. This greatly protracted time course of epidermal injury repair in Plg 

mice as well as in MMP inhibitor-treated wild-type animals appears specifically due to an inability of wound-edge keratinocytes to proteolytically dissect, and migrate through, the fibrin-rich provisional matrix [25]. Consistent with this is the restoration of "normal" healing in mice dually deficient in Plg and fibrinogen [26].

In chronic wounds, where the primary defect is a combination of a deficit in ECM synthesis or an excess of ECM degradation, GN may have a dual role in enhancing wound healing by inhibiting MMP action as well as increasing synthesis of matrix components. To evaluate this possibility, the effects of GN treatment on wound repair rates and integrin expression were assessed in NHKs. GN accelerated repair of partial thickness porcine wounds and stimulated closure of monolayer NHK scrape injuries likely due, in part, to upregulated integrin expression. These findings suggest that GN treatment may provide a novel and cost-effective therapeutic approach to the management of chronic as well as acute cutaneous injuries.

MATERIALS AND METHODS

Partial thickness wound model. All in vivo experiments utilized domestic Yorkshire pigs (approximately 60 lb) in accordance with the guidelines of the Weill Medical College of Cornell University’s Institutional Animal Care and Use Committee. Animals were anesthetized before surgery and wounds created using a dermatome set at a 0.015 inches in depth. A series of shallow, mirror image, partial thickness wounds (5–15 mm) were created using a dermatome set at a 0.015 inches in depth. The left or right side of each mirror image injury pair was randomly chosen to receive either GN (50 μM in 50 μl PBS) or vehicle (50 μl of PBS alone). All wounds were photographed and each was treated with polyurethane dressing (OpSite; Smith and Nephew Med. Ltd., Hull, UK) and further covered with Intersorb dressing (Sherwood Med., St. Louis, MO) and Spandage (Medi-Tech International, Brooklyn, NY). Elliptical biopsies through the center of each wound were designed to include margins of unwounded skin and 5 μm sections were stained with hematoxylin and eosin (H&E). Three criteria (reepithelialization, epidermal thickness, and epidermal maturity) were applied to quantitate the effects of GN on partial thickness healing. These parameters were quantified by image analysis and all evaluations were done with the investigator blinded to the treatment group. The kinetic profile of keratinocyte proliferation during wound healing was quantitated by standard immunohistochemical techniques utilizing anti-Ki-67 antibodies [28]. After results were decoded, each individual GN-treated wound was compared with its mirror image control site. Analysis of variance (ANOVA) for a crossover trial with replication served to determine whether a difference existed between the two treatments with regard to the parameters assessed. This design took into account the different number of paired sites within an animal (otherwise called replications) as well as the fact that each animal received each of two treatments (hence the term “crossover”); P < 0.05 was considered statistically significant.

Cell culture. Skin was obtained from cadaveric donors in accordance with organ donation and consent procedures of the New York Presbyterian Hospital Fire Fighters Skin Bank. Primary cultures of NHKs were maintained under submerged culture conditions and fed every 3 days with fresh medium.

In vitro GN treatment. NHKs were seeded at initial densities of 1 × 10^5 cells/cm². Two days later, GN (100 μM; based upon experimental dose curves) was added to half of the cultures with the percentage of wound closure was assessed using a calibrated ocular grid [31] for periods up to 48 h. By 72 h all cultures were healed.

Data analysis. Data generated from four independent NHK isolates were pooled together for analysis purposes. A two-factor repeated-measures ANOVA, with “group” (GN-treated versus untreated controls over all days combined) and “day” (comparisons between days 2, 3, and 4 of the experiment) as the main effects and including the interaction effect, “group/day” in the model, was initially carried out separately for each of the outcome variables (mean integrin fluorescence, peak channel of integrin fluorescence, and the percentage of integrin-expressing cells). Duplicates were averaged over all replications per day to simplify the analysis. The covariance matrix was assumed to have a compound symmetry structure. Since
Histologic evaluation revealed that a single treatment of partial thickness porcine wounds with GN induced a significantly greater degree of reepithelialization on day 3 postwounding compared to PBS-treated mirror image injuries (53.5 ± 4.8 vs 69.6 ± 6.2; mean percentage of reepithelialization for PBS- and GN-treated sites, respectively; P = 0.03). Indeed, 10/19 GN-treated wounds were ≥80% reepithelialized compared to only 2/19 control wounds on day 3 postinjury. Epidermal thickness (as quantified from the top of the granular layer to the base of the basal layer) was similar in both control (0.07 ± 0.02 mm) and GN-treated (0.07 ± 0.01 mm) cultures.

To determine whether topical application of GN affected local cellular proliferation, sections of healing skin were also stained for mib1 expression; mib1 binds to the nuclear antigen Ki-67 and is expressed in the nucleus of proliferating cells (Fig. 1). Control wounds consistently exhibited a high level of mib1 reactivity within basal layer NHKs (Fig. 1C; arrows). Approximately 70% of basal NHKs expressed Ki-67. Significant numbers of endothelial cells and fibroblasts also expressed Ki-67 in control wounds. While Ki-67 reactivity was elevated in GN-treated wounds (~25% reactivity in basal NHKs) compared to unwounded skin (10% Ki-67+; data not shown), the extent of Ki-67 expression was much lower compared with control wounds (Fig. 1D; arrows). Thus, GN appeared to enhance cellular migration and wound repair despite a relative decrease in cellular proliferation compared to control-treated wounds.

Treatment of NHKs with GN in vitro enhances monolayer wound repair and alters integrin expression patterns. Partial thickness wound repair involves, in large part, an epidermal migratory response. Our previously established in vitro model of wound repair [29–31] was adapted, therefore, to assess the effects of GN on epidermal cell migration. GN treatment significantly accelerated the rate of wound repair in this model compared to control-treated wounds (Fig. 2; Table 1). Consistent with our in vivo observations, increased NHK migration was observed despite the fact that GN dramatically inhibited NHK proliferation (16.7 ± 2.4 vs 30.2 ± 5.5 h population doubling times for control vs GN cultures, respectively), thus effectively reducing the pool of proliferating cells located behind the migrating front [27]. GN inhibited the growth of NHKs in vitro without being cytotoxic, as evidenced by a greater than 90% viability of cells recovered from culture at all time points.

RESULTS

GN promotes partial thickness wound repair in a porcine model. Histologic evaluation revealed that a single treatment of partial thickness porcine wounds with GN induced a significantly greater degree of reepithelialization on day 3 postwounding compared to PBS-treated mirror image injuries (53.5 ± 4.8 vs 69.6 ± 6.2; mean percentage of reepithelialization for PBS- and GN-treated sites, respectively; P = 0.03). Indeed, 10/19 GN-treated wounds were ≥80% reepithelialized compared to only 2/19 control wounds on day 3 postinjury. Epidermal thickness (as quantified from the top of the granular layer to the base of the basal layer) was similar in both control (0.07 ± 0.02 mm) and GN-treated (0.07 ± 0.01 mm) cultures.

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To determine whether GN-enhanced migration was the result of integrin switching or changes in integrin surface expression, the percentage of cells expressing the α2, α3, α5, and β1 integrin subunits as well as the density of expression per cell (as assessed by mean and peak channel fluorescence intensity in GN-treated vs control cultures was quantitated (Table 2). NHKs constitutively expressed α2, α3, and β1 integrin subunits; in addition, greater than 50% of cells also expressed α5 integrin, which is typical of cultured NHKs. GN-treatment caused a small reduction in the fraction of cells expressing α5 integrin, with no appreciable affect on any of the other subunits. Despite the minimal affect on the percentage of integrin-expressing cells, the density of integrin expression per cell was altered by treatment with GN since α3 subunit expression was reduced by 70% in GN-treated cultures. By contrast, relative levels of α5 increased by 26% (Table 2). These differences were observed on all days of cultures (e.g., days 2, 3, and 4) in the presence of GN. No significant differences in the density of the α2 or β1 integrin subunits were observed at any of the time points measured.

DISCUSSION

Wound repair is a complex process that involves "activation" of numerous cell types within the wound bed to effect timely healing [13–15]. Compounds and/or biological agents that accelerate wound repair may be important adjunctive therapies for partial and full thickness injury [32]. Clinical trials with single growth factors have shown some efficacy in tissue repair. However, there are questions concerning the cost-effectiveness of such therapy in terms of the marginal improvement seen in healing rates compared to the expensive nature of the treatment [23], hence the pressing need for alternative, cost-effective agents to enhance wound healing. We have identified GN nitrate as a potential candidate as part of the armamentarium for wound repair. GN is a compound that is stable and relatively inexpensive to manufacture. As a simple inorganic compound it is nonantigenic and can easily be administered topically, orally, or parenterally. GN can readily be maintained in sterile solution, it is biocompatible with many compounds, it can exist in many different formulations, and it has a prolonged shelf-life.

In the present study, we examined whether GN, which has been shown to induce expression of provisional-matrix type ECM proteins (e.g., fibronectin and collagen), could influence the rate of wound repair in both in vivo and in vitro model systems. A single topical treatment with GN accelerated the rate of reepithelialization of partial thickness wound repair in pigs. Indeed, 10/19 GN-treated wounds were more than 80% reepithelialized by day 3 postwounding compared
to only 2/19 mirror imaged paired control wounds. Increased epidermal migration occurred despite the relative suppression of cellular proliferation by GN as assessed by Ki-67 reactivity. Indeed, the epidermal basal layer of healing control wounds exhibited a much greater proportion of Ki-67+ cells (>three fold) compared with the basal epidermal layer of GN-treated wounds.

To better define the mechanism by which GN accelerates epidermal migration, we utilized our previously established in vitro NHK wound repair model to study cell migration and integrin expression in GN-treated cultures. The increased planar motility of GN-treated NHKs correlated with significantly increased density of the $\alpha_5$ integrin subunit per cell (as determined by both mean fluorescence intensity and peak position intensity). Exposure to GN clearly increases surface expression of $\alpha_5\beta_1$ on individual NHKs while not altering the overall fraction of $\alpha_5\beta_1$+ cells. Similarly, $\alpha_5\beta_1$ is expressed at high levels in the migrating epidermal cells as they crawl over the fibronectin-rich provisional wound matrix [33, 34], although in vivo this process
appears to involve a "switch" in integrin expression. In view of the constant correlation between NHK motility and $\alpha_5\beta_1$ receptors [15], it appears likely that this integrin is required for epidermal migration in response to cutaneous injury. In addition to the upregulation of $\alpha_5$ expression per cell, there was a coordinate and dramatic (~70%) decrease in the density of $\alpha_3$ expression per cell in response to GN exposure; $\alpha_3\beta_1$ binds laminin 5 (also known as epiligrin) and is important for the binding of NHKs to the basement membrane. Laminin 5 inhibits human NHK migration as does the classical laminin [35, 36]. Reestablishment of the laminin-rich basement membrane beneath the newly formed epidermis in cutaneous wounds may signal a halt to NHK migration and thus foster a switch from migration to differentiation phenotype. Thus, the induction by GN of decreased $\alpha_3$ expression concomitant with increased $\alpha_5$ expression may foster the accelerated wound repair observed in the more rapid reepithelialization of partial thickness porcine wounds.

Altered integrin expression may be due to a direct effect of GN or up-regulation secondary to an increase in fibronectin and collagen deposition. Indeed, increased expression of fibronectin mRNA in GN treated cultures has been reported previously [8]. ECM composition is influential in regulating cellular gene expression and phenotypic differentiation [13, 37] and may also modulate integrin subunit expression. The

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<td>GN Treatment Accelerates in Vitro Wound Repair</td>
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<th>Percentage wound closure (mean ± SD)</th>
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**FIG. 2.** The rate of repair of control (black bars) and GN-treated (striped bars) scrape-wounded, NHK monolayer cultures; 3–5 day postconfluent NHK cultures were scrape-wounded and their rate of closure was quantitated as a function of their culture in the presence (striped bars) or absence (black bars) of GN. GN treatment accelerated the rate of epidermal wound repair at all time points measured.
mechanism of GN-induced α5 or β1 immunoreactivity in the epithelium is not known but modulation of the TGF-β autocrine loop in the migratory epidermis may be one likely possibility [34]. TGF-β up-regulates synthesis of the extracellular matrix, increases integrin expression, and stimulates keratinocyte motility [33, 34, and unpublished data]. Such autocrine growth factor effects may be relevant to wound closure particularly at early times (12–24 h) postinjury and may involve both cellular contractile changes and gene reprogramming.

GN-dependent augmentation of the ECM accumulation/composition may involve two separate mechanisms (i.e., inhibition of MMP activity by substitution for zinc by gallium and/or enhanced transcription of fibronectin and collagen mRNA), both contributory to an overall increase in the collagen and fibronectin content in the ECM. In chronic wounds, including pressure and venous stasis ulcers, increased levels or activity of MMPs and a corresponding decrease in their specific inhibitors result in excessive proteolysis and degradation of fibronectin and vitronectin [16–23, 38–40]. GN’s positive effects on ECM production and epidermal migration combined with its established inhibitory effects on metalloproteinase activity may make it an extremely useful compound to accelerate the rate of chronic wound repair. Studies are underway to further examine its utility in the chronic wound setting.

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