Increased calcium levels alter cellular and molecular events in wound healing

A. Sank, MD, M. Chi, BS, T. Shima, BS, R. Reich, PhD, and G. R. Martin, PhD,
Bethesda, Md.

Surgical morbidity is dictated directly by wound healing. We have studied the effects of elevated calcium levels using cultured keratinocytes in vitro on two of the rate-limiting steps of wound healing, chemotaxis (directed migration) and adhesion. We found that the increased calcium (10 mmol/L) significantly inhibited both keratinocyte chemotaxis and adhesion (p < 0.05). The calcium effect on adhesion could be partially reversed by pretreatment with the calcium channel blocker verapamil. Based on these data, an animal model was formulated in which topical calcium (5 mmol/L/day) was added to linear incision wounds. This resulted in significantly (p < 0.05) delayed wound contraction characteristic of a chronic or impaired wound. Wound contraction depends on the presence of fibroblasts that synthesize collagen. The chronic wound was characterized by increased collagenase activity (p < 0.05) but little alteration in collagen I synthesis. The addition of verapamil to these chronic wounds resulted in improved wound closure. These studies define the molecular and cellular events occurring as a result of the addition of elevated levels of calcium both in vitro and in vivo. Calcium may play a key role in the pathogenesis of chronic wounds. (SURGERY 1989;106:1141-8.)

From the Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research, National Institutes of Health, Bethesda, Md.

DELAYED WOUND HEALING has an impact on patient morbidity. There are a number of disease states associated with delayed wound healing including diabetes, paraplegia, and chronic renal failure. The specific cellular and molecular events underlying chronic wounds are not clearly elucidated. Normal wound repair is characterized by a cascade of interlinked rate-limiting steps. These rate-limiting steps include clot formation at the incision site followed by chemotaxis and adhesion of endothelial cells, keratinocytes, and fibroblasts to the supporting matrix components. These cells, surrounded by the soft tissue matrix, then proliferate at the wound site before wound contraction and epithelialization. Cellular participation in this reparative cascade is known to be of critical importance, because patients who lack these cells (e.g., those taking chemotherapeutic agents) display delayed wound healing. Keratinocytes, the major epithelial cellular component, are metabolically active and involved in epithelialization and scar formation. The functions of these keratinocytes are known to be sensitive to calcium alterations.3,4

Wound tensile strength is dictated by dermal collagen accumulation. Type I collagen5 is the main dermal matrix component, and together with collagenase6 these synthetic and degradative pathways regulate collagen accumulation at the wound site.

There are a number of possible regulatory steps that, if altered, may result in chronic wound formation. One such regulatory pathway may involve calcium. Calcium is known to affect the normal function of a variety of cells. These properties include cellular mitosis,7 neutrophil exocytosis and superoxide production,8,9 remodeling of embryonic epithelium, and growth factor regulation.10 Since abnormalities of wound healing are found in hypocalcemia (chronic renal failure) and hypercalcemia,11 it is possible that this anion may play a key role in regulating wound repair. To date, the specific cellular effects of elevated calcium levels on soft tissue repair have not been elucidated clearly. This study sought to (1) investigate the role of calcium on the rate-limiting steps of wound healing in cell culture, (2) determine the role calcium plays in altering wound healing, and (3) develop an animal model in which appropriate levels of calcium are added to wounds. This animal model will allow us to study the effects of calcium on wound healing in vivo.

Presented at the Tenth Annual Meeting of the American Association of Endocrine Surgeons, Chapel Hill, N.C., April 17-18, 1989.
Reprint requests: Anthony C. Sank, MD, LDBA, NIDR, NIH, Bldg. 30, Room 426, Bethesda, MD 20892.
11/6/14679
healing in an animal model, and (3) establish therapeutic strategies based on the derived data.

METHODS

The initial experiments were carried out in cell culture and included keratinocyte chemotaxis and adhesion.

In vitro. Human keratinocytes (Clonetics, San Diego, Calif.) derived from neonatal foreskins were grown in culture in the presence of MCDB 153 medium (keratinocyte growth medium [Clonetics]; calcium concentration, 0.1 mmol/L) with added hydrocortisone, epidermal growth factor (10 ng/ml) and bovine pituitary extract. Conditioned medium was prepared by culturing human fibroblasts in Dulbecco's modified Eagle's medium (DMEM) with fresh ascorbic acid (50 mg/ml) for 48 hours. Conditioned medium was stored at -20°C until use in the chemotaxis assay.

Chemotaxis. Keratinocytes suspended in DMEM (at a calcium concentration of 0.1 mmol/L) were added to the top chamber of blind-well Boyden chambers, with a porous filter (8 μm pore, Nucleopore Corp., Pleasanton, Calif.) between the upper and the lower chambers. The surface of the filter was coated with 5 μg of collagen intravenously to improve cell attachment. The cells (keratinocytes) migrating through the filter to its lower surface after a 6-hour incubation period were stained with Diffquick (American Scientific Products, McGaw Park, Ill.) and quantitated by image analysis (Optomax, Inc., Hollis, N.H.). In these studies, various compounds that are assayed for chemotactic ability were placed in the lower chamber. These included the growth factor basic (fibroblast growth factor) and conditioned medium; 3% bovine serum albumin (BSA) in the lower chamber served as a negative control. In separate experiments, various amounts of calcium chloride were added to either the top or bottom compartments of the Boyden chamber. For these experiments, conditioned medium served as the attractant.

Adhesion. Plastic wells (16 mm) were coated with
Table I. Keratinocyte adhesion

<table>
<thead>
<tr>
<th>Matrix component</th>
<th>Adhesion (μm² × 10⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plastic</td>
<td>0.1 ± 0.05</td>
</tr>
<tr>
<td>Collagen I</td>
<td>4.2 ± 1.1</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>3.9 ± 1.2</td>
</tr>
<tr>
<td>Calcium + collagen IV</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Calcium + collagen IV + verapamil</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>Collagen IV + verapamil</td>
<td>4.0 ± 0.8</td>
</tr>
</tbody>
</table>

*Keratinocytes adhere significantly (p < 0.05) more to collagens I and IV than to the negative control (plastic). Calcium (10 mmol/L) significantly decreases adhesion to collagen IV, but this is partially reversed by the addition of verapamil. Keratinocyte adhesion to collagen IV is not altered by the addition of verapamil.

collagens I and IV and allowed to dry overnight in a laminar flow hood. The nonspecific binding of cells to the wells was blocked by the addition of 3% BSA. Keratinocytes (80,000 cells per well), added to each well in the medium used in the chemotaxis studies, were incubated at 37°C in 5% CO₂/95% air for 2 hours. The attached cells were stained with hematoxylin-eosin stain and quantitated with an Optomax image analyzer. Calcium chloride was added to this system at the same concentrations as used in the chemotaxis studies. Pretreatment of the keratinocytes with verapamil (10⁻⁶ mol/L) for 30 minutes was used in separate experiments to block the effect of calcium.

**In vivo**

**Animal model.** Guinea pigs (Hartley strain: 250 to 300 gm) were studied in accordance with an approved National Institutes of Health protocol. Animals were anesthetized with ketamine and xylazine hydrochloride (Rompun) (1 ml/kg of each per animal). After shaving, two 2.5 cm full-thickness incisions were made vertically in the midline of the dorsal skin and collagen sponges (Collagen Corp., Palo Alto, Calif.), cut in the shape of the wound were placed within the wound margins. Calcium chloride (50 to 300 mmol/L) with or without verapamil (1 × 10⁻⁴ moles/L) was applied to the collagen sponges. All wounds were covered with transparent adhesive dressing (Tegaderm; 3M, St. Paul, Minn.) plastic dressings stapled in position. The animals that underwent operation were housed in individual cages throughout the studies.

**Quantitation of wound healing**

These studies sought to document changes between the normal and the calcium-treated wounds.

**Photographs.** Weekly photographs of the wounds were taken with a standard 35 mm camera and flash attachment. Each picture included a centimeter ruler for internal standardization. The total area of the wounds was digitized from the photographs by computer (McMeasure software; Macintosh II computer) and is used as a measure of wound contraction.

**Collagenase assays.** Collagenase activity was quantitated by solid-phase radioassay. Collagen was labeled with ¹²⁵I by the Bolton Hunter method. ¹²⁵I collagen was diluted in collagenase buffer (0.05 mol/L Tris HCl, pH 7.6) to approximately 10,000 cpm/50 μl, and 50 μl was pipetted into each well and incubated at 4°C overnight. Wells were rinsed after the incubation period to remove unbound collagen. Wound tissue was homogenized in 0.15 mol/L NaCl 0.05 mol/L Tris HCl, pH 7.4, at 4°C and centrifuged, and various amounts of supernatant fluid were added to the wells and incubated at 37°C for 18 hours. Buffer alone or certain amounts of collagenase from fibroblasts were added to other wells to serve as positive and negative controls. A standard curve was formulated with known amounts of collagenase standard in buffer, and...
with a densitometer. Known amounts of fibroblast mRNA were included in these studies to serve as a positive control.

Statistics

Data were analyzed by the analysis of variance, with p < 0.05 being considered significant.

RESULTS

Effects of calcium on chemotaxis and adhesion of keratinocytes. Initially, we tested the migratory responses of keratinocytes to attractants in low-calcium medium. Conditioned medium and basic fibroblast growth factor (Fig. 1) induced a significant (p < 0.05) chemotactic response that was dose dependent. When calcium chloride (10 mmol/L) was added to the medium in the Boyden chamber, keratinocyte chemotaxis to conditioned medium and basic fibroblast growth factor was reduced. A similar inhibition was noted when calcium chloride was added to either the top or bottom chamber of the Boyden system (data not shown).

Keratinocytes attached to various matrix components (Table I) including collagens I and IV (50 μg/ml), with 80% of cells attached within 2 hours. We further studied the effect of calcium and the calcium channel blocker verapamil on the attachment of the cells to collagen IV-coated dishes. These studies showed that the addition of CaCl2 (10 mmol/L) inhibited the adhesion of keratinocytes to the collagen matrixes. This effect of calcium was partially reversed by pretreating the cells for 30 minutes with verapamil.

Effects of calcium supplements on experimental wounds. Experimental wounds (Figs. 2 and 3) were created in the dorsally shaved skin of guinea pigs by incising 2.5 cm long full-thickness wounds. A collagen sponge was inserted into the wound and, where indicated, the sponge was supplemented with various amounts of CaCl2 or verapamil plus CaCl2. The calcium chloride supplement grossly interfered with the healing of the wound as judged by wound contraction. Verapamil partially prevented these defects.

Wound contraction. The area of the wound was monitored at weekly intervals, and wound contraction decreased as the wound healed. Calcium delayed wound contraction (Fig. 4) on day 7, which was partially reversed by verapamil (50% inhibition). This inhibitory effect of calcium alone was found to be dose dependent.

Collagenase activity. It is well known that collagenase levels are increased in wound skin presumably to achieve debridement. An excess production of collagenase, however, could occur in the calcium-treated wound (Fig. 5). To test this possibility, we removed...
Increased calcium levels in wound healing

Differentiated Keratinocyte

EGF TGF α

Calcium

Basement Membrane

Fibroblast

Blood Vessel

Macrophage

Collagen

Fig. 5. This diagram summarizes our present concepts of the factors responsible for chronic wound formation. To the left of the diagram are the anatomic layers of the skin, consisting of the epidermis, the underlying basement membrane, and the dermis. In the epidermis the keratinocytes differentiate in response to elevated calcium concentrations. Fibrin is present in the wound clot together with platelets, which, like macrophages, release growth factors. In the dermis there are fibroblasts, blood vessels, and macrophages imbedded in a collagen matrix. The fibroblasts produce collagen whereas collagenase is produced by both macrophages and fibroblasts. Tumor necrosis factor (TNF) and calcium modulate collagenase production. This collagenase, which is present in excess in our animal wound model, breaks down the collagen into smaller fragments. The growth factors, depicted to the right of the diagram, act through calcium-sensitive intracellular pathways on both the epidermis and the dermis. Epidermal growth factor (EGF) and transforming growth factor (TGF) α enhance epithelialization, whereas TGFβ and platelet-derived growth factor (PDGF) appear to act on the dermis. TGFβ has been shown to increase collagen I mRNA expression, which may have therapeutic applications.

wound tissue at various times after wounding, homogenized the tissue, and assayed the supernatant fluid for collagenase activity. Little or no collagenase activity was detected in unwounded skin; however, collagenase levels (Fig. 6) were increased significantly after wounding.

Collagen I gene expression. The expression of this collagen gene was measured in tissue from normal and calcium-impaired wounds as a measure of synthetic activity. RNA extracted from the wounds was probed for collagen α1(1) mRNA by northern analysis. No striking difference in the amounts of message was observed between control and calcium-supplemented wounds.

DISCUSSION

This study demonstrates the important role of calcium in regulating wound healing. The calcium effect appears to be intracellular, because the calcium channel blocker verapamil partially reversed the in vitro and in vivo effects of calcium. In the clinical setting, patients with chronic failure often develop hypocalcemia. These patients, as well as other patients with hypercalcemia, suffer from impaired wound healing. Verapamil applied topically to wounds in these patients may facilitate wound repair. It is also possible that specific cells involved in wound healing respond differently to elevated levels of calcium. This would result in keratinocytes terminally differentiating at a specific concentration of calcium, whereas fibroblasts, for example, may be able to proliferate and migrate at the same levels of calcium.

Wound healing depends on a series of complex interrelated cellular and molecular events. Specific understanding of these events requires dissection of the repair process into its component parts. Our in vitro studies have
examined two of the rate-limiting steps of wound repair, namely cellular chemotaxis and adhesion. These studies could be used with other cell lines such as fibroblasts and endothelial cells to determine the specific role each cell line plays in wound repair. Based on our findings in cell culture, namely that calcium inhibited both keratinocyte chemotaxis and adhesion, we were able to formulate a reproducible impaired or chronic wound model.

Collagen deposition is central to wound contraction and tensile strength formation. This deposition appears to be regulated by a fine balance between synthetic and degradative pathways. Although the collagen I gene expression is identical in normal and calcium-treated wounds, predominantly degradative processes characterize our chronic ulcer model. Furthermore, it has been shown that keloid formation is characterized by an increase in collagen production. Although our collagen I gene data did not show differences between the normal and calcium-treated wounds, there are other possible explanations for delayed repair. There is increasing evidence that growth factors play an important role in wound healing. Recently, Pierce et al. demonstrated that the application of transforming growth factor \( \beta \) to a glucocorticoid-impaired rat wound model reversed the wound healing defect. This evidence, along with that shown by Lawrence et al. suggests that future detailed studies of growth factors may demonstrate the specific etiologic role these growth factors play in chronic wound pathogenesis. The specific mechanisms that regulate growth factors are now the subject of extensive research efforts. Block et al. have shown that the addition of calcium channel blockers (nifedipine, verapamil, and diltiazem) inhibits the effects of platelet-derived growth factor in vascular smooth muscle cells. This evidence supports the role of calcium in modulating growth factors.

These cellular and molecular studies of the effects of elevated calcium levels have provided improved insight into the complex events associated with the challenging clinical problem of delayed wound healing.

In summary, these studies have outlined the specific inhibition of cellular adhesion and migration of epithelial keratinocytes by elevated calcium levels in vitro and in vivo. Therapeutic strategies suggest that the addition of the calcium channel blocker verapamil may be efficacious in management of the chronic wound.

We thank Drs. Hynda Kleinman, Paul Klotman, Yoshi Yamada (National Institutes of Health, Laboratory of Developmental Biology and Anomalies, Bethesda, Md.) for their advice regarding this project and the manuscript, Dr. George Ksander (Collagen Corporation) for providing collagen sponges for the animal experiments, and Drs. Derrick Le Roith (NIAAD, National Institutes of Health) and J. Norton (National Cancer Institute, Surgery Branch) for advice regarding the manuscript.

REFERENCES

DISCUSSION

Dr. Andrew Saxe (Detroit, Mich.). Let me ask the obvious question: Have you examined the effect of low calcium in the system? If you are speculating that this is in part mediated by a parathyroid hormone-like substance, what is the effect of low calcium? Can low calcium speed up some wound-healing events? And have you measured the culture supernatant for parathyroid hormone-like substance?

Dr. Sank. Regarding the low calcium, we grow the cells (keratinocytes) in a medium called MCDB-153, which has a calcium concentration of 0.1 mmol/L. If we increase the calcium concentration up to 1.2 mmol/L, there is some alteration of keratinocyte chemotaxis, which occurs in the presence of a calcium concentration of 1.2 to 1.8 mmol/L. This has not only been work from our lab but also from Dr. Yuspa’s at the NIH. So there is some switching, which occurs between 1.2 and 1.8 mmol/L of calcium, that suddenly changes the cells from being proliferative and active to differentiate and no longer be useful in wound healing. There are some data showing that in chronic ulcer tissue, at the interface between the normal skin and the ulcer, there is an increase in calcium levels. This suggests that the calcium may play a role in inhibiting the proliferation of a variety of cells.

About measurements in the supernatant, we have not measured any parathyroid hormone-like substance. We would like to answer a number of questions regarding parathyroid hormone and keratinocytes in culture. Studies are in progress.

Dr. Burney (Ann Arbor, Mich.). I have treated three patients with chronic ulcers associated with ectopic calcification. None of these people had abnormalities of their calcium metabolism but had it as a result of dermatomyositis. In two of the three, we simply removed the ectopic calcifications and were able to get the wounds to heal. These chronic ulcers associated with calcifications may or may not be related to the phenomenon that you are talking about. Obviously, if you have calcium deposits in your wounds, you locally increased calcium concentration. What is the relationship between the normal calcium concentration in wound tissue and the calcium concentration that you are achieving by adding topical calcium? How much additional calcium are you really adding, and how much is that changing the calcium concentration in the tissues?

Dr. Sank. That is obviously an important question. As a correlation between our calcium studies and the clinical situation, there is a well-established association between chronic renal failure, hypercalcemia, and poor wound healing. In the transplant literature, there are also cases of hypercalcemia associated with wound-healing problems.

Regarding the amounts of calcium we added to the wounds, we add 5 mmol/L of calcium chloride to the wound per day, which is about twice the upper limit of normal serum calcium. There are studies from John McPherson at the Collagen Corporation, Palo Alto, Calif., detailing the rate of release of a compound added to a collagen sponge placed in the wound. And the reason we use the calcium sponge is that it adds calcium alone without the sponge, the calcium is rapidly lost from the wound site. The calculated delivery of calcium to the wound by the sponge is 60% of the added dose, which is 3 mmol/L/day or what Harrison’s textbook of medicine describes as the upper limit of calcium in the serum. The data we would like in our guinea pig model is exactly what the calcium levels are at the wound site. There are a number of investigators at the Johns Hopkins Medical Center using a dye called fura-2 to estimate intracellular calcium concentrations. As yet, we have not done these studies.

Dr. Kenneth H. Cohn (Brooklyn, N.Y.). If you extrapolate your data to patients, the ones who have the most trouble with wound healing are not the ones who have failed parathyroid reexplorations, but the ones with Cushing’s disease. Have you looked at the effects of exogenous steroids and seen if they are quantitatively or qualitatively similar to the effects of hypercalcemia?

Dr. Sank. One of the problems in performing gene-expression studies in human patients is that one of these studies requires a large amount of tissue. Now there is new machinery available called the PCR (polymerase chain reaction) which is a gene-amplifying device. This innovative machinery allows us to take small amounts of human tissue and then perform a variety of molecular studies on this tissue. We can now study...
the genes expressed in a variety of tissues in the body and do not need large amounts of tissue. The patients we are studying with this technique are those with hyperparathyroidism, those taking large doses of steroids, and others with generally impaired wound healing. We are looking at the levels of genes expressed in the impaired (chronic) wound compared to the patient's own normal skin. There are a number of exciting studies going on with this PCR device, which became available about 1 year ago, that we believe will allow detailed molecular studies of altered soft tissue repair in patients.

Dr. Gary B. Talpos (Detroit, Mich.). The clinical implications are interesting. Localized acidosis seen in ischemia might be better treated by topical bicarbonate when revascularization can no longer be performed. In addition, the variable response of Raynaud's phenomenon to calcium channel blockers also ties into your work nicely.