Vanadate and the Absence of Myofibroblasts in Wound Contraction

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Hypothesis: Fibroblasts, not myofibroblasts, are responsible for wound contraction. Only myofibroblasts express a smooth muscle actin for which vanadate blocks its expression. Wound contraction in vanadate-treated rats will proceed normally in the absence of myofibroblasts.

Design: Laboratory study using rats.

Methods: Wound healing in rats receiving vanadate parenterally, an inhibitor of tyrosine phosphate phosphatases, was investigated. For 21 days, treated rats received drinking water containing vanadate, 0.2 mg/mL, in isotonic sodium chloride solution, and the control rats received isotonic sodium chloride solution alone. On day 7, 4 square, full-excision wounds were made dorsally and measured, then 2 polyvinyl alcohol sponges were placed ventrally in subcutaneous pockets.

Results: After 2 weeks, the wound area in the rats receiving vanadate measured 7.1 ± 1.8 U (mean ± SD), and the wound area in the control rats measured 7.2 ± 2.2 U. The control rats' granulation tissue (GT) had myofibroblasts, or α-smooth muscle (α-SM) actin-positive fibroblasts, whereas the vanadate-treated group's fibroblasts were devoid of α-SM actin. By Western blot analysis, GT homogenates in the vanadate-treated group contained less α-SM actin. By electron microscopy, control rats' GT showed classic myofibroblast populations, and the collagen fiber bundles were randomly organized. In contrast, the wounds in the vanadate-treated group showed unencumbered fibroblast populations and neatly ordered, parallel collagen fiber bundles. By polarized light microscopy, the GT of the vanadate-treated group displayed orderly collagen fiber bundles.

Conclusions: The differentiation of fibroblasts into myofibroblasts requires the dephosphorylation of selected tyrosine phosphate residues. In the absence of myofibroblasts, the rate of rat wound contraction is normal, and collagen fiber bundles have a more orderly arrangement. Myofibroblasts are not required for wound contraction.

The wound healing process advances by an overlapping sequence of events referred to as "lag," "proliferative," and "remodeling" phases of repair. The lag phase is characterized by inflammation, which checks infection and initiates the proliferative phase of repair. The proliferative phase is characterized by the migration and division of fibroblasts and the synthesis and deposition of a new, vascularized connective tissue matrix. The remodeling phase of repair involves the organization of that new connective tissue matrix into a scar. The connective tissue organization in scar differs from dermis; a parallel arrangement of scar collagen fiber bundles replaces the basket-weave collagen fiber bundles of dermis. With few exceptions, dermal loss is replaced with scar and not dermal regeneration. The amount of scar deposited in full excisional wounds in rats is limited because of wound contraction, in which normal skin is pulled into the defect. Skin is pulled into the wound site by forces generated through the continued reorganization of the newly deposited collagen fibers in granulation tissue (GT), rather than through cell contraction. The requirement of a specialized fibroblast, the myofibroblast, that has been proposed to generate those processes is the subject of this report.

Cell "crawling" is required for the migration of fibroblasts into the wound site during the early proliferative phase of repair. Cell crawling involves polymerized actin organized into a filamentous network at the cell's leading edge. Actin-binding proteins play important roles in the polymerization of actin into highly branched networks seen in the leading edge of ambulatory fibroblasts. In sedentary fibroblasts, actin filaments are organized into cytoplasmic microfilaments that aggregate into thick stress fibers. These actin bundles in stress fibers demonstrate minimal branching and terminate at focal adhesion plaques near the plasma mem-

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focal adhesions act as switches in the transfer of information and the character of GT deposited in polyvinyl alcohol (PVA) sponge implants in rats ingesting vanadate. For 1 week, rats in the experimental group received vanadate in their drinking water, and the control group received isotonic sodium chloride solution in their drinking water before open wounds were made and PVA sponges were implanted. Two weeks later, the rats were killed, the wound sizes were recorded, and tissue specimens were biochemically and histologically analyzed. The following questions are addressed: Does an inhibitor of tyrosine phosphate turnover block myofibroblast formation? Are myofibroblasts critical for wound contraction? Are there differences between myofibroblasts and fibroblasts in organizing collagen fiber bundles within GT?

**MATERIALS AND METHODS**

Adult male Sprague-Dawley rats (weight, 375-400 g) were purchased (Charles River Labs, Wilmington, Mass). The rats were weighed and then placed in pairs in cages. The treatment group rats (n = 8) were given sodium orthovanadate (Sigma-Aldrich Corp, St Louis, Mo), 0.2 mg/mL, in sodium chloride, 150 mmol/L, in their drinking water for 1 week before wounding and for 2 weeks after wounding, when the experiment was terminated. Control rats (n = 7) received sodium chloride, 150 mmol/L, in their drinking water during the same period. The salted water was necessary to make the vanadate palatable. Unlike other animals, rats tolerate the long-term ingestion of sodium chloride solution in place of water.

Rats were placed under anesthesia with an intraperitoneal injection of pentobarbital sodium (Nembutal), 50 mg/kg of body weight, and their backs and abdomens were shaved. On the back of each rat, 4 full-thickness excision wound squares of 15 × 15 mm were made with scissors. The wound edges were tattooed and photographed with a ruler in place. An incision was made on the abdominal surface, and subcutaneous pockets were made on each side of the midline. A PVA sponge (Unipoint Laboratories, High Point, NC) was cut into disks of 3 mm thick and 12 mm in diameter. Sponge disks were boiled in distilled water, blotted with sterile gauze, and each weighed to the nearest milligram. These were placed into the subcutaneous pockets, and the entry wound was sutured closed. The rats were returned to their cages after complete recovery from anesthesia and remained on their drinking water regimen.

Two weeks later, the rats were killed with an overdose of pentobarbital and then weighed. The healing wounds were rephotographed with a ruler in place, and the healed wound sites and PVA sponges were harvested. The sponges and wounds were bisected. Half were processed for biochemical analysis, and the other half were processed for histological evaluation. Tissues to undergo biochemical analysis were homogenized in lysis buffer (0.1% sodium dodecyl sulfate; Tris hydrochloride, 10 mmol/L; at pH 6.8), boiled, cleared by centrifugation, and stored frozen (−80°C) until analyzed. The wound and sponge halves used for histological analysis were divided into pieces for paraffin and frozen sections. Only specimens from open wound tissue were processed for transmission electron microscopy (TEM; Philips Technologies, Cheshire, Conn). The paraffin sections were fixed in 10% formaldehyde, embedded, sectioned, and stained with hematoxylin-eosin or Sirius red/fast green, as previously described. Specimens for frozen sectioning were placed in cassettes, submerged in tissue cryo-embedding liquid (Fisher Scientific, Springfield, NJ), frozen in liquid nitrogen, and stored at −80°C. Frozen sections were cut 4 to 6 µm thick with a cryostat, fixed in buffered 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 detergent (Sigma-Aldrich Corp), and immunostained with monoclonal antibody directed to α-SM actin and β-actin (Sigma-Aldrich Corp). The α-SM actin–antibody complex was identified by a fluorescein-conjugated second antibody (sheep antimouse F(ab)2; Jackson ImmunoResearch Labs, Inc, West Grove, Pa) directed to mouse IgG, and filamentous actin was demonstrated by rhodamine-phalloidin staining (Molecular Probes, Inc, Eugene, Ore). The wound tissue specimens for TEM were cut into 1-mm cubes and fixed for 2 hours in Karnovsky fixative, then postfixed in 1% osmium tetroxide or 1.5% potassium ferrocyanide, dehydrated in ethanol, stained with 1% aqueous uranyl acetate, embedded in Spurr resin (Polysciences, Inc, Warrington, Pa), sectioned, and examined by TEM.

To measure wound areas, photographic slides taken immediately following wounding and 2 weeks later were projected. Their sizes were equalized by the included ruler, so all images were at the same magnification. The images were then traced on plain white paper. The tracings were cut out and weighed to the nearest 0.1 g. The mean ± SD weight from the wound tracings was calculated.

For Western blot analysis (immunoblotting), the protein concentrations were determined for the cleared homogenates using the Bradford method. Equal amounts of protein were run by sodium dodecyl sulfate–polyacrylamid gel electrophoresis with a Tris hydrochloride–glycine buffer on a mini-Protein II cell system (Bio Rad Labs, Hercules, Calif) following instructions from the supplier. Protein transfer was made to a PVDF membrane (Millipore Corp, Bedford, Mass). The membrane underwent sequential stainings with primary antibodies directed to α-SM actin and β-actin. The membrane was treated with 5% dried milk to block nonspecific protein-binding sites before incubating with primary antibody. After being incubated with primary antibody, the membrane was rinsed and then incubated with an antism IgG peroxidase-conjugated secondary antibody. The membrane was finally washed before developing with an electrochemiluminescence system (Amersham Life Sciences, Arlington Heights, Ill) following the manufacturer’s instructions.
RESULTS

Rats ingesting sodium chloride solution or sodium chloride solution containing vanadate, 0.2 mg/mL, for 3 weeks had similar weight gains. During the 2 weeks after wounding, the group given sodium chloride solution alone (control group) gained $21 \pm 5$ g (n = 7), and the vanadate-treated group gained $15 \pm 3$ g (n = 8) (all data are expressed as mean ± SD). The difference between the 2 groups' weight gain was not significant ($P > .05$; Student $t$ test).

The full-excision open wounds healed mostly by wound contraction. Figure 1 shows representative wounds made on the backs of rats from the 2 experimental groups immediately and 2 weeks after wounding. Wounds from both groups contracted about 75% during the 2 weeks. The noncontracted wound area was determined by weighing tracings, and 1 area unit equaled 1 g. The initial wound size averaged 27.6 U for both groups, and at 2 weeks, the control wounds contracted to $7.2 \pm 2.2$ U (n = 12), whereas the wound size of the vanadate-treated group was $7.1 \pm 1.8$ U (n = 12). Administering vanadate parenterally did not alter wound contraction. In both treated and control groups, open wounds closed by wound contraction.

The weights of PVA sponges at the time of implantation and harvesting at 2 weeks were recorded as a measure of GT deposited. The GT weight of sponges from the control group was $63 \pm 36$ mg and from the vanadate-treated group, $77 \pm 40$ mg. The amount of GT deposited in the PVA sponge implants was similar between groups; ie, there was no significant difference ($P > .05$; Student $t$ test). The ingestion of vanadate did not alter the amount of GT deposited in PVA sponge implants.

Vanadate treatment, however, altered the structure of cells and the organization of collagen fibers bundles in GT. In normal dermis, fibroblasts do not exhibit cytoplasmic stress fibers, but in GT they do. Rhodamine-phalloidin–stained stress fibers within fibroblasts and smooth muscle cells of blood vessel from a 2-week-old

Figure 1. Wounds immediately after wounding in (A) a control rat and (B) a vanadate-treated rat. Those same wounds 2 weeks later from control rats maintained on sodium chloride solution (C) and from the vanadate-treated rat (D).
Figure 2. Frozen sections of healing wounds were fluorescently stained with either rhodamine-phalloidin or with $\alpha$-smooth muscle ($\alpha$-SM) actin antibody. A, Section from a control rat shows fluorescent-staining stress fibers in fibroblasts. B, Section from a vanadate-treated rat shows the absence of fluorescent stress fibers in fibroblasts. Smooth muscle cells are present in blood vessel. C, Section from a control rat shows $\alpha$-SM actin-stained stress fibers in myofibroblasts. D, Section from a vanadate-treated rat shows the absence of $\alpha$-SM actin staining. Filaments are contained within smooth muscle cells of blood vessels (original magnification, $\times$100 for all figures).
healing open-wound GT are shown in Figure 2. In contracting wounds from control rats, prominently stained stress fibers in the myofibroblasts and in the smooth muscle cells of vessel walls were evident (Figure 2, A). In contrast, the GT of open wounds from vanadate-treated rats (Figure 2, B) was devoid of stained stress fibers within fibroblasts. Vanadate blocked the development of stress fibers forming in fibroblasts but had no effect on stress fibers within smooth muscle cells of blood vessels. Although the degree of wound contraction was identical in the open wounds of treated and control rats, a major structural difference was that vanadate ingestion prevented the appearance of stress fibers in the fibroblasts of the GT of contracting wounds.

Myofibroblasts have been characterized in GT and hypertrophic scar by their prominent cytoplasmic stress fibers, which contain \( \alpha \)-SM actin.8 Myofibroblasts were readily identified in control rat GT by stress fibers showing \( \alpha \)-SM actin (Figure 2, C). On the other hand, in the fibroblast populations of GT from vanadate-treated rats, \( \alpha \)-SM actin stress fibers were absent (Figure 2, D). The absence of \( \alpha \)-SM actin–staining fibroblasts was not due to flawed immunostaining because the smooth muscle cells of the vessel wall did contain that antigen (Figure 2, D).

Granulation tissue from 2-week-old PVA sponge implants was examined to confirm the open wound findings. The PVA implants from control rats contained a high density of myofibroblasts (Figure 3, A). In contrast, GT from PVA sponge harvested from the vanadate-treated rats contained fibroblasts devoid of \( \alpha \)-SM actin (Figure 3, B). Like the GT of open wounds, the smooth muscle cells in the vessel wall showed \( \alpha \)-SM actin, demonstrating an internal control for immunostaining. In all slides examined from vanadate-treated rats, the GT of both open wounds and PVA sponge implants was devoid of myofibroblasts, but all blood vessel smooth muscle cells contained \( \alpha \)-SM actin.

Western blot analysis was used to determine the expression of 3 different cytoskeletal proteins in PVA sponge homogenates: the focal adhesion plaque protein vinculin; \( \alpha \)-SM actin; and the microfilament protein \( \beta \)-actin. Twenty micrograms of protein from GT homogenate, from either control or vanadate-treated rats, was analyzed and compared. As shown in Figure 4, A, the ingestion of vanadate specifically reduced the levels of \( \alpha \)-SM actin compared with those in controls, due to the absence of myofibroblasts. Vanadate did not affect the levels of vinculin, indicating no alteration in its expression in focal adhesion plaques (Figure 4, B). By the expression of equivalent densities of \( \beta \)-actin bands (Figure 4, C), equal quantities of cell microfilaments were applied to the polyacrylamide gels. It appears that the expression of \( \alpha \)-SM actin is specifically reduced in GT from vanadate-treated rats.

Biopsy specimens of 2-week-old contracting wounds were examined by TEM. Granulation tissue from contracting wounds from control rats showed typical myofibroblast cell populations. Characteristic, numerous cell-cell associations and indentations of the cells' nuclei were seen (Figure 5, A). These myofibroblasts showed aggregated microfilaments contained in stress fibers in an area immediately below the plasma membrane of the cell,
Figure 4. Western blot analysis of \( \alpha \)-smooth muscle (\( \alpha \)-SM) from granulation tissue. Tissue homogenates of protein, 20 \( \mu \)g, from vanadate-treated or control rats underwent immunoblot analysis. A, Following vanadate treatment, levels of \( \alpha \)-SM actin are reduced in lane V (vanadate) compared with those in lane C (control). B, The densities of \( \beta \)-actin bands in lanes C and V of tissue homogenates were equivalent. C, The levels of vinculin, a focal adhesion protein, were equivalent between the 2 treatment groups. The arrows in all 3 indicate the location of the 66 000 molecular weight marker.

Figure 5. Transmission electron microphotographs of granulation tissue from 2-week-old contracting open wounds from a control rat (A, original magnification \( \times 4600 \), and B, original magnification \( \times 6800 \)). Cells are attached to one another, the collagen fiber bundles are arranged randomly, and there is an area just beneath the plasma membrane of the cell that is devoid of subcellular organelles. Sections from a vanadate-treated rat are shown (C, original magnification \( \times 4600 \), and D, original magnification \( \times 7700 \)). Cells do not touch one another, the collagen fiber bundles are arranged in a parallel manner, and just beneath the plasma membrane of the cell are numerous subcellular organelles (arrow).
which was devoid of other subcellular organelles (Figure 5, B). In addition, the extracellular compartment contained collagen fiber bundles that were randomly arranged. In contrast, the GT from vanadate-treated rats contained cell populations displaying minimal cell-cell associations and the absence of cytoplasmic stress fibers, and the area just beneath the plasma membrane of the cell contained subcellular organelles (Figure 5, C). The arrangement of collagen fiber bundles was striking because they were organized in a parallel manner (Figure 5, D). The ingestion of vanadate appeared to promote the arrangement of collagen fibers in distinctively ordered parallel bundles.

By light microscopy, hematoxylin-eosin–stained sections of healing open wounds showed no obvious differences in the cell types, cell density, or orientation of blood vessels within the GT between the 2 treatment groups (not shown). The PVA sponge implants from both treatment groups showed similar levels of GT ingrowth, density of cell populations, number of blood vessels, and character of connective tissue. By Sirius red–polarized light microscopy, however, differences in the packing and organization of collagen fiber bundles were apparent between treatment groups. The collagen fiber bundles of GT from a control rat’s PVA sponge implants showed modest intensity of greenish yellow birefringence, and the collagen fiber bundles were finely organized (Figure 6, A). The implants harvested from vanadate-treated rats demonstrated greater greenish yellow birefringent intensity, and the collagen fiber bundles were organized in an orderly parallel manner (Figure 6, B). The greater birefringence in the vanadate-treated rats compared with the control rats was the more ordered arrangement of the collagen fiber bundles. These differences in birefringence paralleled the TEM findings, in which more ordered collagen fiber bundles were evident in the vanadate-treated group.

**COMMENT**

The ingestion of vanadate in drinking water for 3 weeks apparently was not toxic to adult rats because no differences in weight gain were noted. Despite ingesting sodium chloride solution in place of water for 3 weeks, both groups of rats gained weight at equivalent rates. Likewise, the ingestion of vanadate neither inhibited nor stimulated the closure of open wounds or the amount of GT deposited in PVA sponge implants. At the microscopic level, however, the long-term ingestion of vanadate altered both the structure of cells and the organization of collagen fiber bundles in GT. Parenteral vanadate inhibits the conversion of fibroblasts to myofibroblasts, which have been proposed to be required for wound contraction.9 The findings presented here support the hypothesis that myofibroblasts are not required for wound contraction.

Vanadate added in vitro to cultured fibroblasts prevents the appearance of cytoplasmic stress fibers and large focal adhesion plaques.10 Human fibroblasts in monolayer culture normally show prominent stress fibers, but the inclusion of vanadate prevents their appearance. Vanadate inhibits phosphatases that remove phosphate groups from selected phosphorylated tyrosine residues in proteins. Vanadate also down-regulates p125 focal adhesion kinase, which is associated with the retarded appearance of both stress fibers and focal adhesions.11 This implies that the removal of selected tyrosine phosphate groups is involved with the appearance of stress fibers and the expression of \( \alpha \)-SM actin within fibroblasts of GT.

The in vivo ingestion of vanadate does not prevent stress fibers from developing in the smooth muscle cells of new
blood vessels. This implies that the development of stress fibers in fibroblasts requires signals different from those required for these structures to appear in smooth muscle cells. With vanadate ingestion, α-SM actin expression occurred in smooth muscle cells of new vessels but not in fibroblasts developing into myofibroblasts. Although ingested vanadate does not alter smooth muscle cell structure, it reportedly enhances the proliferation and the contractility of vascular smooth muscle cells. Others report that phosphorylation of the tyrosine residues in focal adhesion proteins can be related to cell shape, microfilament organization, and cell locomotion. Transmission electron microscopic studies reveal that vanadate affects the organization of microfilaments in treated ovarian granulosa cells that show fragmented actin filaments.

More important, the persistence of selected tyrosine phosphate residues in fibroblasts of GT promotes the better organization of collagen fiber bundles. Evidence supports a relationship between the phosphorylation of tyrosine residues and cell function in wound healing. The binding of cells to extracellular matrix is associated with increased phosphorylation of focal adhesion–associated tyrosine kinase. Mouse fibroblasts receiving high concentrations of platelet-derived growth factor display alterations in the levels of tyrosine phosphate residues in focal adhesion proteins, show the absence of cytoplasmic stress fibers, and demonstrate better attachment to their surrounding matrix.

It has been proposed that cell “tractional forces” describe the mechanical force that organizes collagen fiber bundles required for the evolution of scar integrity, the contraction of open wounds, and the advancement of scar contractures. The organization and orientation of collagen fibrils into scar matrix occur through forces generated by resident cells. Fine microfilaments are present in fibroblasts, generating the tractional forces involved in the translocation of collagen fibrils, whereas fibroblasts with stress fibers are poor at translocating collagen fibrils. Parenteral vanadate prevents fibroblasts from developing into myofibroblasts, while promoting the more orderly organization of collagen fiber bundles.

By both TEM and polarized light optics, collagen fiber bundles were more organized in the wound specimens of the vanadate-treated group. Vanadate therapy does not change the cell density of GT, as revealed by hematoxylin-eosin–staining patterns. The maturation of fibrils in rat GT and the development of stress fibers apparently occur through the dephosphorylation of selected tyrosine phosphate residues. Hence, a change occurs in the interaction of cells and collagen fiber bundles when these tyrosine residues lose phosphate. Vanadate preserves selected tyrosine phosphate groups and prolongs the interaction of cells with collagen fiber bundles, producing a more orderly connective tissue matrix.

The closure of open wounds in tight-skin mice by wound contraction was shown to proceed in the absence of myofibroblasts. It was concluded from that study that myofibroblasts do not actively participate in wound contraction. The hypothesis for wound contraction is that collagen is reorganized by the cytoskeletal contractile unit of the fibroblast, where fine microfilaments attached to small focal adhesion structures linked to αRI integrin, the collagen receptor, translocates collagen fiber bundles. The mechanism for the reorganization of collagen fibers into thicker fiber bundles is the translocation of collagen fibrils over the cell surface. When collagen fibers reach a point where there is sufficient resistance to the further translocation of collagen fiber bundles, the collagen is released from its attachment to αRI integrins, which causes the intracellular clustering of focal adhesions into large plaques, the aggregation of microfilaments into stress fibers, and the expression of α-SM actin.

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