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Charged Beads Enhance Cutaneous Wound Healing in Rhesus Non-Human Primates

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Enhanced cutaneous wound healing by positively charged cross-linked diethylaminoethyl dextran beads (CLDD) was studied in a standardized incisional wound model in 20 adult and 20 geriatric *Macaca mulatta* (rhesus) partitioned equally over five time periods. Physiologic saline served as a control. Soft-tissue linear incisions were prepared between and 1 cm inferior to the scapulae. There were four incisions per rhesus; each incision was 1.5 cm long with 1 cm of undisturbed tissue between incisions, and both the experimental CLDD and physiologic saline treatments were administered to each rhesus. The incision treatments were either CLDD and soft-tissue closure with 4-0 BioSyn sutures or sterile physiologic saline and closure with 4-0 BioSyn sutures. The hypothesis was CLDD would enhance cutaneous wound repair. Verification of the hypothesis consisted of clinical examinations and histologic and tensiometric evaluations on biopsy specimens at 10 and 15 days, whereas 5-day and 2- and 4-month groups were assessed clinically and biopsy specimens were assessed histologically.

The clinical course of healing for all groups was unremarkable. At 10 days, incisions in adult rhesus treated with CLDD had a 30-percent greater tensile strength compared with the physiologic saline-treated incisions ($p = 0.01$), whereas for geriatric rhesus, the CLDD treatment proved to be 15 percent greater in tensile strength compared with the physiologic saline cohort ($p = 0.11$). By day 15, incisions in adult rhesus were 26 percent stronger than the saline treatment group ($p = 0.07$), and the difference was 36 percent ($p = 0.02$) for the geriatric rhesus. From 5 through 15 days, histologic observations revealed a gradual decrease in quantity and integrity of CLDD, with no remnants of CLDD at either 2 or 4 months. Macrophages and multinucleated giant cells were localized in the dermis and were associated with the CLDD. These cells decreased commensurately with the decrease of CLDD beads.

The data suggest that CLDD can enhance significantly the tensile properties of healing cutaneous wounds in both adult and geriatric rhesus. Moreover, if the wound healing is enhanced in geriatric patients, this finding may

be clinically germane to conditions where wound healing is compromised, such as in diabetics and patients on steroids.

In contemporary health care, economic and effective therapies to enhance cutaneous wound healing have taken center stage. Although the efficacy of recombinant human (rh) growth factors is acknowledged to benefit the cutaneous wound-healing cascade, persuasive data are wanting, and pecuniary considerations are constraints. Therefore, cost-efficient, clinically effective alternatives have been sought. An example of a compelling alternative is electrical stimulation. However, electrical stimulation applied to cutaneous wounds has a complex and contradictory legacy. The notion that an electrical stimulation could enhance cutaneous wound repair is derived from cutaneous injury data, where the epidermis of the skin becomes electronegative with respect to the dermis. A "current of injury" to damaged cutaneous tissue purportedly provokes a current, $22 \mu\text{A}/\text{cm}^2$, that terminates when the tissue heals. The electrical current may result from deformation of the crystalline structure of collagen in the extracellular matrix, thus producing a wound-stimulating piezoelectric effect.¹ Alterations in the extracellular matrix may in turn regulate cells and gene expression through the intracellular cytoskeleton,^{2,3} leading to secretion of vulnerary factors for tissue repair.

Furthermore, as a result of cutaneous wounding, the "current of injury" may be stimulated by fluxes in fluid flow at the injury locus, producing electrokinetic-generated streaming potential.¹ Purportedly, streaming potential can modulate blood flow. For example, negative electrical stimulation can increase blood flow.⁴ Angiogenesis (i.e., the growth of branching capillaries) and blood flow are pivotal for cutaneous wound repair⁵ and are controlled through interactive dynamics among the extracellular matrix, cells, and the signaling factors expressed by cells. Macrophages are influential cells that direct events during the wound-healing cascade^{6,7} through expression of several signaling molecules, including platelet-derived growth factor, epidermal growth factor, transforming growth factor, fibroblast growth factor, and insulin-like growth factor.^{8,9} At the completion of wound healing, the "current of injury" ceases, and resident cell populations become consistent with the pre-injured phenotype. It is speculative whether a toggling interaction among cells, extracellular matrix, signaling molecules, and the "current of injury" sustains the wound healing cascade and with renewal of the epidermis-dermis, if a "current of injury" off-switch down-regulates cell activity.

Relevant to electrical currents, charges, and polarity and cutaneous healing is the population of cells affiliated with the wound-healing continuum and their predictable appearance during wound repair.¹⁰ The macrophage is a marquis cell affiliated with tissue repair,^{6,7} and a positive charge seems to solicit vectorial macrophage migration.¹¹ Furthermore, the "current of injury" and electronegativity between the dermis and epidermis could establish a "charged field gradient" influencing macrophage navigation toward the more electropositive dermis. Localization of macrophages within the dermis may provide a critical mass of cells to secrete signaling molecules necessary to restore the dermis and the epidermis. Fibroblasts also respond to electrical charge stimulation, and evidence suggests an enhancement of fibroblast DNA and collagen synthesis,¹ further underscoring the beneficial utility of therapeutic electrical current and charges.

Unfortunately, anecdotal, equivocal clinical results, and contradictory experimental observations have marred the acceptance of electrical stimulation for therapeutic wound repair. Moreover, it has been difficult to ensure a lo-

calized effect from a designated polarity. Therefore, a therapeutic with a known charge density, with polarity that could be controlled and applied locally, could provide clinical efficacy. Consistent with this notion was the application of "ionically charged" beads that could be targeted to a wound site. We previously hypothesized ionic charges could be exploited to fulfill the physiologic roles speculated for "electrical fields" to stimulate and to enhance wound repair. In a reported study, we previously proved this hypothesis in a rat model and determined positively charged cross-linked diethylaminoethyl dextran beads (CLDD) enhanced cutaneous wound healing.¹² Consequently, the next logical step was to validate the effectiveness of positively charged CLDD in *Macaca mulatta* (rhesus), a species highly relevant to man. Therefore, the general hypothesis for the current study was that positively charged CLDD would enhance cutaneous wound healing in the rhesus. Moreover, in light of the growing geriatric population in the United States and the clinical observations of a lag in wound healing in the elderly, a geriatric cohort of rhesus was included. The hypothesis was the CLDD would benefit the geriatric rhesus by enhancing cutaneous repair. The long range goal for the therapeutic CLDD is application as a clinical adjunctive treatment for patients who are likely candidates to have a delayed cutaneous healing response, such as the geriatric, diabetic, or individual on steroids.

MATERIALS AND METHODS

Preparation of CLDD

Positively charged cross-linked diethylaminoethyl dextran beads (DEAE Sephadex A-25, Pharmacia) were prepared by treating the DEAE with sodium periodate according to a method previously reported.^{13,14} In brief, 150 g of vacuum-dried diethylaminoethyl beads were hydrated with 24.72 g of sodium periodate in 2.5 liters of water. After 90 minutes, beads were washed with 5 M sodium chloride and acidified with 1 M hydrochloric acid followed with a water wash to remove excess acid, yielding a size range of beads 40 to 120 μm . Beads were suspended in a polyethylene glycol mixture (97% polyethylene glycol 300:3% polyethylene glycol 200,000) at a ratio of 0.1 g of CLDD beads to 10 ml of polyethylene glycol. Known amounts of CLDD/polyethylene glycol were

sterilized with 21.5 to 40 kiloGray of gamma irradiation, aseptically inserted into sterile squeeze tubes, and maintained in an aseptic manner until surgical implantation.

Animal Groups, Surgery, and Incision Model

Healthy female *M. mulatta* rhesus from the Oregon Regional Primate Center were identified, and a cohort of 20 was selected as the adult group, ranging in age from 4 to 11 years old. An additional group of 20 rhesus were geriatric; they were 18 to 20 years old.

Preoperative handling and general anesthesia were managed in the usual fashion. The surgical site was prepped and draped in a sterile manner.

Operatively, each rhesus received four linear incisions between and 1.5 cm inferior to the scapulae. The incisions were 1.5 cm in length and 1 cm apart through the epidermis, dermis, and panniculus adiposus without disturbing underlying muscular fascia. Incision margins were elevated, and the tissue plane between the panniculus adiposus and muscular fascia was advanced with blunt dissection for an additional 5 mm. No electrocautery was used.

Either 0.25 cc of topical CLDD or an equivalent volume of physiologic saline was administered to alternating incisions. CLDD was backloaded into a 1.0-cc syringe with no needle and applied both directly in the wound and underneath the adjacent edges of the wound. The sterile, 0.9% physiologic saline (McGaw Inc., Irvine, Calif.) was administered in a similar fashion. Incisions were closed with simple interrupted sutures (4-0 BioSyn, US Surgical, North Haven, Conn.). Neither antibiotic salve nor dressing was applied.

Following therapy, rhesus were returned to their individual cages for postsurgical recovery and were given free access to food and water. Their movement was not restricted. Postoperative analgesia was not required. Over the course of the study, incisional sites were assessed by routine clinical examination for dehiscence, erythema, and drainage.

Biopsy

At the designated times after incisional wounding (5, 10, or 15 days and 2 and 4 months), rhesus were returned to the operating room, anesthetized, prepped, and draped in a sterile fashion. An elliptical portion of epidermis, dermis, and subcutaneous adipose tissue encompassing the original incisions was

dissected carefully, and the resulting deficit was closed using subcuticular and vertical mattress sutures.

Using a template with surgical blades, the biopsy specimen was partitioned into individual specimens for histology and tensiometry for the 10- and 15-day groups, whereas only histology specimens were recovered for the 5-day and 2- and 4-month groups. Standardized strips of tissue that included 15 mm on each side of the incisional wound were trimmed carefully (total length of 30 mm) and were wrapped immediately in foil, placed on dry ice, and stored at -80°C . Specimens designated for histology were fixed immediately in 70% ethanol.

Tensiometry for 10 and 15 Days

There were four incisional wounds per rhesus that were treated with either physiologic saline or CLDD. Consequently, tensiometric measurements were accomplished on a designated specimen of tissue from each incisional wound providing eight test specimens for each temporal period (10 and 15 days).

In preparation for tensiometric testing, specimens were thawed at a constant rate and standard manner and were allowed to stabilize to room temperature before tensile testing.

Tensile strength of the wounds was measured with a Tensiometry 10 (Monsanto, St. Louis, Mo.) at a crosshead speed of 10 mm/min. A recording was made for each tissue sample subjected to tensile loading and tensiometry data for physiologic saline and CLDD at each time period (i.e., 10 and 15 days) and age group (i.e., adult and geriatric). Data were reported as a mean plus one standard deviation with significance established at $p = 0.05$.

Histology

Following fixation from 70 to 100% ethanol, specimens were processed for glycol methacrylate and sectioned to 4.5- μm thickness, and alternating sections were stained with either Goldner-Masson trichrome or hematoxylin and eosin. Histologic sections were examined with bright field light microscopy (Zeiss Axio-phot Microscope, Zeiss Instruments Co., Inc., New York, N.Y.).

Statistics

Statistical analyses were done to determine treatment and age-dependent effects of physiologic saline and CLDD on the mean tensile

strength of the cutaneous wounds. Data were analyzed by an analysis of variance and Fisher's protected least significant difference test to determine differences between treatments and times within each age and interactions of age, treatments, and times. Significance was established at $p = 0.05$, and data were presented as mean tensile strength (in newtons) plus one standard deviation.

RESULTS

Tensile Strength

Only 10- and 15-day time periods were assessed. At 5 days, cutaneous tissues are too immature to respond to tensiometric assessing; therefore, it is not possible to measure and detect differences between treatments. At 2 and 4 months, cutaneous tissue maturity mitigates against tensiometric discrimination between treatments.

The 10-day tensiometry results indicated that the CLDD-treated wounds for the adult rhesus

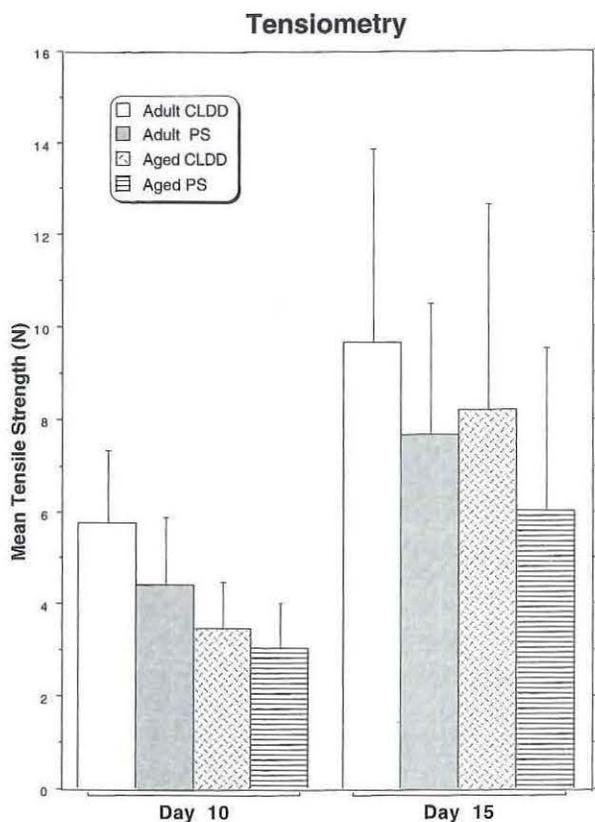


FIG. 1. Tensiometric data (10- and 15-day) are presented for adult and aged rhesus. Each bar represents a mean of 10 rhesus, and error bars represent 1 standard deviation. Statistical significance was established at $p = 0.05$. CLDD, positively charged cross-linked diethylaminoethyl dextran beads; PS, physiologic saline.

had a 30-percent increase in breaking strength compared with the physiologic saline-treated wounds (Fig. 1). The CLDD-treated wounds for the geriatric rhesus had a 14-percent increase in breaking strength compared with the physiologic saline-treated wounds.

At 15 days, for the adult rhesus, incisional wounds treated with the CLDD yielded a 25.8-percent increase in breaking strength for incisional wounds compared with the physiologic saline-treated wounds (Fig. 1). Geriatric rhesus administered the CLDD had a 36.3-percent increase in breaking strength compared with the physiologic saline-treated wounds.

Irrespective of treatments and time, the cutaneous wounds appeared similar from days 5 through 15. By 2 months, histologic evidence of an incision was extremely difficult to confirm, even in the geriatric group. By 4 months, there was no histologic evidence of a cutaneous incision.

Observations for the 5- through 15-day specimens included an epidermal thickening at the incision with subjacent hyperplasia of connective tissue stroma and numerous robust fibroblasts. In the dermis of the rhesus treated with CLDD, there seemed to be a more organized, vectorially oriented collection of collagenous fibers, with less birefringence than for the physiologic saline treatments (Fig. 2, below). CLDD beads were localized to the dermis, and bead degeneration increased from day 5 through 15, with no evidence of the CLDD beads at either 2 or 4 months (Figs. 2 through 4).

Contiguous to the beads were either macrophages or multinucleated giant cells (Figs. 3, above, and 4). The numbers of these cells decreased concomitantly with bead degeneration from 5 through 15 days. Lymphocytes and occasional neutrophils and monocytes also were observed.

DISCUSSION

Healing of cutaneous wounds may not be problematic for healthy individuals. However, with an increasing geriatric population in the United States, a commensurate burden in health care challenges will be generated. It is highly likely that cutaneous wound healing in the elderly would benefit from an effective, cost-efficient therapeutic. Consistent with this concept was the development of positively charged CLDD beads to enhance cutaneous wound healing. Moreover, the design of this

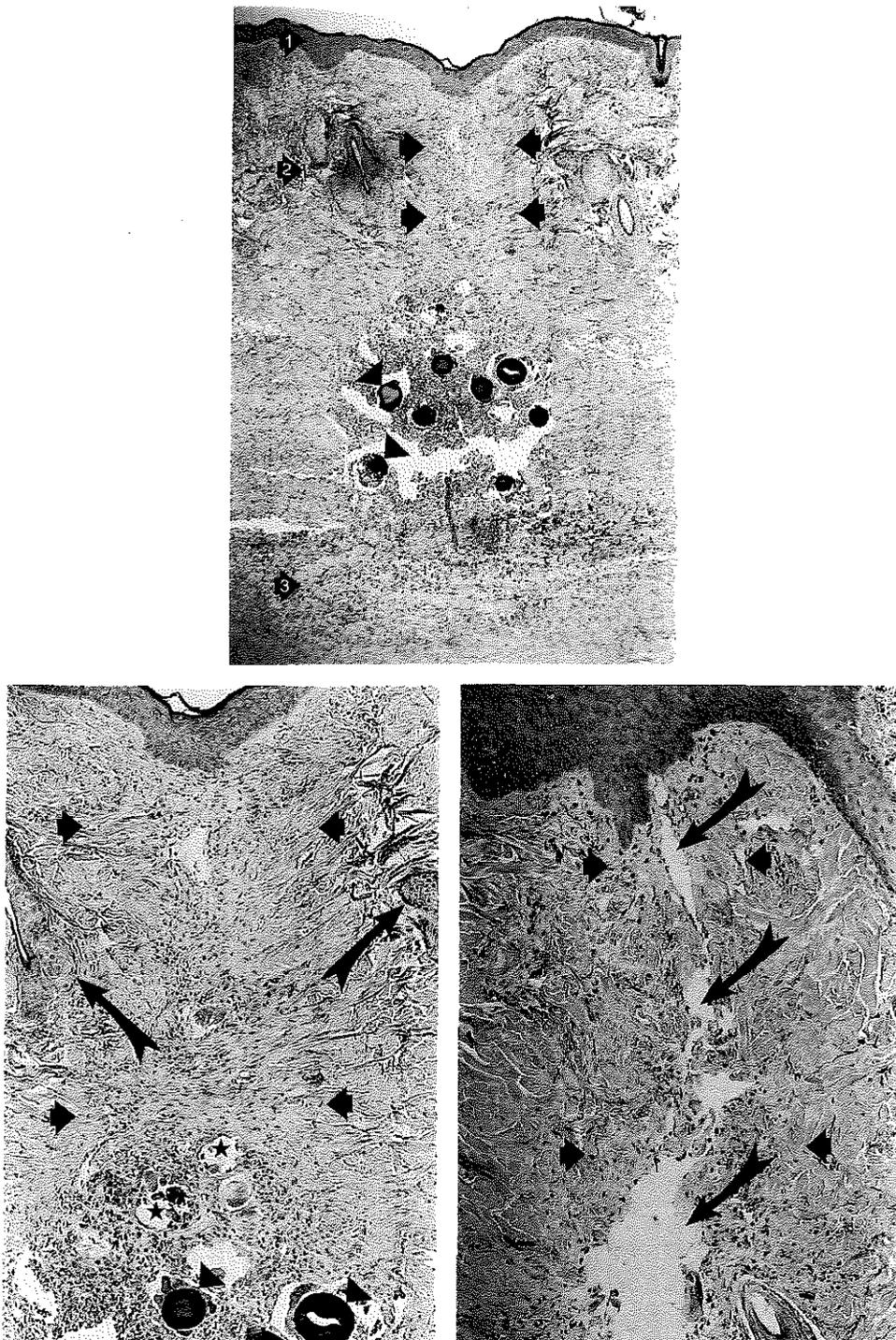


FIG. 2. (Above) The histologic appearance of an incisional wound at 10 days posttreatment with CLDD. Arrows with 1, 2, and 3 delimit epidermis, dermis, and subdermis, respectively. Black arrows (without numbers) indicate the incisional wound zone still visible in the dermis; however, the epidermis is intact. The CLDDs are observed in the transitional region at the base of the dermis (▼) (2.5× magnification, Goldner trichrome stain). (Below, left) A slightly higher magnification than panel above. Black arrows indicate the incisional wound zone visible in the dermis. CLDDs are observed at the base of the dermis (▼), and CLDD remnants are identified by the star. The curved arrows indicate sebaceous glands typically found in the epidermis and are associated with hair follicles (5× magnification, Goldner trichrome stain). (Below, right) The incisional wound counterpart of previous panel (below, left) shown at the same magnification. Black arrows indicate the incisional wound zone visible in the dermis. (5× magnification, Goldner trichrome stain).

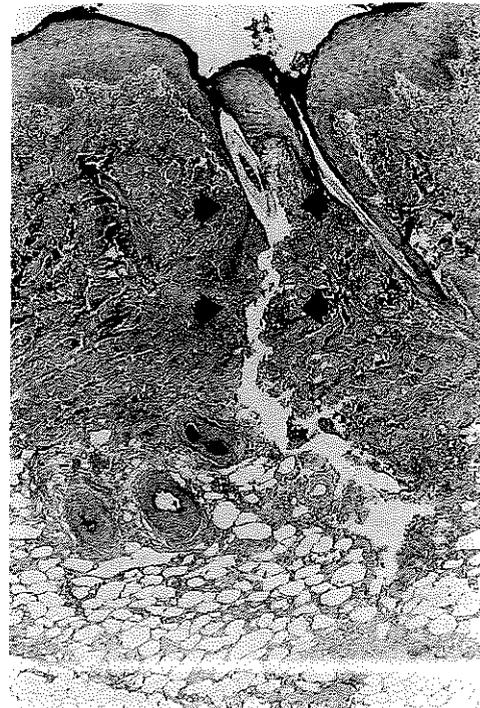


FIG. 3. (Above) Appearance of the incisional wound at 10 days posttreatment with CLDD. Arrows with 1, 2, and 3 delimit epidermis, dermis, and subdermis, respectively. Black arrows (without numbers) indicate the incisional wound zone in the dermis. The epidermis is intact, and keratinized component is apparent (dark-staining band). The CLDDs are observed in the transitional region at the base of the dermis (▼) admixed with round cell inflammatory infiltrate (2.5× magnification, Goldner trichrome stain). (Below, left) A slightly higher magnification than panel above. CLDDs are observed at the base of the dermis (▼), and the round cell inflammatory infiltrate is identified by the star. The curved arrow indicates the sebaceous gland-hair follicle complex (5× magnification, Goldner trichrome stain). (Below, right) The incisional wound counterpart of the previous panel is shown at the same magnification. Black arrows indicate the incisional wound track visible in the dermis. Collagen bundle orientation is random, and distinct gaps are present (5× magnification, Goldner trichrome stain).

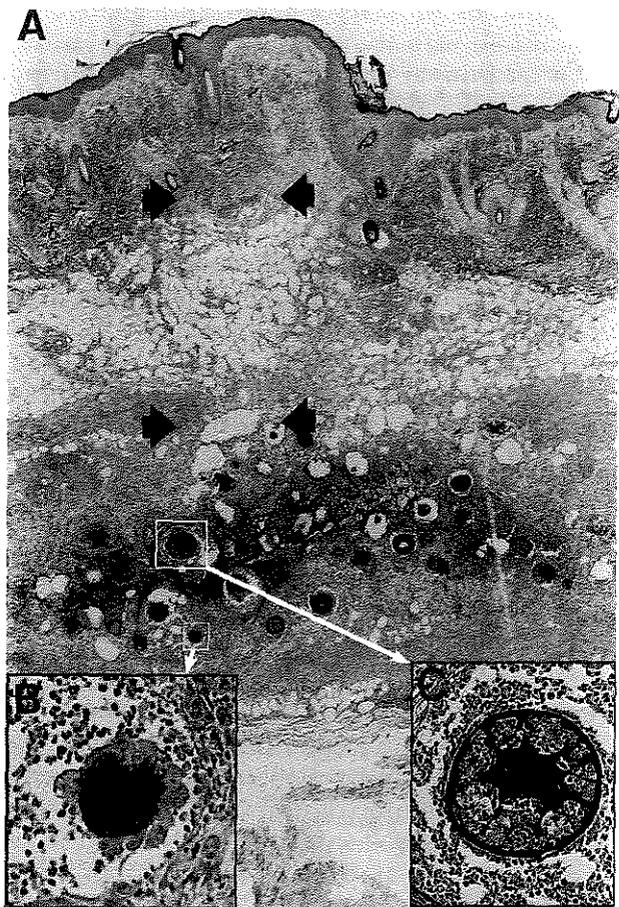


FIG. 4. (Panel A) Appearance of the incisional wound at 10 days posttreatment with CLDD. Black arrowheads indicate the incisional wound zone in the dermis ($2.5\times$ magnification, Goldner trichrome stain). (Panel B) A partially degraded CLDD enveloped by histiocytic-appearing cells ($10\times$ magnification, Goldner trichrome stain). (Panel C) A partially degraded CLDD enveloped by and containing small round lymphocytic-appearing cells ($10\times$ magnification, Goldner trichrome stain).

study focused on optimizing clinical relevance to the human population; thus, the rhesus was selected as a candidate wound model. The rational approach we followed was to build on the significant bank of preclinical data^{1,2,13,28,29,34} that clearly indicated the benefit of positively charged CLDD. Consequently, we compared the positively charged CLDD with a "common surgical practice": irrigation of a wound site.

Results strongly suggest CLDD therapy enhanced tensile strength for 10- and 15-day temporal groups in adult and geriatric rhesus. Although there was more variability (i.e., a greater standard deviation) in the 15-day cohort than the 10-day cohort, statistical analyses of the tensiometric data clearly indicated a

significant (36 percent) improvement for the geriatric rhesus treated with CLDD versus physiologic saline. Biologic variation and the cellular and stromal variables of cutaneous wound healing may have more significant impact on cutaneous healing outcome at 15 days than at 10 days.

The greatest increase in tensile strength was achieved at 15 days in the geriatric rhesus treated with CLDD, where the CLDD-treated incisional wounds were 36 percent stronger than the wounds administered physiologic saline. This is a highly relevant clinical finding fortifying the notion that a cost-effective, topically applied "biomaterial" could improve cutaneous incisional wound repair. Although growth factors have been administered to cutaneous wounds to enhance healing, variability in responses coupled with their expense underscores the appeal of the CLDD therapeutic.

Lynch¹⁵ has applied combinations of platelet-derived growth factor and insulin-like growth factor delivered in a methyl cellulose carrier to partial-thickness cutaneous wounds and observed enhanced epithelialization. Several reports have noted the benefits of epithelial growth factor in promoting cutaneous healing,¹⁶⁻¹⁹ whereas other studies have demonstrated ineffectiveness with epidermal growth factor.^{20,21} Mustoe and others have shown combinations of platelet-derived growth factor-BB, transforming growth factor-beta, and fibroblast growth factor improve wound-breaking strength in a rat incision model.²²⁻²⁵ In addition, recent data imply that systemic administration of growth hormone (but not insulin-like growth hormone) to malnourished, diabetic, steroid-treated, hypophysectomized, and normal animals improves the incisional wound tensile strength (reviewed by Robertson et al.²⁶). Despite the provocative potential of growth factors, they have not caught on as clinical therapeutics. Difficulties with delivery of the factors, variability in concentrations of growth factor among different studies, ambiguities in assessment criteria, and expense have deterred widespread clinical acceptance. An appealing alternative is the biomaterial known as CLDD. In the characterized rhesus model, CLDD proved to be an effective, safe, and inexpensive therapeutic.

According to tensiometric data, the CLDD treatment significantly enhanced the breaking strength of incisional wounds in adult rhesus at 10 days. By 15 days, incisional wounds in the

adult rhesus treated with physiologic saline had a comparable tensile strength to the CLDD treatments. Data from the 10-day geriatric cohort, although not statistically significant, suggested an increase in wound tensile strength with CLDD. However, at 15 days, the geriatric rhesus treated with CLDD had a statistically significant 36-percent increase in wound tensile strength above physiologic saline treatment. We hypothesize this is the result of delayed wound healing in the aged rhesus. That is, a temporal delay in appearance of wound-healing elements required additional time to enable CLDD efficacy. Therefore, with additional time, the effectiveness of the CLDD was realized. Consequently, by day 15, the beneficial affects of CLDD were apparent with the geriatric population, whereas the positive influences of CLDD were detected by 10 days for the adult population.

Histologically, for 10- and 15-day wounds, the epidermal and dermal layers of the CLDD and physiologic saline cohorts seemed to be indistinguishable. There were macrophages and multinucleated giant cells associated with CLDD remnants, with occasional lymphocytes, neutrophils, and monocytes in the dermal stroma. The histologic impression was consistent with a low grade, localized reaction around the CLDDs. Grossly, the incisional wounds treated with CLDD seemed clinically normal.

Mechanistically, cutaneous wound-healing enhancement by CLDDs is unclear. It is recognized that macrophages are highly important for cutaneous wound repair,²⁷ and it has been shown during dermal wound healing that electrical current plays a defining role in the directional migration of these cells and other phenotypes.⁴ Moreover, it seems that macrophages preferentially migrate toward a positive charge,^{11,28,29} and positively charged materials invoke a favorable wound-healing response in rat incisional wounds.¹² It is well known that expression of macrophage-derived growth factors stimulate fibroblasts and optimize the quantity and organization of collagenous stroma.^{6,8,30-32} Although the tensiometric data clearly portrayed the superiority of the CLDD to physiologic saline, histologic observations did not present a striking cellular and morphologic picture at any of the temporal periods. There was a decrease in charged beads from days 5 through 15, and they were absent by 2 months. Similarly, by histologic observation,

the number of macrophages and giant cells diminished with the decrease in CLDD beads.

An additional observation was that during injury repair, macrophages were found in the dermis. Moreover, topically applied CLDD particles localized to the dermis. As a consequence of the colocalization, macrophages may recognize CLDD as "foreign" and initiate a cascade of activities to remove the beads. When the macrophage response is insufficient to accomplish this deed, development of a multinucleated phenotype (i.e., giant cells) ensues to accomplish the task. Furthermore, attachment of macrophages to CLDD beads could alter the shape of the cell^{2,33,34} (i.e., mechano-transduction) and promote expression of vulnerary growth factors to promote cutaneous healing. However, it is not clear why positive charges preferentially attract macrophages and why and how the macrophages are activated by positive charges. No attempt was made in this study to elucidate the mechanisms and signaling factors responsible for the enhancement of cutaneous wound repair in response to the CLDD. Future studies will address the mechanistic components of this highly significant phenomenon.

In summary, at 10 days, incisions in adult rhesus treated with CLDD had a 30-percent increase in tensile strength compared with saline treatment ($p = 0.01$), whereas for geriatric rhesus, the CLDD treatment provided a 15-percent increase in tensile strength compared with the physiologic saline ($p = 0.11$). By day 15, adult rhesus had a 26-percent increase in tensile strength compared with saline treatment ($p = 0.07$), and the difference was 36 percent ($p = 0.02$) for the geriatric rhesus. From 5 through 15 days, a gradual decrease in quantity and integrity of CLDD was observed histologically. No remnants of CLDD were detected at either 2 or 4 months. Therefore, data suggest that CLDD can enhance significantly the tensile properties of healing cutaneous wounds in both the adult and geriatric rhesus. Moreover, if the wound healing is enhanced in geriatric patients, this finding may be clinically germane to conditions where incisional cutaneous wound healing is compromised, such as in diabetics and patients on steroids.

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