ELECTRO- AND MAGNETOBIOLOGY, 19(1), 1–19 (2000)

EFFECTS OF ELECTRICALLY GENERATED SILVER IONS ON HUMAN CELLS AND WOUND HEALING

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ABSTRACT

A method of producing local antibiotic effects by means of an iontophoretic technique using free silver ions has been evaluated *in vitro* and *in vivo* for more than two decades. The antibiotic properties of the technique have proved useful in both animal and human studies. In the course of determining the optimal clinical methodology for infected open wounds, a significant growth stimulation property resembling local tissue regeneration was noted. This has been traced to either the apparent production of dedifferentiation of normal mature cells or the stimulation of preexisting stem cells in the wound, resulting in the production of large numbers of progenitor cells. This process has now been studied in detail, and the results are presented herewith.

INTRODUCTION

The sparse circulatory pattern of bone makes it difficult to achieve an adequate level of systemic antibiotic in the treatment of osteomyelitis, and such infections have been notoriously difficult to treat. It appeared that a broad-spectrum local antibiotic with the capacity to infiltrate bone without relying on simple diffusion from a circulating drug level would be useful, and an iontophoretic technique was considered. Silver ions have been known to be a useful antiinfective agent since the late 1880s, when Crede (1) introduced the use of silver nitrate for the prophylaxis of gonorrhea opthalmicum. Silver ions are produced in limited numbers from such compounds or from silver foil; a metallic silver electrode made positive (anode) in a simple DC electrical circuit and applied to an open wound should emit such ions in larger numbers and, additionally, cause them to

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migrate along the voltage gradient into the exposed tissues. Although this procedure is not classical iontophoresis, it appeared it might be adaptable for clinical use.

Beginning in 1973, *in vitro* studies demonstrated that such ions were an effective antibiotic with a very broad spectrum and favorable quantitative evaluations compared with synthetic antibiotics (2–5). Animal studies indicated equal efficiency in experimental osteomyelitis in rabbits with no acute deleterious side effects (6). In all studies, precautions were taken to avoid overt electrolysis, which would have introduced erroneous results *in vitro* and produced tissue damage in clinical use. The level of voltage required to produce measurable electrolysis in human soft tissue with silver wire electrodes was determined to be approximately 1.1 V (7). Accordingly, applied voltage in all studies was limited to 0.9 V or less.

The first clinical studies were cautiously begun in 1975 using a variety of silver materials including silver wire, silver foil, and a commercially available silvered nylon fabric. A standardized technique was evolved using the silver nylon fabric placed as the anode in open osteomyelitic lesions with the application of 0.9-V DC between the fabric and a cathode of carbon-impregnated rubber. The electrical current was provided by a simple battery-operated DC generator limited to 0.9-V output. Initial treatments were limited to 4-h periods twice a day. As bacteriologic monitoring revealed useful results and no overt local or systemic side effects were noted, the treatment time was gradually extended to be continuous, with once daily electrode removal and replacement with a new silver nylon anode. Detailed results of these initial studies have been reported (8,9), and the technique was found to be a useful and safe clinical treatment for recalcitrant, open osteomyelitis (10).

As the clinical study progressed, improvements in electrical generator design and the silver nylon fabric were made, with a resulting increase in the antibacterial effect and the appearance of several unexpected clinical phenomena.

A profuse, sterile exudate began to appear in the wound during the second and fourth day of treatment (Fig. 1). Shortly thereafter it became evident that an enhancement of wound healing was occurring beyond that which could be attributed to infection control. Open cavities began to fill with abundant, healthy granulation tissue, producing rapid coverage of exposed bone surfaces. This was followed by the rapid ingrowth of full-thickness, innervated skin and healing of bony non-unions. These observations have been under evaluation since 1979.

MATERIALS AND METHODS

In Vitro Study

Cultures of granulation tissue from human wounds were grown using Dulbecco's modification of Eagle's basic medium with 10% fetal calf serum, HEPES buffer, and nonessential amino acids at 37°C in a positive-pressure clean bench. No antibiotics or growth factors were added to the medium. Plastic Petri dishes (10 cm; Carolina Biological Supply, Burlington, NC) were used for routine cultures. Glass microscope coverslips were placed in each dish for cell adhesion and recovery. Cultures were examined daily using phase contrast microscopy with an Olympus CK inverted microscope equipped for photomicroscopy.

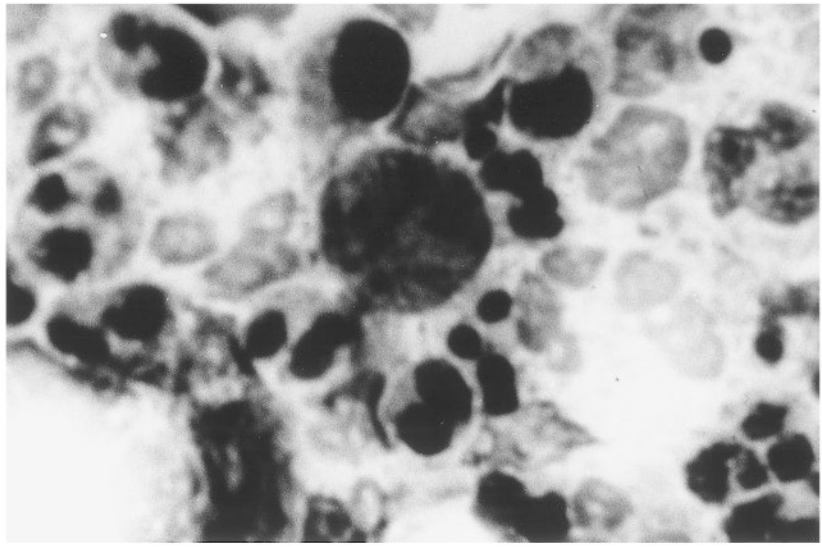


FIGURE 1. Smear of exudate from wound treated with iontophoretic silver for 9 days and stained with Wright's stain. A number of large mononuclear cells with deep basophilic staining are visible. (Original magnification ×200.)

Photographic Methods

Phase contrast photomicrographs were taken with an Olympus OM1 camera on Kodak Technical Pan film developed by appropriate chemical techniques. Standard photographic techniques were used for printing, and no digital manipulation was employed. Unless otherwise noted, 35-mm negatives were enlarged only to fill-frame 5 × 7-in. photographic paper. Cells were stained with either Giemsa-Jenner or Wright's stain using standard techniques.

Current Exposure

For controlled exposure of cells to electrical current, modified tri-well, 20-cm plastic dishes were used (Fig. 2). This modification permitted limiting the exposure of the cells in the positive chamber to the free silver ions emitted from the anode without also exposing the cells in the negative chamber. Both positive and negative chambers were exposed to the same electrical current and voltage drops, whereas the cells in the third, control chamber were not so exposed. Using this method, aliquots of 3T3 mouse fibroblasts (ATCC CCL 96) were seeded into each chamber and permitted to grow for 24 h, at which time 0.1 V was applied across the two silver electrodes for 24 h using a simple battery-operated DC generator. After an additional 24 h of incubation, the coverslips were removed, and the cells were fixed and stained.

Clinical Study

All patients were advised of the experimental nature of the treatment and told that they could terminate treatment any time they wished. The entire treatment method was

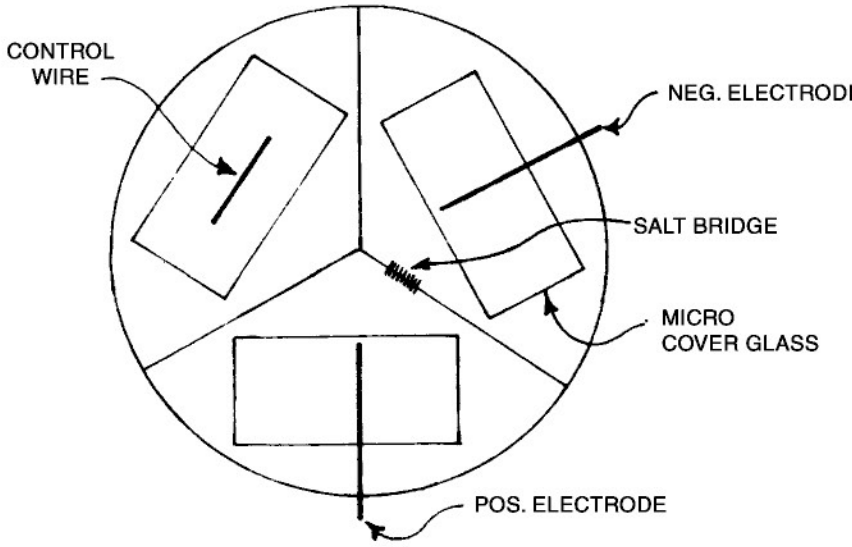


FIGURE 2. Modified plastic tri-well Petri dish providing for exposure of cells separately to positive and negative electrical environments as well as a control, nonelectrical environment. In this study, the electrodes were 99.99% pure silver and were placed over each glass coverslip. Cells in the positive chamber were exposed to the free silver ions given off by the silver anode; these ions were excluded from the negative chamber.

fully demonstrated and explained; if it was physically possible, the patients were required to perform their own daily dressing changes under close supervision. This protocol received Institutional Review Board approval to comply with the ethical guidelines of the 1975 Declaration of Helsinki.

Initially, all treatments were given as described in detail in prior publications (8–10) Further laboratory evaluation of the treatment method and the materials has resulted in some alterations. The electrical generator has been changed to provide a variable voltage scalable to the total treatment area with 0.2 V applied to wounds of 1–2 in. square and linearly increasing to a maximum of 0.9 V applied to wounds of 10 in. square. The silver nylon used as the anode is now manufactured by Omnishield (Clarks Summit, PA). This fabric contains no trace elements by X-ray spectroscopy. The silver deposit is unique in nature, consisting of silver crystals averaging 50 nM in diameter, (11).

Infected, traumatic wounds do not lend themselves well to objective analysis such as case-controlled studies, and no such attempt has yet been made. However, in ten cases of open, infected wounds containing a non-united fracture, a modified open bone grafting technique was used, which permitted a semiquantitative analysis of granulation tissue growth rate. This surgical treatment (12) normally involves converting the existing wound into an ovoid saucerization extending down to and exposing all involved bone and adjacent soft tissue. This wound is then packed with bone graft material and permitted to heal slowly with no specific local treatment other than daily dressing changes. The clinical success rate of this procedure averages less than 50%, and the maximum growth rate of granulation tissue is between 0.15 and 0.2 cm^2/day (13).

In this series, treatment with silver iontophoresis was started immediately after sau-

cerization. The silver nylon anode was applied to the base of the wound covering the exposed bone and adjacent soft tissues only. Contact with the wound edges was avoided to prevent stimulating any amount of wound closure that would limit subsequent bone grafting. In addition, any ingrowth of soft tissue was debrided to maintain wound size. After all basal soft tissues and bone were completely covered with a layer of granulation tissue, the entire wound was packed with cancellous bone grafts, and the silver anode was immediately reapplied, now over the entire wound, including the bolus of bone graft and skin edges. Treatment was continued until all exposed tissues were again covered with granulation tissue and skin closure occurred. Since the primary surgical wound was deliberately made ovoid, measurements of wound area were reasonably accurate, and two determinations of rate of growth of granulation tissue were possible. These are reported as pregraft rate (the rate at which granulation tissue entirely covered the bone and base of the wound) and post-graft rate (the rate of granulation tissue coverage over the exposed bone graft material).

RESULTS

In Vitro Study

Wright-stained smears of the exudate from the silver-treated wounds (Fig. 1) revealed it to be hypercellular, consisting of 75–80% neutrophils with the remainder composed of pleomorphic, basophilic, primitive appearing large cells. The pathology report indicated that it resembled abnormal, active bone marrow with many blast forms but with many normal cell types missing. Since the patients treated were all over the age of 26 and the treatment sites had no access to any active bone marrow, it appeared possible that these cells were being produced by the dedifferentiation of some preexisting mature cell type in the wound. Since fibroblasts are the commonest cell type in such wounds, their reaction to electrically generated silver ions *in vitro* was determined using modified, triwell Petri dishes (Fig. 2).

Prior to the silver ion exposure, the fibroblasts in all chambers were normal in morphology and firmly attached to the glass substrates. During the 24 hs of treatment, the cells in the silver anode chamber became round, separated from the substrate, and began clumping together as free-floating masses with a variety of blast-like morphologies similar to those observed in the exudate. Over the following 24 hs, the clumping became more evident while cellular morphology remained constant (Fig. 3). Fibroblasts in the cathodal and control chambers demonstrated no morphologic or functional changes.

Exposure of similar cultures to the same electrical parameters administered via a variety of other metallic electrodes (gold, platinum, copper, stainless steel) resulted in either negligible cytologic alterations or direct toxic effects in the positive chamber.

Clumped cell groups from the silver anode chamber were transferred to standard plastic Petri dishes and cultured for several weeks. These cells remained apparently viable but totally quiescent during this period of subculture, demonstrating no morphologic alteration or mitotic activity. At the end of 2 weeks they all gradually reverted back to typical 3T3 fibroblast morphology and resumed their standard rate of mitotic activity.

In an attempt to obtain a few human fibroblasts to confirm these observations, small (approximately 1-mm^2) explants of granulation tissue were collected from wounds treated with the silver technique for 1 week or more, from four male donor patients ranging in age from 30 to 42 years. The explants were washed in two changes of culture medium

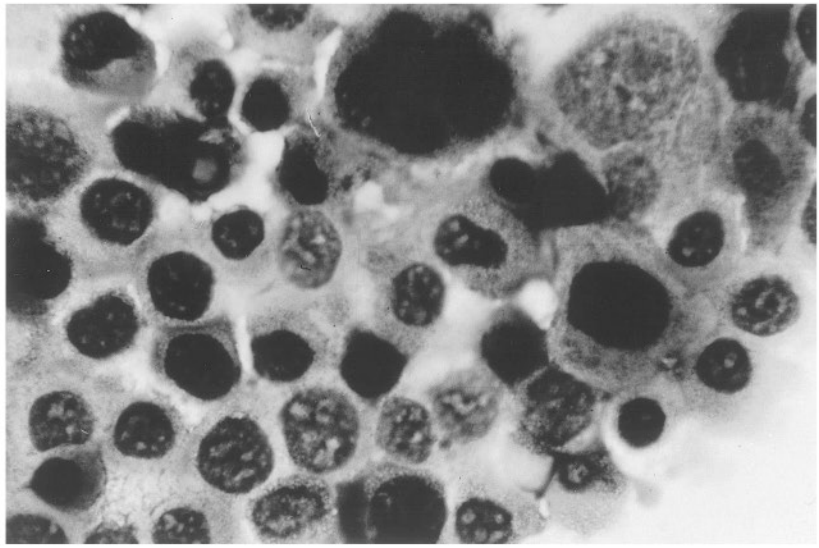


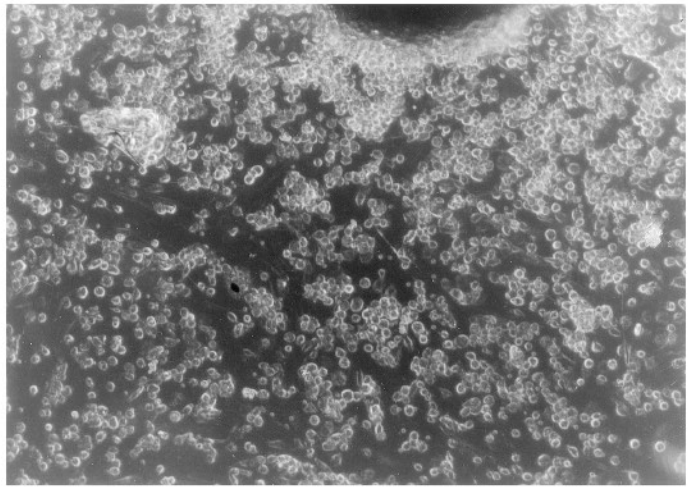
FIGURE 3. Culture of 3T3 fibroblasts exposed to electrically generated silver ions in a Petri dish, modified as in Figure 2, for 24 h. All cells are rounded and free-floating with basophilic nuclei. A number have morphology similar to that shown in Figure 2. (Giemsa-Jenner stain, original magnification ×200.)

and cultured, attached to glass coverslips, in 10-cm Petri dishes using a standard technique without applied electrical current. The cultures were incubated and examined daily by phase contrast microscopy. The production of large numbers of round, free-floating cells was observed, beginning between the second and third day of incubation. The growth started from the explant (Fig. 4A) and proceeded outward (Fig. 4B), until by the fourth to fifth week the glass slip was covered with a mass of tightly packed cells (Fig. 4C).

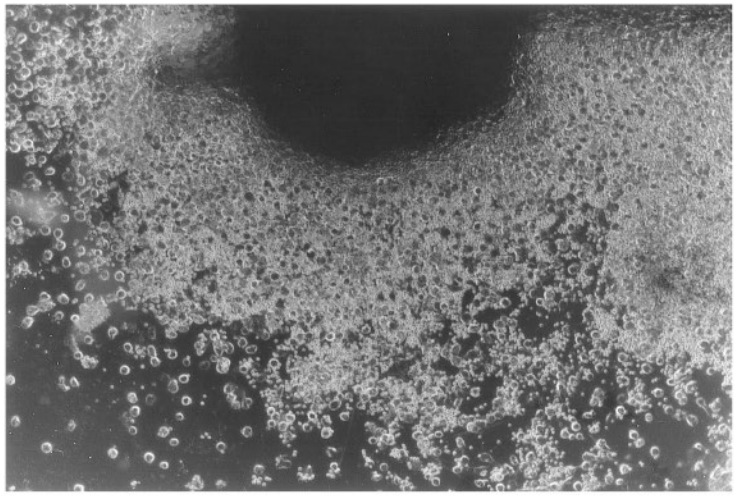
During the the first 2 weeks of incubation, most of these cells were round and mononuclear, with active appearing nuclei; they were also variable in size and motile when viewed with phase contrast (Fig. 5). These cells were strongly basophilic with Wright's or Giemsa-Jenner stain (Fig. 6). No mitotic figures were observed, and amitotic divisions were rarely seen.

Individual round cells were observed to expel their nuclei along with a thin rim of cytoplasm (Fig. 7). Many of these ''free'' nuclei were observed to become actively motile with ruffled borders, pseudopodia, or filopodia often forming large, free-floating clumps

FIGURE 4. (A) Culture of granulation tissue from 42-year-old patient treated for 14 days with iontophoretic silver. Culture is 6 days old. A fairly uniform population of round cells may be seen radiating out from the explant at top center. (Phase contrast, original magnification ×40.) (B) Same culture at 12 days. The density of cells in the vicinity of the original explant is greater and spread further out into the culture. (Phase contrast, original magnification ×40.) (C) Same culture at 32 days of incubation. The cell population is dense throughout the field. The original explant is not shown as the cell population in that area was too dense for good definition. A number of normal-appearing fibroblasts are visible. (Phase contrast, original magnification ×40.)



(A)



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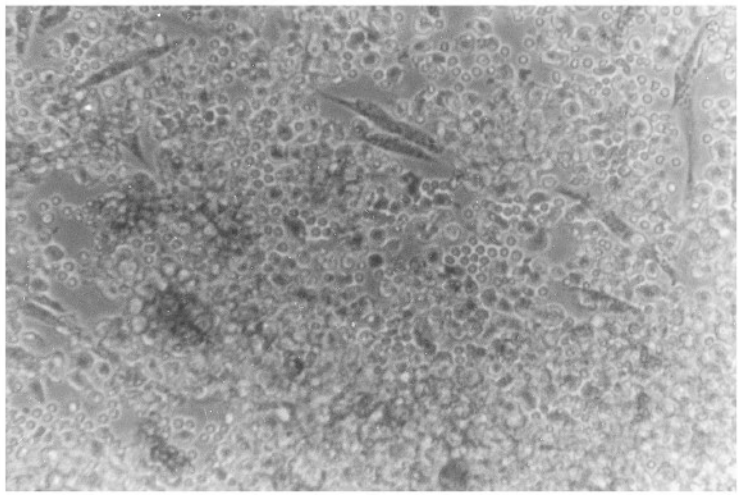

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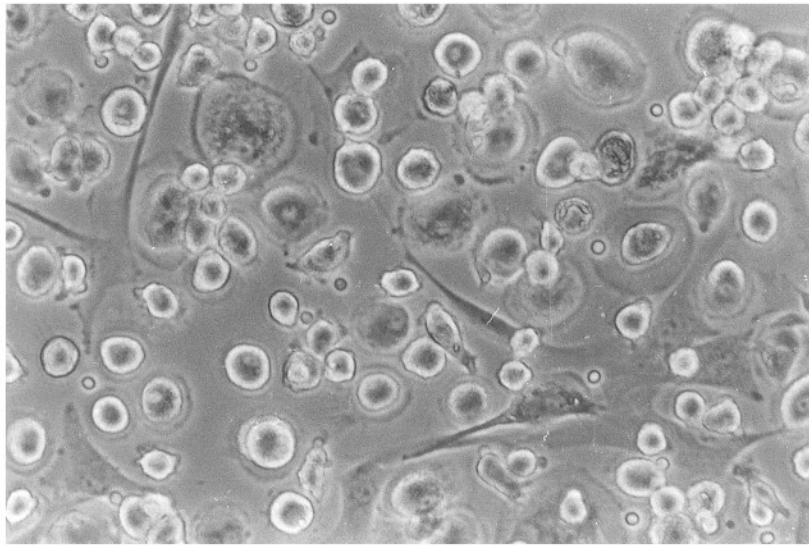

FIGURE 5. Granulation tissue culture from 36-year-old patient treated with iontophoretic silver for 12 days. Culture is 6 days old. Most cells are mononuclears, with variable nuclear-cytoplasmic ratios. A number of large mononuclear cells and a few normal-appearing fibroblasts are visible. (Phase contrast, original magnification ×100.)

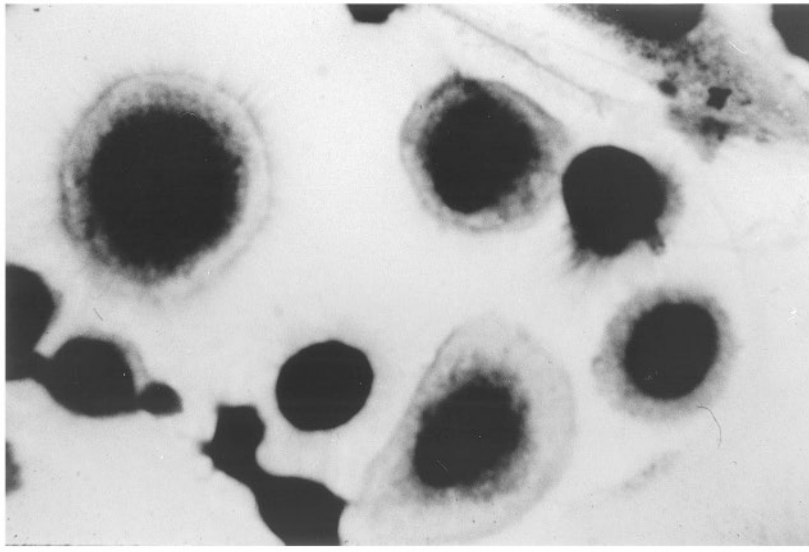

FIGURE 6. Giemsa Jenner-stained cells from granulation tissue culture from a 32-year-old patient treated with iontophoretic silver for 12 days; the culture was 8 days old. Since all cells were free-floating, many were lost in processing. A portion of a relatively normal fibroblast is visible in the upper right corner. Several large mononuclear cells are also visible. One such in the upper left shows several filopodia. The remainder of the cells are ''free'' nuclei with only a thin rim of cytoplasm. One such, immediately below the fibroblast, also demonstrates a few filopodia. (Original magnification ×200.)

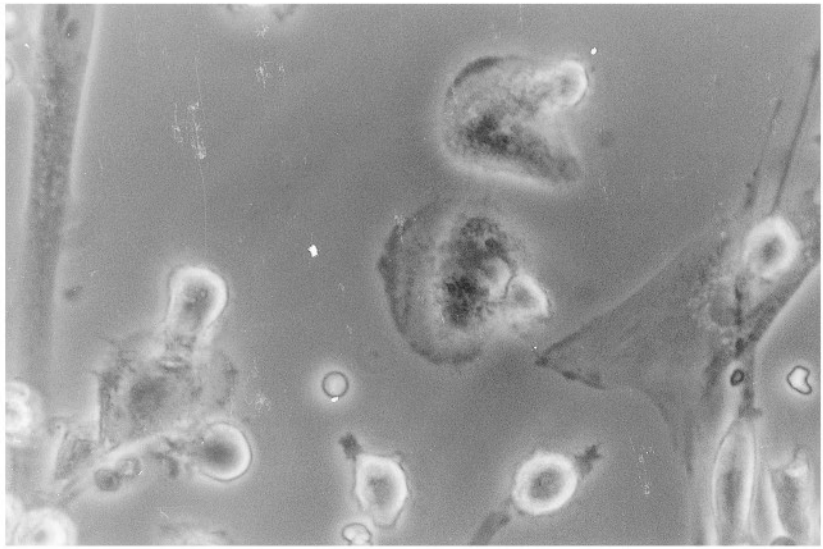

FIGURE 7. Granulation tissue culture from a 32-year-old patient treated with iontophoretic silver for 12 days; the culture is 6 days old. At top center a cell in the process of expelling its nucleus is visible. At the center a larger cell is being penetrated by a free nucleus. To its left lower border a small, nonstructured vesicle is visible. This is apparently the residual cell membrane and contents from a cell that has already expelled its nucleus. At the left lower border, another larger cell is being penetrated by two free nuclei. The free nucleus at the upper border of the larger cell is actively penetrating the host cell while the other free nucleus is contacting the host cell's membrane with multiple pseudopodia. (Phase contrast, original magnification ×200.)

(Fig. 8). The free nuclei were observed to contact other cells actively, to attach to their membrane, and to enter the host cell (Figs. 9 and 10). This activity resulted in the penetration of many cells by multiple nuclei, which then formed large nuclei within large host cells (Figs. 9 and 10). After the first week of culture, phase contrast observations of any individual active culture showed all these activities ongoing at the same time; hence many of the figures show multiple activities. With continuous observation, it appeared that filopodia formation preceded cell-cell contact and was then followed by pseudopod formation on the part of the motile free nucleus. The larger cell contacted the smaller one and then developed flattening of its membrane at the point of contact, followed by the appearance of a pit on the membrane and shortly thereafter penetration of the larger cell. Various aspects of this behavior may be seen in Figures 7–10.

At the end of approximately 4 weeks, all new cell production ceased, the remaining cells all slowly reverted to normal fibroblast morphology over the following week to 10 days, and all activity ceased. If maintained in culture these cells continued in a totally quiescent state, and over the following approximately 2 weeks all appeared to undergo senescence and die.

If, during the earlier stages of the growth phase, the original explant of granulation tissue was removed from the culture, new cell production stopped, with similar reversion to normal fibroblast morphology. If the removed explant was immediately placed in a new culture medium, the production of new cells again occurred but on a shorter time scale and with less cell production.

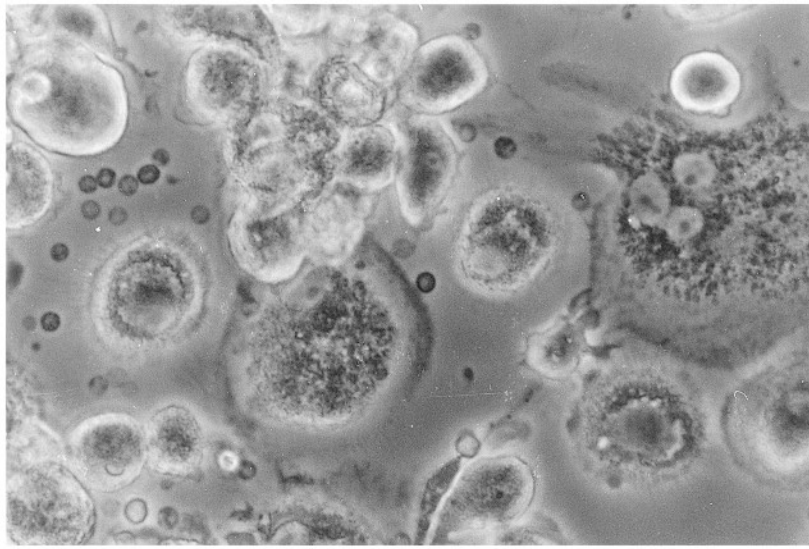

FIGURE 8. Same culture as in Figure 5 at 14 days. A clump of previously expelled nuclei is visible at the top center. Immediately below it is a large cell with what appears to be a poorly organized single nucleus. At right center an even larger cell is visible with a large nucleus containing several newly penetrated free nuclei. At center left several small vesicular structures residual from the nuclear expulsion are visible. (Phase contrast, original magnification ×200.)

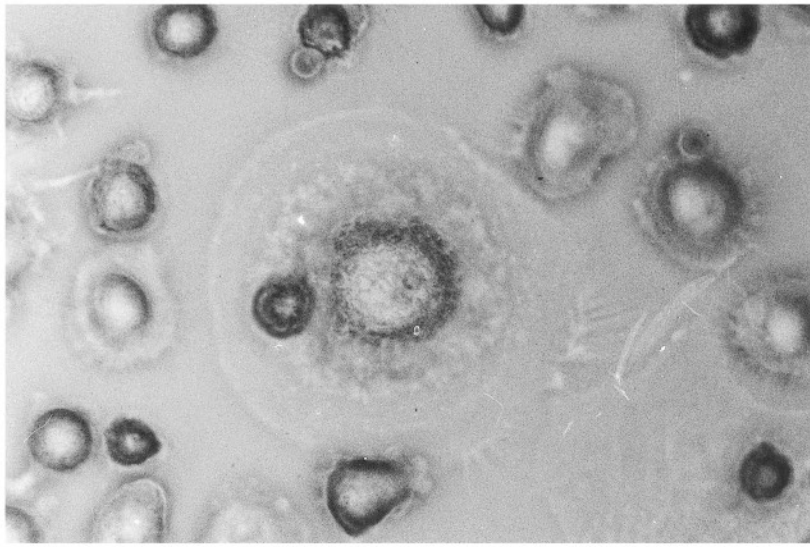

FIGURE 9. Granulation tissue culture from a 43-year-old donor treated with iontophoretic silver for 16 days. Culture age is 11 days. A large cell with a central well-organized single large nucleus is visible at the center field. Two free nuclei are visible immediately adjacent to it at 1 o'clock. Both display ruffled borders, and one appears to be beginning to penetrate the large cell, which displays a flattening of its membrane in the area. A number of filopodia are visible radiating from the membrane of the large cell immediately below this flat area. A third free nucleus with ruffled borders is in contact with the large cell and is visible at 6 o'clock. The free nucleus adjacent to the nucleus of the large cell was superimposed on it and is not internal. (Phase contrast, original magnification ×200.)

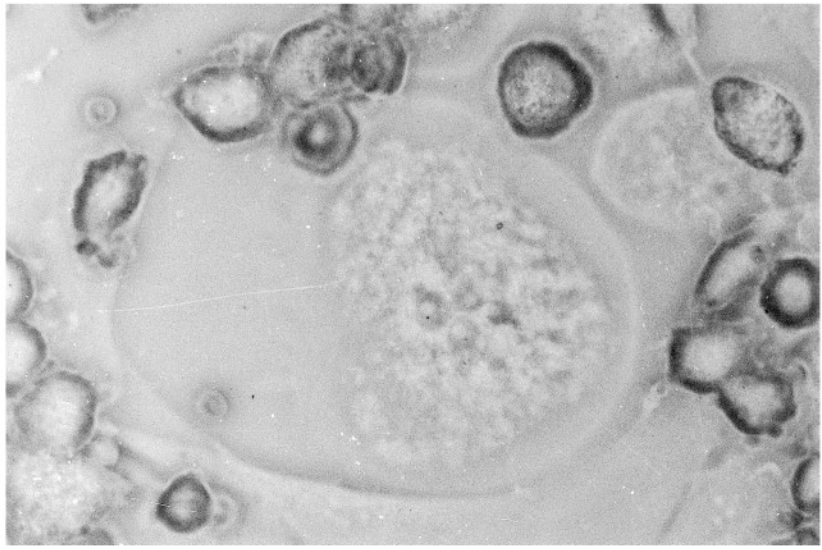

FIGURE 10. Culture of granulation tissue from a 32-year-old patient treated with iontophoretic silver for 20 days; the culture age is 14 days. A very large cell with a large, poorly organized, excentric nucleus is visible. Several smaller free nuclei are in the process of penetrating the large cell. One such, located at 9 o'clock on the large cell, shows a well-defined ruffled border, one pseudopod of which is actively penetrating a well-developed pit on the large cell's membrane. (Phase contrast, original magnification ×200.)

Explantation of small aliquots of the cellular wound exudate itself into culture media yielded no cell multiplication, mitotic or amitotic. The cells began degenerating soon after explantation and within a week the culture was essentially devoid of living cells.

Clinical Study

Over 100 patients have been treated with silver iontophoresis with no local or systemic side effects, including argyria, being observed. The subgroup of ten patients on whom open bone grafting was done permitted an analysis of the rate of granulation tissue growth per day with the silver therapy. Except for case #10, all had chronic, open osteomyelitis with bone non-unions. All healed satisfactorily with healing of the non-union and return of function. Two cases had a small, residual, intermittently draining sinus due to small, retained sequestrae that did not impair function. Wound size remained constant during the pre-graft period as granulation tissue growth was limited to the base of the wound and the exposed bone. Rates of granulation tissue growth were calculated for the pre-graft and post-graft periods as noted in the Methods section (Table 1). As noted, the average rate for the pre-graft period was slightly more than 1 cm^2/day, and that for the post-graft period was slightly less than 1 cm^2/day. These rates should be contrasted with the average rate of granulation tissue growth in non-silver-treated open bone grafts of 0.1 cm^2/day (13).

Table 1. Granulation Tissue in Patients Treated with Open Bone Grafts and Silver Ion Iontophoresis[a]

Patient no.	Age (yr)	Clinical data	Wound size (cm^2)	Days to complete granulation tissue coverage		Rate of coverage (cm^2/day)	
				Pre-graft	Post-graft	Pre-graft	Post-graft
9	52	Osteomyelitis, non-union, femur intermittent, drainage 30 yr	50	38	48	1.3	1.0
10	46	Retained intraosseus shrapnel, tibia intermittent drainage 20 yr	15	19	16	0.8	0.9
11	31	Osteomyelitis, non-union tibia intermittent drainage 10 yr	32	21	35	1.5	0.9
12	64	Postoperative osteomyelitis tibia intermittent drainage 7 yr	16	41	14	0.3[b]	1.1
14	25	Osteomeylitis, non-union tibia, intermittent drainage 3 yr	24	12	35	2.0	0.7
19	55	Osteomyelitis non-union tibia drainage 30 yr	20	14	21	1.4	2.0
20	61	Osteomyelitis, non-union tibia drainage 21 yr	30	47	46	0.6	0.6
23	30	Osteomyelitis, non-union tibia drainage 9 yr	40	56	48	0.7	0.8
24	42	Postoperative osteomyelitis non-union tibia, drainage 14 months	35	27	48	1.3	0.7
28	18	Postoperative osteomyelitis delayed union tibia, continuous drainage 4 months	45	52	48	0.8	0.9

[a]Only cases that had a follow-up time of 1 year or greater are included. Prior to entering this program all cases except #10 and #28 had several attempts at debridement and either metallic fixation or bone graft with no success. At last follow-up all cases demonstrated bone union sufficient for full weight bearing. Cases #9 and #14 had persistent drainage from a residual sinus tract after skin healing.

[b]Granulation tissue growth in the pre-graft period was delayed by the extrusion of several sequestrae from the wound.

DISCUSSION

The growth-promoting effect of the silver iontophoretic technique is substantiated by the measured rates of growth of granulation tissue in the open bone graft series, which were approximately five to six times faster than that reported for non-silver-treated grafts. It should be noted that granulation tissue growth in normal open wounds begins at the wound periphery and proceeds toward the center, with only a minor contribution from open soft tissue and none from the exposed bone. When the silver iontophoretic treatment is added, all the soft tissues as well as the vascular supply of the bone become major contributors of granulation. As a result, the establishment of a ''lawn'' of granulation tissue over the entire wound occurs much earlier.

Previous reports on the clinical use of electrically generated silver ions for their antibacterial properties, (8,9) indicated evident accelerated granulation tissue growth, although this was not quantified at the time. This and the associated rapid healing of bone

non-unions were unexpected, because the concepts then current held that a positive electrical polarity at a wound site was associated with a diminution or actual cessation of growth, whereas an electrically negative environment was required for normal growth (14,15).

All other attempts at clinical utilization of a wide variety of electrical parameters as wound healing enhancers have been disappointing (16). The use of a silver material as the positive anode appears to be the sole exception. The clinically observed accceleration of granulation tissue, skin, nerve, and bone growth is probably attributable to some action of the silver ions, possibly in concert with the applied electrical voltage.

Several other workers have reported increases in growth rates with the use of silver anodes in a variety of experimental wounds. In 1983, Alvarez *et al*. (17) reported a significant increase in the healing rate of dermatome-produced wounds in mini-pigs using silver nylon anodes, but they ascribed this result to the passage of the electrical current alone.

Subsequently, Chu and his colleagues (18–22) reported on an extensive series of experimental animal burn and surgical wounds treated with silver nylon anodes using a slightly different technique. Their electrical generator was current controlled rather than voltage controlled (as in this study), and the silver nylon anodes were sutured in place over the wound for the duration of the experiment. The use of animals, however, enabled them to assess the results of the treatment with histologic examination, data unobtainable in clinical studies.

They first (18) noted a significant antibacterial effect, which they attributed to the introduced silver ions, but they made no observations of growth enhancement. Their second report (19) noted rapid reepithelialization of silver-treated scald wounds compared with untreated controls and the ability to use regrown skin from silver-treated wounds repeatedly as donor skin grafts. Histologically the treated wounds revealed an essentially normal epidermal and dermal appearance with normal numbers of active hair follicles. Untreated control wounds revealed far fewer hair follicles and an extensive subepidermal inflammatory fibrotic reaction. Their 1991 paper (20) reported on the healing rates of silver-treated split-thickness skin grafts on second-degree burn wounds. They noted that skin grafts on control animals required at least 7 days to become partially adherent and 14 days to become fully adherent. Silver-treated grafts began to adhere on day 1 and were fully adherant by day 4. Histology demonstrated a nearly normal appearance of the treated grafts, with a normal number of hair follicles, whereas control grafts showed subepidermal fibrosis and only a few hair follicles. A 1995 paper (21) reported enhanced survival of meshed composite skin grafts with the silver treatment compared with non-silver-treated control grafts. This paper also presented the results of a comparison between identical scald wounds treated with silver anodes or with simple dressings of silver nylon (without electrical current) and control wounds treated with vaseline gauze dressings. Wound size and contraction were practically identical with both silver treatments, and both were equally superior to Vaseline gauze dressings alone. A 1996 paper (22) reported that silver fabric used either as an anode or cathode and applied immediately after the experimental burn significantly reduced wound edema There were no observations on wound healing included in this report.

While Chu and his colleagues contend that their results are produced by the electrical current alone, their own data (21) appear to refute this concept. Despite these differing opinions on the responsible agent, the histologic data reported by Chu and his colleagues support the observations of accelerated growth and tissue regeneration with silver anodes observed in the clinical series reported herewith.

However, Chu *et al*. have not reported the production of exudate or any evidence

of cellular effects. This may be due to the differences in technique or the fact that they did not search for cellular events during the healing phase, centering their histologic studies on the outcome rather than the process.

While it is postulated that the evident enhancement of healing is the direct result of the cellular changes reported in this paper, these changes raise many questions. Are they simply the result of a toxic effect of the electrically generated silver ions? Do they represent the clonal expansion of preexisting stem cells in wound tissue or are they actually the result of a dedifferentiation process induced by this agent? Finally, are they the result of the electrical current alone, the silver ions alone, or a combination of the two?

The 3T3 fibroblast study provides some answers to several of these questions. First, the alteration from typical fibroblast cells firmly attached to the substrate to a population of free-floating cells with typical blast cell morphology would appear to indicate their apparent dedifferentiation. If this is so the period of cellular inactivity following the exposure may be ascribed to the lack of inducer signals in the simple cell culture environment. The subsequent resumption of normal morphology and activity indicates the lack of any lasting toxic effects from the silver ions. The failure of other nontoxic metal ions to produce a similar alteration with the same electrical parameters strongly indicates that the electrically generated silver ion is the agent responsible for the observed cellular changes.

While the fibroblast results indicate the possibility that a similar dedifferentiation process occurs in the silver-treated wounds, the possibility of a clonal expansion of preexisting stem cells cannot be ruled out. It is now known that progenitor and pluripotent stem cells are present in muscle (23), bone and bone marrow (24), and fibrous tissue (25). Most recently Young *et al*. (26) have identified a number of bioactive factors that influence these cell populations, with platelet-derived growth factor-BB (PDGF-BB) being the most potent stimulator of their proliferation. It is possible that the electrically generated silver ions produce the same result through some similar action or via the stimulation of production of PDGF-BB or some similar agent. However, it would appear unlikely that any agent similar to a growth factor was produced in the granulation tissue cultures, since they received no treatment other than media changes.

The rapid and extensive production of new cells in the granulation tissue cultures was unexpected. The morphology of the cells produced was similar to that of human hematopoetic (27) or embryonic stem or progenitor cells (28) produced in culture by a variety of other methods. The large number of cells produced appears to exceed the cell population present in the original explant and may be compatible with an *in vitro* clonal expansion of either newly dedifferentiated cells or preexisting stem cells.

It would appear reasonable to postulate that the cell behavior noted in the granulation tissue cultures is also present in the treated wound itself. If so, it is highly unusual and appears to have little relationship to that observed in normal amphibian blastema formation. Such events as ejection of the nucleus could superficially be ascribed to a toxic effect preliminary to cell death. However, the survival of these ''free'' nuclei, and the accompanying perinuclear cytoplasm, is evident by their high level of activity while in culture.

The appearance of filopodia and the apparent intercellular ''communication'' as well as the merging of the ''free'' nuclei to form larger cells with a single large nucleus and the subsequent penetration of these cells by additional ''free'' nuclei (which results in very large, single cells with the DNA content of multiple nuclei) would appear to be of value in subsequent tissue reconstruction. The eventual return of these cells to normal fibroblast morphology in culture is attributed to the lack of any ''instructions'' in the

culture medium. Although these activities are abnormal, some analogous cell behavior in nucleated erythrocytes has been noted in heart regeneration in salamanders (29), a process that proceeds with great rapidity.

In these cultures, all new cell production was centered in and around the explant proper. Very few amitotic and no mitotic divisions were noted in any portion of the culture. It would therefore appear that new cell production was derived from a nidus of cells remaining in the explant. The cessation of new cell production following removal of the original explant from an active culture would appear to substantiate this. However, histology of freshly excised, washed, granulation tissue explants revealed a matrix of fibers, a few capillaries, free erythrocytes, and a few cells identical to those in the exudate. Histologic sections of explants removed at the time of cessation of new cell growth demonstrated primarily a matrix of collagen fibers with a simple infiltrate of cells similar to those in the culture media with no nidus formation. Lacking a nidus of competent cells, the clonal expansion dependency on the presence of the original explant appears to indicate an inductive factor associated with some, as yet unidentified, factor in the explant itself. The lack of this factor in the cultures of the cellular wound exudate alone may explain their failure to proliferate. The 3T3 cells ultimately resume normal mitotic activity at the end of the silver exposure, while the human granulation tissue culture cells die, possibly because the 3T3 cells come from a theoretically immortal transformed cell line, while the human cells have a limited life time.

The above observations appear to indicate that the cellular events in human wounds treated with iontophoretically introduced silver ions are not the result of a toxic effect or of the activation of some growth factor or equivalent agent. It appears probable that the cells produced represent either the activation of a preexisting stem cell population or, based on the 3T3 fibroblast study, more likely the dedifferentiation of mature cells in the wound.

What role, if any, the electrical factors play in these processes is unclear at this time. There is, however, a considerable body of evidence indicating a relationship between DC electrical currents and both spontaneous and induced regeneration in a variety of species.

Initially, Becker (14) reported that electrical potentials in regenerating salamander limbs had a different polarity compared with scarification healing in frog limbs. Smith (30) subsequently reported stimulation of a remarkable level of limb regeneration in adult frogs by applying a negative polarity current to the amputation stump. He later reported (31) that, also in the adult frog, electrode placement in accord with significant structures in the original embryonic development of the limb was maximally effective in stimulating regeneration. Becker and Murray (32) reported the production of cytologic changes compatible with dedifferentiation in frog nucleated erythrocytes exposed *in vitro* to a very low range of DC current. Harrington and Becker (33) later reported that these electrically changed cells demonstrated significant increases in RNA and protein content, and that both the morphologic and chemical changes could be inhibited by puromycin or cyclohexamide. Later, Becker and Murray (34) reported that fractures of the long bones in frogs demonstrated a negative polarity along with apparent dedifferentiation of the erythrocytes in the fracture hematoma. These cells later formed the bone ''callus'' (a blastemal structure) responsible for healing the fracture.

In 1972, Becker (35) and Becker and Spadaro (36) reported a modest amount of regeneration in forelegs of weanling rats amputated through the mid-humeral level and exposed to negative polarity, very low-voltage electrical current from a platinum cathode.

Organized, multitissue growth to the distal humeral joint was noted; however, the regenerate progressed no further than the joint line and resembled an aborted attempt possibly due to insufficient blastema formation.

In the only study reporting spontaneous regenerative growth in human appendages, Illingworth (37) noted that amputated fingertips in children younger than 4 years regenerated if the wound was not sutured closed. Later, Illingworth and Barker (38) noted that electrical measurements during this regrowth were similar to those reported during regeneration of salamander forelegs. More recently, Angrist *et al*. (39) have reported that normal human dermal wound healing is accompanied by a biphasic DC current similar in polarity to that in salamander limb regeneration.

It would appear that negative electrical polarity plays an important role in amphibian regeneration by stimulating the cellular dedifferentiation that results in blastema formation. The role of a similar factor in mammalian healing processes is less distinct. The abortive limb regeneration produced in mice (35,36) by negative polarity may be ascribed to the stimulation of stem cells in the bone marrow. This appears likely because the negative platinum electrode was inserted directly into the marrow cavity.

The wound healing produced by the induction of a positive polarity in the wound using the silver iontophoretic technique appears to be entirely unrelated to all other types of regeneration, either normal or electrically stimulated. It may be based entirely on the cellular effect of the electrically generated silver ions and may not involve any of the ancillary electrical factors present in normal regeneration. It is evident that these observations provide far more questions than answers and that any attempt to understand fully the mechanisms involved would be speculative at this time.

Tissue regeneration remains a subject of intense interest in both basic science and clinical medicine. Of the many theoretical approaches to restoration of some measure of local regeneration in the human, Peacock (40), Gross (41), and Stocum (42) have contributed extensively in this area. However, despite much effort, to the best of the author's knowledge there have been no reports other than the present one of significant enhancement of normal growth rate or production of overt tissue regeneration in the human or other mammals.

CONCLUSIONS

Exposure of mammalian fibroblastic cells *in vitro* to free silver ions emitted from a silver anode results in their transformation into blast or stem cell morphology, which appears to be the result of a dedifferentiation process and not of a toxic effect. Exposure of normal human cells in wounds to the same electrically generated free silver ions results in the production of an exudate containing approximately 10–15% of cells with the same primitive morphologic characteristics. Healing rates in these wounds are significantly accelerated and are accompanied by enhanced healing of bone, soft tissue, nerve, and skin, with replacement of missing tissues by histologically normal tissues.

Explantation of granulation tissue from such wounds during the healing phase into standard culture media results in the production of large numbers of new cells, all with characteristics of primitive or stem cells. It is believed that this *in vitro* process is the same as that ongoing in the silver-treated wound. The responsible agent for these cellular effects is believed to be the electrically generated silver ion, although some ancillary effect of the iontophoretic current itself cannot be ruled out at present.

These observations are currently believed to represent either the dedifferentiation of mature human cells and their subsequent clonal expansion, or the production of an expansion of preexisting stem cells in the tissues by the action of iontophoretically introduced free silver ions. Regardless of the mechanism(s) involved, the treatment results in the *in situ* production of large numbers of primitive cells in human wounds with no evidence of toxic effects.

It is presently unclear whether these observations represent Stocum's criterion: "A great advance would be the ability to induce the production of progenitor cells in situ by dedifferentiation of differentiated cells" (43). However, should the observations reported there be confirmed, the ability to produce dedifferentiation and clonal expansion *in situ* of adult human cells would make possible a number of clinical applications not heretofore attainable.

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