It has been recognized that the rate of cutaneous wound healing declines with age, yet the molecular processes that affect this decline remain poorly understood. The purpose of this study was to compare reepithelialization and contraction rates, and growth factor profiles in full-thickness wounds in swine of various ages. Multiple full-thickness excisional wounds were created on the dorsum of 24-month-old (n = 2), 4-month-old (n = 2), and 2-month-old (n = 2) Yucatan Minipigs. The extent of reepithelialization was shown to decrease with increasing age in a manner that was statistically significant among the 2-month-old (79%), 4-month-old (48%), and 24-month-old pigs (22%). Enzyme-linked immunosorbent assay results showed that endogenous vascular endothelial growth factor concentrations in the 2- and 4-month-old animals peaked on day 4, reaching levels of 482 pg/ml and 420 pg/ml, respectively. In the 24-month-old pigs the vascular endothelial growth factor concentration peaked later (day 6), and was present at a lower level (229 pg/ml). On day 4 the vascular endothelial growth factor levels in the older pigs reached only 120 pg/ml, representing a four-fold decrease in concentration compared to the younger pigs. A comparison of platelet-derived growth factor-BB concentrations across the age groups showed similar patterns in the 2- and 4-month-old pigs (peaks of 77 and 91 pg/ml on days 2 and 3, respectively), and levels in the 24-month-old were below the sensitivity level (31.5 pg/ml) of the assay. Transforming growth factor-β1 levels across the age groups did not differ in a manner that was statistically significant, and all age groups peaked on day 9. Wound contraction showed no statistical differences among the age groups from days 3 to 9. On day 11, however, wound contraction in 2-month-old pigs was about 10% faster than in 24-month-old pigs (p < 0.05). These data suggest a possible new algorithm for treating wounds in aged skin, by which exogenous growth factors can be added to the wound microenvironment in doses and at times that match the growth factor profiles observed in wounds made in younger skin. (WOUND REP REG 2001;9:371–377)

People aged 65 or greater currently represent about 13% of the population of the United States; yet by 2030 they are expected to constitute 20% of the population, or seventy million individuals. These figures remind us that even small advances in our understanding and treatment of the commonly observed phenomenon of delayed healing in the wounds of the elderly have the potential to make a large impact on the lives of a great many individuals. Given the high morbidity and mortality that is frequently associated with wounds that fail to heal predictably in this population, it is disconcerting that the cellular and molecular mechanisms behind this delay in healing remain poorly understood.

Modern clinical comparisons of healing rates in older versus younger patients began with Carrell and DuNouy’s measurements of wound closure and contraction in 20-, 30-, and 40-year-olds during World War I. In 1932, Howes
and Harvey studied stomach wounds in rats, and noted that the “velocity” of fibroblast growth and wound strength was decreased in older rats.³

Since these early studies, investigators have continued to show that various aspects of the wound healing process are hindered by increasing age. As Swift et al. pointed out,⁴ delays in reepithelialization and decreases in collagen synthesis and organization have been studied in humans and rats by Butcher and Klingberg,⁶ Holt et al.,⁶ and Tan et al.⁷ Recent work by Khorramizadeh et al. indicates that a slower rate of collagen production, a greater collagenase activity, or expression of collagenase mRNA by aging fibroblasts could contribute to attenuated wound healing in elderly patients.⁸ Other investigations into age-related delays in healing have examined differences in blastema growth in juvenile versus adult rabbit-ear punch wound models,⁹ differences in healing in transcorneal freeze wounds in young and old rabbits,¹⁰ the prolongation of healing in split-thickness skin donor sites in patients over 60 years old versus younger patients,¹¹ and the relationship between age, inflammatory response, and cellular adhesion molecules.¹²

One of the more promising approaches to the study of wound healing involves the correlation of age-related changes to alterations in wound growth factor concentration.¹³⁻¹⁵ In our own investigation into this area, we utilized a swine wound model developed by this laboratory, in which external vinyl chambers were applied over full-thickness surgical wounds created on the dorsum of pigs.¹⁶⁻¹⁸ By injecting a saline solution containing antibiotics into the chambers, an incubator-like environment was created that has been shown to improve the rate of reepithelialization.¹⁷, ¹⁸ In the current study, on each of the first 11 days post-wounding, we analyzed fluid from multiple wounds in 2 pigs from each of 3 age groups (2, 4, and 26 months old). This model gave us the unique ability to accurately measure growth factor concentrations in the wound microenvironment on a daily basis, and with minimal disruption of the healing process. We were then able to profile the timing and extent of vascular endothelial growth factor (VEGF), transforming growth factor-β1 (TGF-β1), and platelet-derived growth factor-BB (PDGF-BB) release during the first eleven days of the healing process in each age group, and relate these findings to the extent of reepithelialization.

The chamber model has also been used as a means of delivering recombinant growth factors into the wound microenvironment,¹⁹ making it a potential tool for the treatment of wounds containing suboptimal levels of certain growth factors. Thus, in future studies we will utilize our analysis of the temporal profiles of growth factors in order to deliver them into the wound microenviron-

ment at doses that are optimal for each phase of the wound-healing process.

**MATERIAL AND METHODS**

Six domesticated female Yucatan Minipigs (Charles River Laboratories, Wilmington, MA) in three age groups (2 pigs per group) were used: 2 months old (7 to 9 kg), 4 months old (15 to 20 kg), and 24 months old (68 to 80 kg). Following arrival, the pigs were allowed to acclimate themselves to our animal facilities for 7 days prior to surgery. The pigs were penned individually to minimize the risk of chamber rupture. The environment was controlled to remain within a temperature range of 70 to 75°F, 65% humidity, and a light cycle of 12 hours on and 12 hours off. The animals were fed 1.25 to 2.2 pounds of standard swine diet, depending on age, and received an uninterrupted supply of clean, fresh water. Staff veterinarians examined the animals daily for illness or discomfort. A Panepinto sling (Britz-Heidbrink, Inc., Wheatland, WY) was used to hold the pigs during the initial surgery and for daily maintenance of the wound chambers and fluid. All animal procedures were approved by the Harvard Medical Area Standing Medical Committee on animals.

**Full-thickness wound preparation**

Pigs were given a combination of Tiletamine-Zolazepam (4–6 mg/kg I.M.) and Xylazine (2.2 mg/kg) as a sedative, placed in the Panepinto sling, and placed on 3L of oxygen. Intubation followed, and a mixture of oxygen (3L) and Isoflurane (1 to 3%) was administered as a general anesthetic. Pulse oximetry, rectal temperature, and respiratory rate were monitored. The porcine dorsum was clipped with standard fine-tooth animal clippers. Special care was taken to avoid mechanical trauma during the preparation process in order to avoid the activation of tissue repair mechanisms prior to experimental wounding. The skin was surgically prepared with successive applications of 7.5% providone iodine scrub, 10% providone iodine scrub, and 70% isopropanol. The skin surface was then defatted with trichloroethane. Each 2.5 cm² wound site was outlined with a sterile surgical marker and template. The number of wounds created on each pig depended on the animal’s size (6 wounds on the 2-month-olds, 8 wounds on the 4-month-olds, and 26 wounds on the 24-month-olds). The wound sites were evenly distributed over the dorsum of the pig. The outlines of the wounds were re-traced using a tattoo gun (Special Electric Tattoo Marker, Huck Spaulding Enterprises, Inc., Voorheesville, NY). The tattoo provided us with an indelible outline of the wounds that allowed us
to measure the extent of wound contraction on days 0, 3, 6, 9, and 11, and reepithelialization on Day 11. Multiple full-thickness excisional wounds were created using a "#11 scalpel. The number of wounds created on the animals of each age group was determined by the number of chambers that could be placed side-to-side in two rows on the dorsum. Thus, 6 wounds were created on the 2-month-olds, 8 wounds on the 4-month-olds, and 28 wounds on the 24-month-old pigs. Incisions were made immediately interior to the tattoo marks and to a uniform depth. A thin layer of medical adhesive (No. 7730, Hollister, Libertyville, IL) was applied to the skin surrounding the wound, and an adhesive-backed vinyl chamber (No. 689, PA Medical Corporation, Colombia, TN) was placed over each wound. The circular edges of the chambers were sealed with strips of Tegaderm (3M Health Care, St. Paul, MN). 2.5 ml of saline containing 100 units/ml of penicillin and 100 μg/ml of streptomycin were injected into each chamber using a 3cc sterile syringe and 25-gauge needle. Air pockets within each chamber were aspirated, and the injection site was sealed with cellophane tape. After recovering from anesthesia, the pigs were penned separately in custom-made, smooth-sided stainless steel cages.

**Daily animal maintenance**

After each 20- to 24-hour interval post-surgery through day 11, the pig was sedated and placed in the Panepinto sling. 3L/min of oxygen was delivered via a nose cone. Pulse oximetry, respiratory rate, and rectal temperature were monitored. The pig was examined for signs of wound infection (elevated body temperature, elevated respirations, putrid or cloudy wound fluid), or other illness. Wound fluid from individual chambers was collected daily and replaced with an injection of 2.5 ml of fresh saline-antibiotic solution into each chamber. All wound fluid was stored at ~80°C. If chamber leakage resulted in the collection of less than 2.5 ml of wound fluid, the sample for that wound that day was discarded. Chambers with leaks were removed with a medical adhesive remover (Hollister, Libertyville, IL), replaced, and sealed with Tegaderm. In addition, on day 3, 6, 9, and 11 post-surgery, all chambers were removed and the tattoo outlines of the wounds were traced using a sterile, clear plastic sheet and ultra-fine-point indelible marker. New chambers were applied and 2.5 ml of fresh saline-antibiotic solution was injected into the empty chambers in the manner described above. This maintenance schedule was followed until day 11 post-surgery, at which time the 2- and 4-month-old pigs were euthanized via an intravenous administration of 5 grams of thiopental sodium. The study of the 24-month-olds was continued until day 15, as the large number of wounds created in these animals allowed us to take an adequate number of biopsies at both day 11 and day 15. Uniform 5-millimeter-wide biopsy strips were taken from each wound in a cephalic-to-caudal direction that spanned the entire wound plus a margin of 5 mm of unwounded tissue on each end. The biopsies were immediately placed in formalin solution and sent to the Pathology Laboratory at the Brigham and Women's Hospital for routine histological processing with slides stained with Hematoxylin and Eosin.

**Growth factor assays**

To determine the levels of VEGF, TGF-β1, and PDGF-BB in the daily collected wound fluid of different age groups of pigs, equal amounts of wound fluid from each wound of a pig were combined and followed by enzyme-linked immunosorbent assay (ELISA) analysis with colorimetric sandwich ELISA kits (VEGF, TGF-β1, and PDGF-BB, R&D Systems, Minneapolis, MN). The ELISA plates were read by a V Max Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA) using SoftMax Pro Software (v. 3.0). Results were averaged for each age group, and graphical comparisons and statistical significance were generated using Student’s t-tests.

**Reepithelialization and contraction measurements**

Each Hematoxylin and Eosin-stained biopsy slide was scanned (Epson Perfection 636U with slide attachment, Epson American Inc., Long Beach, CA) into a computerized image (jpeg format). The image was loaded into Paintshop Pro (v 6.02, Jasc Software, Eden Prairie, MN) which allowed us to measure wound length using pixels as units. The progression of each reepithelialized “tongue” (the reepithelialized tissue from each side of the wound appears as an isthmus or tongue in cross-section) was marked. Because the wounds were outlined using tattoo ink during the initial procedure, the lateral margins of the reepithelialized “tongue” were easy to identify and measure. The extent of reepithelialization was determined using the following formula:

\[
\%\text{ reepithelialization} = \left( \frac{\text{Sum of length of 2 reepithelialization tongues}}{\text{Total length of wound}} \right) \times 100
\]

The extent of wound contraction was measured using clear plastic sheets containing the wound tracings from days 0, 3, 6, 9, and 11. These transparent sheets were scanned into 24-bit bitmap computer images using the Epson scanner. Scion Image software (Scion Corporation, Frederick, MD) was used to measure the area of each wound tracing, and a contraction percentage was
computed for each wound on each day using day 0 as “0% contracted.”

**Statistical analysis**
To determine significance for the ELISA data, a Student’s t-test was used to compare the growth factor concentration for a given age group on a given day with the concentrations of the other age groups on that same day. Similar analyses were used to compare the reepithelialization data for the three age groups on day 11, and the contraction data for days 0, 3, 6, 9, and 11. Microsoft Excel 2000 was used for all calculations. All tests were two-sided, and a $P$ value of 0.05 or less was considered to indicate statistical significance.

**RESULTS**
Using data collected and averaged from biopsies on day 11, we determined the mean (± SD) reepithelialization for the three age groups (2 pigs per group) to be: 79 ± 13 percent for 2-month-old animals, 51 ± 14 percent for 4-month-old pigs, and 21 ± 14 percent for 24-month-old animals (Figure 1). A clear, inverse relationship between age and extent of reepithelialization was evident and significant ($P<0.05$). Although wound contraction measurements from days 0, 3, 6, and 9 showed no statistical differences across the age groups (Figure 2), on day 11 the wound contraction of full-thickness wounds was faster in 2-month-old pigs than in 24-month-old pigs ($p < 0.05$).

**Growth factor concentrations in wound fluid**
To investigate whether the levels and kinetics of growth factors expression in the wound microenvironment differ among three different age groups during the healing process, the amount of growth factor present in daily collected wound fluid was examined by ELISA. It was found that peak concentrations of VEGF (Figure 3A)}
were reached on day 4 in both the 2- and 4-month-old pigs (482 pg/ml and 419 pg/ml, respectively). The VGEF level peaked in the 24-month-old pigs on day 6 with a concentration of 229 pg/ml. Collectively, the data show that the peak in VEGF concentration of the 24-month-old pigs was delayed by 2 days and diminished by a factor of 4 when compared to the younger age groups on day 4 (P<0.05). Moreover, the levels of VEGF detected on days 1, 2, 3, 5, 8, 9, and 11 in 2-month-old pigs and on days 1 and 3 in 4-month-old pigs were also statistically different from that of 24-month-old pigs (p < 0.05).

TGF-β1 levels (Figure 3B) peaked at day 9 in all age groups (9643 pg/ml in the 2-month-olds, 14160 pg/ml in the 4-month-olds, and 6151 pg/ml in the 24-month-olds). Statistical analysis of those data indicates that there is no age-related difference in TGF-β1 concentration.

PDGF-BB levels (Figure 3C) were similar in both of the younger age groups, peaking on day 1 for the 2-month-old (100 pg/ml) and on day 2 for the 4-month-old (129 pg/ml). PDGF-BB levels in the 24-month-old were undetectable. Because the minimal concentration of PDGF-BB that can be detected with this ELISA kit is at 31.5 pg/ml, the amount of PDGF-BB present in the wound fluid of 24-month-old pigs could either be very close to 31.5 pg/ml or much lower than that of 31.5 pg/ml.

**DISCUSSION**

Our choice of the Yucatan Minipig as the subject of this study was based upon the similarities that exist between its skin and that of humans. The thickness of the dermis and epidermis, the relative lack of hair, and the presence of a papillary dermal layer, subdermal fat, rete ridges, and apocrine sweat glands are characteristics that the Minipig’s integument shares with humans.

Analysis of VEGF, TGF-β1, and PDGF-BB levels in the microenvironment of porcine full-thickness wounds revealed patterns of expression and concentration that differ by age groups. Measurements of wound reepithelialization also indicated an extent of healing that declined with increasing age. Taken together, these data suggest age-related delay in healing that is linked to delayed and diminished growth factor release.

Wound healing involves the release of many growth factors during the complex phases of inflammation, proliferation, and remodeling. In our investigation we chose to study VEGF, TGF-β1, and PDGF-BB because of the central roles they play in repair, and because commercial ELISA kits were available that allowed us to measure these factors in a porcine model. Many of the other kits currently on the market are better suited to the study of other species, such as rodents, which have less porcine homology. For example, the R&D Quantikine ELISA for KGF left us unable to profile this growth factor. Perhaps products with greater porcine homology will become available in the future, allowing us to expand our study.

VEGF is vital for vascular morphogenesis. Its direct actions include increasing endothelial cell permeability, growth, and migration. Indirectly, it up-regulates expression of plasminogen activator and plasminogen activator inhibitor-1, which also play important roles in wound healing. This study found that VEGF peaks on day 4 for younger pigs (older pigs have a four-fold lower VEGF expression on this day), whereas it takes 6 days for peak expression in the older pigs at a mean concentration two-fold lower. Wound biopsies taken on day 11 showed that reepithelialization of full-thickness wounds in the older pigs approached only 41% of the extent of reepithelialization attained in the 4-month-old pigs, and only 27% of the reepithelialization reached by the 2-month-olds. These data suggest that it is not only the absolute expression of VEGF that is critical to wound healing, but also the timing of expression, and both of these aspects of growth factor release are adversely affected by increasing age. Our findings of delayed and diminished VEGF presence in older pigs is consistent with recent studies by Swift et al. which found delayed reepithelialization, collagen synthesis, and angiogenesis in the wounds of aged mice contained significantly less fibroblast growth factor-1 and VEGF than wounds in younger mice. TGF-βs are capable of provoking a spectrum of biological responses. They are expressed in a variety of cell types, such as monocytes, fibroblasts, and keratinocytes, and are present at high levels in platelets. The findings that TGF-βs affect the growth and differentiation of a variety of cell types that are involved in wound healing, and that TGF-βs are also secreted by these cells suggest an important role for this growth factor in the wound healing process. Indeed, studies by Beck et al. show that local and systemic administration of TGF-β1 enhances wound repair in aged rats. Specifically, it was shown that in a rat incisional wound model, single administration of recombinant human TGF-β1 increased wound breaking strength of aged rats to levels similar to that of normal young adult rats. Interestingly, although it was determined that the concentrations of TGF-β1 peaked on day 9 in the wound fluid of all age groups, no statistically significant difference was seen in TGF-β1 peak concentrations across the age groups. This finding suggests that the local concentration of TGF-β1 has no direct effect on the observed differential rates of excisional wound healing in the porcine wound chamber model in the age groups studied. It should be noted that although similar levels of TGF-β1 were detected in the local wound envi-
environment, the response to TGF-β1 signaling may vary significantly among the different age groups, which could be caused, for example, by the various levels of TGF-β1 receptors expressed in cells involved in the healing process among the three age groups.

PDGF is a major mitogenic factor for fibroblasts and keratinocytes. PDGF can be produced by many different types of cells, including endothelial cells and keratinocytes. PDGF may play an important role in wound healing based on the observations that it is released into the wound environment by platelets soon after injury, and the synthesis of PDGF and its receptor is induced in wounds. Moreover, PDGF-BB can function synergistically with TGF-α or insulin-like growth factor I to accelerate wound healing in porcine partial-thickness wounds. Studies by Ashcroft et al. found a delay in appearance of PDGF A and B isoforms, as well as PDGF-α and β receptors, in acute incisional wounds in aging mouse colonies. Our attempt to create a temporal profile for PDGF-BB was limited by an ELISA kit, which had a lower readable limit of 31.5 pg/ml. The samples taken from the 24-month-old pigs contained concentrations of PDGF below this limit for all 11 days of study. For statistical purposes, we assigned PDGF-BB values of 31.5 pg/ml to each study day for the 24-month-old pigs, and this produced analyses that were not statistically significant. Future development of more sensitive ELISA kit may allow us to investigate the changes that occur in the PDGF-BB profile with increasing age.

Taken together, the profiles of VEGF, TGF-β, and PDGF-BB generated by this study suggest an interesting temporal relationship over the first 11 days of healing. Across the age groups, PDGF-BB reached it speak early in the wound repair process (day 1 or 2), fell to diminished concentrations during days 5 and 6, and then rose again. TGF-β rose initially on day 1 along with PDGF-BB. After falling on day 2, it rose again and reached its highest levels on day 9 (Figure 3). VEGF reached its peak between the other two growth factors (Figure 3, days 4 and 6). The interrelationship between these factors will continue to be examined in future studies.

Future applications of the data obtained in this study will make use of the chamber model as a treatment modality. Having identified when and by how much a wound in an older pig is lacking a certain growth factor, or combination of growth factors, one can now deliver growth-promoting genes into the wound microenvironment with either a direct in vivo gene transfer approach such as microseeding or through ex vivo gene transfer. In particular, by cloning a growth-promoting gene under the control of a genetic switch, such as the tetracycline-inducible gene switch that was recently developed, the timing of gene expression can be readily set by addition of tetracycline to the local wound environment (unpublished data). Moreover, by changing the tetracycline concentration in the wound environment, the level of growth factor gene expression can be finely adjusted according to therapeutic needs. This regulated delivery of growth factors in the wound microenvironment should maximize their biological effect and decrease the toxicity resulting from uncontrolled expression.

ACKNOWLEDGMENT
This work was supported by Public Health Service Grant 2R01GM5144905 from the National Institutes of Health.

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