

# Wound Splinting Regulates Granulation Tissue Survival<sup>1</sup>

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**Purpose.** Fibroblast survival within an *in vitro* collagen matrix is dependent on matrix anchorage to a rigid substratum. The purpose of this study was to determine whether granulation tissue survival *in vivo* also is dependent on matrix anchorage. We hypothesized that splinting an excisional wound (i.e., anchoring the wound edges) would promote granulation tissue survival and that desplinting a splinted wound would produce granulation tissue apoptosis.

**Methods.** Eighteen Wistar rats (3 months, 350 g) underwent excisional wounding (2 × 2 cm, dorsal skin) with immediate wound splinting (a metal template affixed with sutures) on day 0. On day 6, rats (*n* = 6 per group) underwent splint removal (desplinted), splint removal with circumferential incision of the wound edge (desplint/release), or no intervention (splinted); sacrifice of all animals was on day 7. Frozen sections of granulation tissue were stained with TUNEL or H&E; data were analyzed with ANOVA and the unpaired *t* test.

**Results.** The cross-sectional and surface area of the desplinted and desplint/release granulation tissue both decreased compared to the splinted granulation tissue (\**P* < 0.05). The nuclear density of the desplint/release granulation tissue was 25% less compared to the splinted granulation tissue (\**P* < 0.05). The desplinted and desplint/release apoptotic rates were twice and >10× greater than the splinted apoptotic rate, respectively (\**P* < 0.05).

**Conclusions.** The rate of cell death in a splinted wound (an *in vivo* equivalent of an anchored FPCM) is minimal to nil, which is consistent with our hypothesis. Desplinting and releasing the wound edge of a previously splinted wound (the *in vivo* equivalent of a detached FPCM) results in granulation tissue regression and a large increase in apoptosis. Desplinting a wound alone results in changes somewhat intermedi-

ate to the splinted and desplint/release conditions. Loss of wound anchorage acutely promotes granulation tissue apoptosis. © 2003 Elsevier Science (USA)

**Key Words:** granulation tissue; wound healing; wound contraction; splinting; survival; apoptosis.

## INTRODUCTION

An organism's response to a full-thickness traumatic cutaneous defect is to close the wound. This healing response includes synthesis of a temporary wound matrix (granulation tissue), wound contraction, and wound epithelialization. Under some pathologic conditions, such as with keloids [1] or burn wound contraction [2], there is dysregulation of granulation tissue which produces morbidity. An understanding of the regulation of granulation tissue survival potentially could lead to improved therapies for such pathologic conditions. Evidence from animal [3] and cell system [4–7] models suggest that physical anchorage of the extracellular matrix promotes granulation tissue survival. The purpose of this study was to determine whether survival of granulation tissue in a splinted wound model was dependent on immobilization of the wound margin. Such dependence *in vivo* would provide further evidence supporting the *in vitro* observations that matrix anchorage can regulate wound cell survival.

## MATERIALS AND METHODS

**Animal model.** The use of animals in this research was approved by our institutional animal care and use committee. The excisional wound model has been described previously [3]. Under isoflurane (AErrane; Fort Dodge Animal Health, Fort Dodge, IA) inhalation anesthesia, a full-thickness (dermis + panniculus carnosus) wound (2 × 2 cm template) on the rat dorsum was created and immediately splinted by affixation of a stainless-steel square (2 × 2 cm internal dimension) with a continuous 5-0 nylon suture which looped over-and-over around the splint and skin edge (Fig. 1A). All wounds were

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dressed with polyurethane (Tegaderm; 3M Health Care, St. Paul, MN) and protected with a circumferential (thorax level) plaster of paris wrap, which did not cause respiratory embarrassment. This anesthetic, wounding, and dressing technique has been used in >300 rats with 1 mortality (an anesthetic overdose). Preliminary experiments involving 29 rats were performed to optimize the parameters of the wound splinting model.

**Experimental design.** Eighteen animals were wounded with immediate application of a metal splint followed by the Tegaderm/plaster dressing. On postwounding day (PWD) 6, six rats (splinted group) underwent a dressing change only (down to but not including the Tegaderm), six rats (desplinted group) had their splints removed with placement of a fresh Tegaderm/plaster dressing, and six rats (desplint/release group) underwent splint removal followed by wound edge release (a circumferential incision of the dermis/granulation tissue interface using a No. 15 scalpel blade) and a fresh dressing. All animals then were sacrificed 24 h later (i.e., PWD 7) by CO<sub>2</sub> asphyxiation.

**Histology and TUNEL.** After sacrifice an *en bloc* excision of the granulation tissue (with splint, if present), deep paraspinal musculature, and 2 cm of surrounding skin was performed. Each specimen was fixed in a  $\geq 20\times$  volume of 3% paraformaldehyde in PBS (made fresh) for 24–48 h (one to two exchanges); splinted wounds were fixed with the splint in place. Specimens then were dehydrated in 30% sucrose for 24 h prior to being embedded in Tissue Freezing Medium (Triangle Biomedical Sciences, Inc., Durham, NC). Each specimen was sectioned transversely in two locations. Frozen sections (5  $\mu$ m thickness) from each location were stained with H&E or TUNEL (the latter with propidium iodide counterstain, PI) as previously described [3]. At least two fluorescent micrographs were captured from each section using a Nikon (Melville, NY) Optiphot-2 fluorescent microscope with a digital camera (Spot camera; Diagnostic Instruments, Brentwood, MO) interfaced to a personal computer. The absolute number of TUNEL-positive figures was quantified from analysis of digital images (at least four per wound) using NIH Image software (public domain, <http://rsb.info.nih.gov/nih-image/>); the apoptotic rate was defined as (number of TUNEL-positive figures  $\div$  the number of PI-labeled nuclei)  $\times 100$ .

**Wound area and nuclear population density measurement.** Wound surface area was determined by tracing each wound onto a transparency immediately prior to sacrifice (animals under anesthesia) and measuring the area of the scanned tracing (300-dpi resolution) with NIH Image. Wound cross-sectional area was determined by scanning an H&E section of each wound (transverse section at greatest breadth) at 2700 dpi (SprintScan 35 Plus; Polaroid, Cambridge MA) and measuring the granulation tissue area with NIH Image. Nuclear population density was measured by analyzing PI-labeled images (minimum of four per wound) with NIH Image.

**Statistical analysis.** Data were analyzed with ANOVA; if the null hypothesis was void, then between-group comparisons were performed with the unpaired *t* test. Statistical significance was defined as  $*P < 0.05$ .

## RESULTS

The initial (PWD 0) wound surface area was defined as the area of the template, or 4 cm<sup>2</sup> (Fig. 1A). By PWD 7, the surface area of the splinted granulation tissue (Fig. 1B) had decreased to approximately 93% of the initial area; 1 day after desplinging or desplint/release (also PWD 7), the granulation tissue surface area had decreased to 35 or 15% of the splinted area, respectively (Table 1). For comparison, unsplinted wounds contract to 25% of the starting surface area during the same interval (data not shown). Granulation tissue

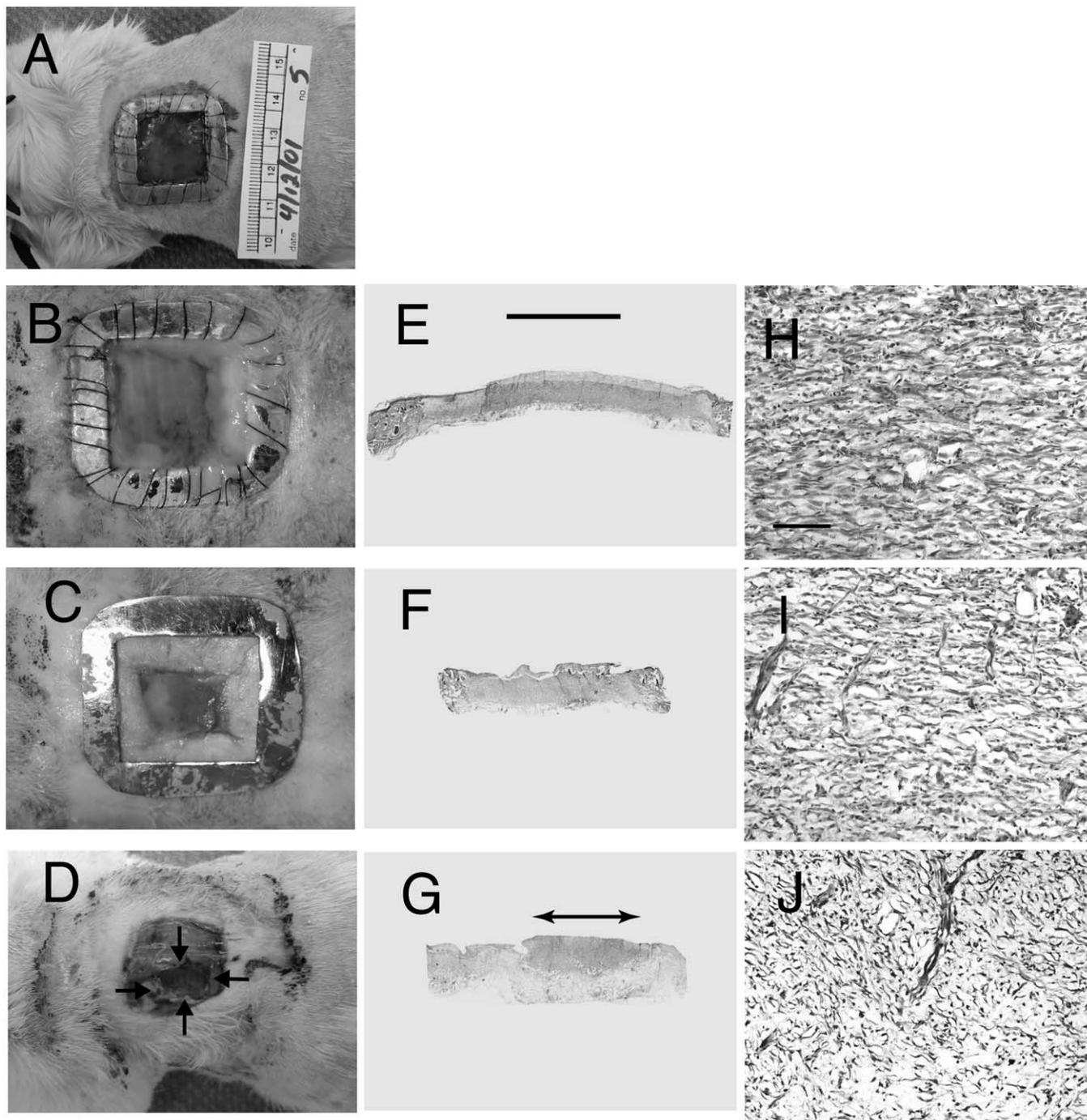
contraction after 24 h of desplinging or desplint/release (i.e., PWD 6–7) is shown in Figs. 1C and 1D, respectively. Most (80–90%) of this contraction occurred in the first few minutes after the intervention; in addition, the skin edge of desplint/release wounds retracted back to the original dimension of the excision almost immediately after release (data not shown). After the circumferential incision of the dermal margin, the desplint/release granulation tissue was freely mobile upon a base of loose, filmy connective tissue (Fig. 1G).

The shape of the splinted granulation tissue in cross section on PWD 7 (Fig. 1E) was elongated and rectangular. One day after splint removal (PWD 7), the cross-sectional shape still was rectangular, but shorter in lateral dimension (Fig. 1F). One day after desplint/release (PWD 7), the granulation tissue developed an ovoid cross-sectional shape (Fig. 1G). The cross-sectional area of the granulation tissue for the desplinted and desplint/release wounds on PWD 7 (1 day after intervention) was 66 and 42% of the area of the splinted wounds, respectively (Table 1). Subjective comparison of microscopic H&E morphology revealed that the staining of the desplinted (Fig. 1I) and desplint/release (Fig. 1J) granulation tissue was progressively less intense compared to the splinted granulation tissue (Fig. 1H). In addition, the extracellular matrix and cells of the splinted granulation tissue are aligned along the transverse axis (i.e., parallel to the direction of contraction), whereas this polarization is absent in the desplint/release wound.

TUNEL of frozen sections of the splinted granulation tissue produced only rare labeled nuclei (Fig. 2A). There were slightly more TUNEL-positive figures in the desplinted sections (Fig. 2B), and the desplint/release granulation tissue sections contained numerous labeled nuclei (Fig. 2C). Examination of PI-labeled sections did not reveal any obvious difference with respect to nuclear distribution or population density (Figs. 2D–2F). As expected, the desplinted apoptotic rate was slightly but significantly greater than the splinted rate (Fig. 3; also compare Fig. 2A vs 2B); the desplint/release apoptotic rate, however, was an order of magnitude greater than the splinted rate (Fig. 3; also compare Fig. 2A vs 2C). Quantification of the nuclear population density using PI-labeled images revealed that the desplint/release density was 20% less than the splinted density (Fig. 3).

## DISCUSSION

Previous studies on wound splinting in animals demonstrated that splint removal from a splinted wound results in rapid contraction of the granulation tissue; wound edge release performed in addition to splint removal results in granulation tissue contraction and retraction of the dermal margin [8–10]. These observations were reproduced in the present study. In addi-



**FIG. 1.** The effect of splinting, desplinting, and desplint/release on gross and microscopic wound morphology. A–D, dorsal photographs. A: Splinted wound on PWD 0, immediately after the splint has been sewn in place. Ruler is in centimeters. B: Splinted wound on PWD 6, immediately prior to intervention. C: Desplinted wound 24 h after splint removal (i.e., PWD 7). Splint has been placed back over the wound to demonstrate the degree of contraction which has occurred. D: Desplint/release wound 24 h after splint removal and wound edge release (i.e., PWD 7). Arrows indicate margin of granulation tissue. E–G, whole wound cross-sectional micrographs (H&E) which correspond to B–D. Bar = 5 mm; double-headed arrow in G indicates lateral extent of granulation tissue. H–J, higher power micrographs (H&E) of the granulation tissue from the central region of the wound; images correspond to B–D. Bar = 120  $\mu$ m.

tion, regression of granulation tissue after desplinting was demonstrated using physical and biochemical measurements of the wound matrix [9, 11]. If granulation tissue regression is defined by either a decrease in

tissue volume and/or a decrease in cell population density, then in this study regression was apparent 24 h after desplint/release, as demonstrated with wound surface and cross sectional area (Table 1) and with PI

TABLE 1

**Granulation Tissue Surface Area and Cross-sectional Area on PWD 7 (1 Day after Desplinting or Desplint/Release), Given as Mean  $\pm$  SD**

	Surface area, mm <sup>2</sup>	Cross-sectional area, mm <sup>2</sup>
Splinted	371 $\pm$ 2	13.1 $\pm$ 2.7
Desplinted	88 $\pm$ 30*	8.7 $\pm$ 1.0*
Desplint/release	38 $\pm$ 19***	5.5 $\pm$ 1.2***

\*  $P < 0.05$  compared to splinted.

\*\*  $P < 0.05$  compared to deslnted (ANOVA and unpaired  $t$  test).

counterstaining (Figs. 2 and 3). It was demonstrated that the regression occurred in conjunction with a 10-fold increase in granulation tissue apoptosis in the deslnt/release wounds (Figs. 2 and 3).

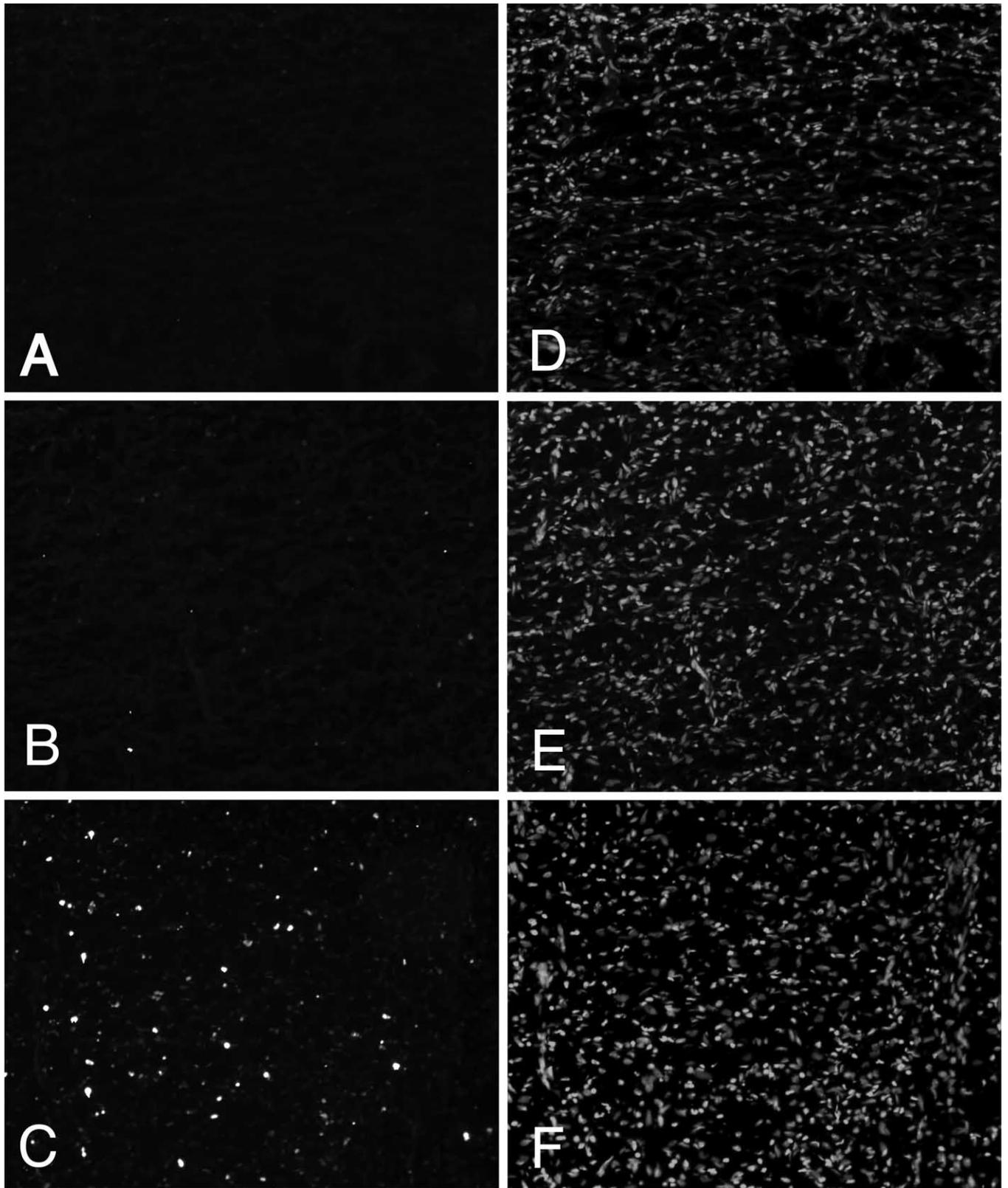
Wound splinting may be viewed as an extreme form of matrix anchorage. In an otherwise unsplinted wound, the granulation tissue is attached to a dermal edge that can have a varying degree of mobility; in our rat excisional wound model, dermal mobility in an unsplinted wound is relatively high, such that it closes easily by contraction (data not shown). Releasing the dermal edge in an unsplinted wound induces a 2- to 3-fold increase in granulation tissue apoptosis [3]. In the present study we have decreased dermal mobility (or, in other words, enhanced anchorage of the granulation tissue) by splinting the wound at the initial time of wounding. The removal of the wound splint combined with the wound edge incision induces a radical decrease in the lateral (i.e., the plane of contraction) anchorage of the granulation tissue *matrix*; the cells within this matrix, of course, are still embedded therein. Our intention with this maneuver was to demonstrate that a release of lateral wound anchorage would produce a large increase in apoptosis compared to the anchored wound—and the increase that we did observe was greater than one order of magnitude, or higher than that seen with simple release of the margin of an unsplinted wound [3].

The data of this study support one of our main hypotheses on healing: that mechanical stress is an important positive modulator of granulation tissue survival. Although mechanical stress was not directly measured in this study, the presence of stress in the splinted granulation tissue may be inferred by the relatively rapid contraction of the granulation tissue and retraction of the dermal edge which both occurred after the deslnt/release intervention and also by the alignment of the fibroblasts in the plane of contraction within the splinted granulation tissue. While mechanical stress probably does not play much of a role in the acute formation of granulation tissue (i.e., during the first several days after wounding in which various cells proliferate within the wound, presumably secondary to

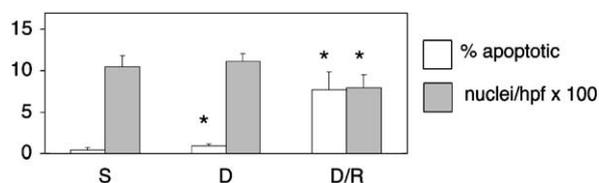
cytokine stimulation), the data of this study and previous work [3] implicate a role for mechanical stress in the promotion of survival within established granulation tissue. This would suggest that investigation into the role of mechanical stress during the phase of granulation tissue “chronicity” would yield insight into important healing mechanisms. An alternative hypothesis to explain the cell death in this model is alteration of cell shape induced by an increase in cell–cell crowding after release of wound anchorage. Cell shape is an important regulator of survival *in vitro* [12]. The data collected herein, however, cannot differentiate between the roles of cell shape vs wound anchorage in the induction of granulation tissue apoptosis.

It could be argued that a circumferential incision at the lateral edge of the granulation tissue (i.e., releasing a wound) will devascularize the wound. The subsequent granulation tissue regression then could be attributed to wound ischemia. Based on previous histologic examination [3], however, the wound vasculature in this model derives from the loose connective tissue inferior to the granulation tissue and not from the lateral interface of the dermis and granulation tissue. This anatomical arrangement also was apparent after multiple gross inspections which revealed that the granulation tissue was based on an inferior pedicle of loose, filmy tissue that, when placed on stretch, demonstrated an abundance of small vessels which coursed directly into the granulation tissue (data not shown). Wound ischemia from a lateral incision (which left the inferior “vascular” pedicle undisturbed) therefore would be unlikely. It also could be argued that the deslnt/release wound is not analogous to the detached collagen matrix, because the former remains attached inferiorly after the releasing incision, while the latter floats freely in the tissue culture medium after detachment. As described above, however, the attachment of the granulation tissue to the underlying paraspinal musculature was quite loose. This “attachment” offered no observable resistance to granulation tissue contraction.

The mechanism by which granulation tissue survival is dependent on matrix anchorage is speculative. In epithelial or endothelial monolayers, cell survival is anchorage dependent; a cell separated from its substratum undergoes a specialized form of apoptosis, or *anoikis* [13]. Anchorage-dependent survival of granulation tissue, as probed in the present study, exists in a different context from the monolayer examples—the wound cells are not separated from their substratum; rather, the anchorage of the matrix to the substratum is disrupted. Data from a cell system model, the fibroblast-populated collagen matrix, demonstrated that apoptosis induced by disruption of matrix anchorage is associated with inhibition of focal adhesion kinase and protein kinase B [14], which suggests that the



**FIG. 2.** TUNEL assay in splinted, desplinted, and desplint/release wounds on PWD 7 (1 day after desplinting or desplint/release). A–C, TUNEL images. D–F, corresponding PI counterstain. A & B: splinted wound. B & E: desplinted wound. C & F: desplint/release wound.



**FIG. 3.** Effect of wound splinting (S), desplinting (D), and desplint/release (D/R) on granulation tissue apoptosis (% apoptotic = apoptotic rate) and nuclear population density (nuclei/hpf = nuclei per high power field) on PWD 7 (1 day after desplinting or desplint/release). Error bars = standard deviation; \* $P < 0.05$  compared to S, ANOVA and the unpaired  $t$  test.

mechanism of anchorage-dependent survival in a three-dimensional collagen matrix actually may be similar to that in an anoikis-susceptible monolayer [15, 16].

Although the TUNEL assay can be misleading in certain situations [17, 18], the predominant trend in the literature supports its utilization as a primary assay for apoptosis. We have optimized the use of TUNEL in our tissue culture and animal models [3, 4], and we routinely have obtained clean and reproducible results. For these reasons, and also because it is less cumbersome in tissue sections compared to other methods, the TUNEL assay remains our primary technique to quantify apoptosis *in situ*.

In the wound splinting model, anchorage of the wound matrix to the dermal margin promoted survival of the cells in the granulation tissue, while removal of the anchorage resulted in an acute 10-fold increase in apoptosis. This result is analogous to the finding in the fibroblast populated collagen matrix, an *in vitro* wound model. Both models are consistent with the hypothesis that mechanical stress is a positive modulator of fibroblast survival in a three-dimensional matrix. Importantly, the animal model in this report provides a means to investigate the regulation of cell survival in the wound matrix, a topic which has been limited mainly to *in vitro* investigation.

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