Granulation tissue regression induced by musculocutaneous advancement flap coverage

Mark A. Carlson, MD, Michael T. Longaker, MD, *and* **Jon S. Thompson, MD,** *Omaha, Neb, and Stanford, Calif*

Background. *Clinical experience suggests that granulation tissue may be inhibited by coverage with a musculocutaneous flap. We hypothesized that coverage of an open wound with a musculocutaneous flap would result in regression and apoptosis of the wound's granulation tissue.*

Methods. *In the first experiment, 32 rats underwent excisional wounding; 16 underwent musculocutaneous flap coverage of their granulation tissue on postwounding day 8, and then 16 rats (8 controls + 8 flaps) were killed on both postwounding days 10 and 12 (2 and 4 days after the flap procedure, respectively). In the second experiment, 18 rats were wounded, and on postwounding day 5 the rats underwent flap coverage (n = 6), wound edge release/mobilization (the first step of the flap procedure) without flap coverage (n = 6), or dressing change only (n = 6); all rats were killed on postwounding day 6 (24 hours after the secondary intervention). Apoptosis was quantified with the terminal deoxynucleotidyl transferase–mediated nick-end labeling assay.*

Results. *Placement of a musculocutaneous flap over an 8-day-old excisional wound in the first experiment increased the apoptotic rate in the granulation tissue from 0% to 1% (controls) to 5% to 10% at both 2 and 4 days after flap coverage (P < .05). Cell population density decreased 50% in the flap-covered granulation tissue compared with the controls (P < .05). In the second experiment, circumferential release of the granulation tissue resulted in an equivalent increase in granulation tissue apoptosis over controls compared to that induced by the full flap procedure.*

Conclusions. *Coverage of established granulation tissue with a musculocutaneous flap resulted in histologic regression of the wound's granulation tissue after 2 to 4 days of flap coverage and induced at least a 5-fold increase in the apoptotic rate of the granulation tissue. Releasing the wound edge increased granulation tissue apoptosis to a level equivalent to that produced by the musculocutaneous flap procedure, suggesting that alteration of the wound's mechanical environment is responsible for the acute induction of apoptosis in this model. (Surgery 2002;131:332–7.)*

From the Department of Surgery, University of Nebraska Medical Center, Omaha, Neb, and the Department of Surgery, Stanford University School of Medicine, Stanford, Calif

THE PRECISE ROLE OF granulation tissue in dermal healing is speculative, but its probable functions include (1) to serve as a provisional matrix in which further matrix proteins can be secreted; (2) to maintain a barrier to infection; (3) to provide a scaffold for epithelialization; (4) to serve as a reservoir for nutrients and growth factors for the wound; and (5) to contain contractile cells that effect wound contraction.[1-3] Wound contraction is a cell-driven event.[4-7] Given the potential of granulation tissue cells and wound contraction to cause morbidity in such conditions as keloid scarring or burn wound contracture, we have been interested in the regulation of cell survival in the granulation tissue matrix.

Wound cells are eliminated during the resolution phase of healing by apoptosis.[8] Coverage of a bed of granulation tissue with a full-thickness skin graft or a musculocutaneous flap (MCF) can inhibit the granulation tissue and induce wound cell apoptosis[9,10]; granulation tissue apoptosis also occurs under the advancing edge of wound epithelium.[11,12] Granulation tissue regression, therefore, may be induced by paracrine signaling between a
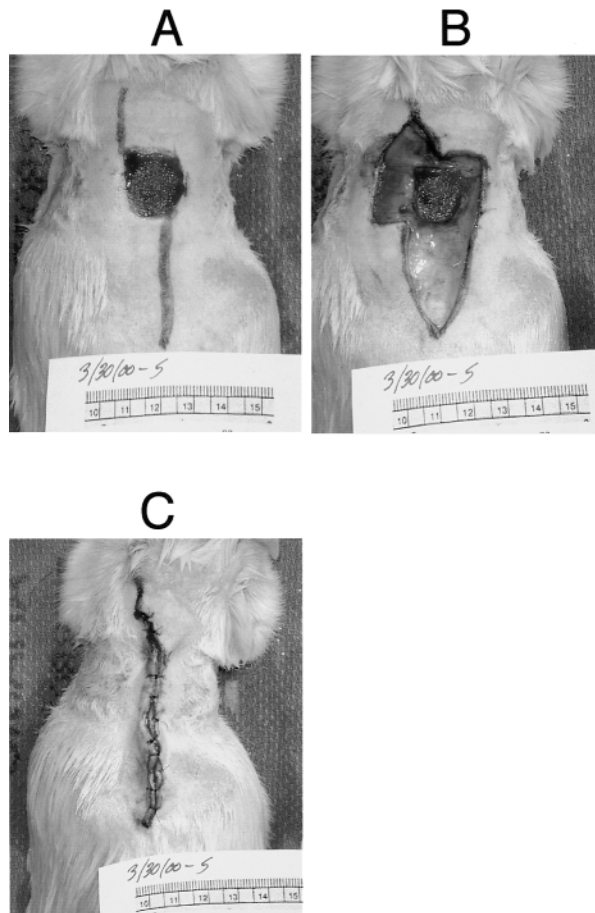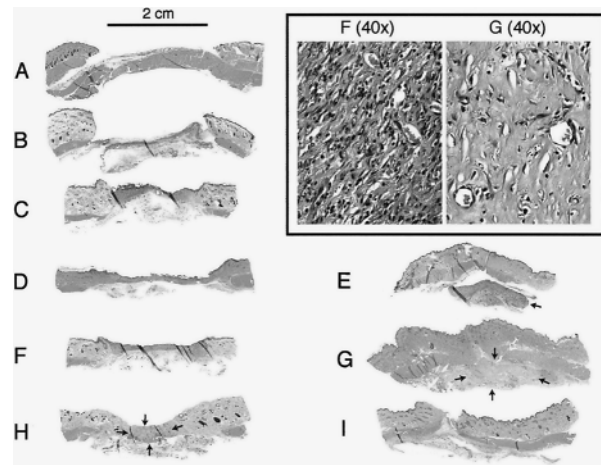
Fig 1. Technique of MCF placement. **A**, Granulation tissue at PWD 8. Proposed surgical incision is traced. **B**, Appearance immediately after surgical incision and skin edge mobilization but before MCF coverage. **C**, Appearance after the MCF has been advanced and sewn in place.

Fig 2. Sequence of whole wound micrographs (transverse plane) from control and MCF animals. **A**, Control, PWD 0; **B**, control, PWD 3; **C**, control, PWD 6; **D**, control, PWD 8; **E**, MCF, PWD 8 (immediately after flap placement); **F**, control, PWD 10; **G**, MCF, PWD 10 (2 days after flap placement); **H**, control, PWD 16; **I**, MCF, PWD 16 (8 days after flap placement). *Inset*: Higher power view of granulation tissue from control **(F)** and MCF **(G)** animals 2 days after flap placement. *Arrows* indicate granulation tissue.

biologic wound cover (eg, a flap) and the granulation tissue. Alternatively, mechanical loading may be critical for cell survival, as is the case with fibroblasts in an in vitro wound model, the fibroblast-populated collagen matrix.[13,14] We extrapolated from this model and hypothesized that granulation tissue regression and apoptosis after wound coverage with an MCF are a result of a reduction of mechanical loading on the granulation tissue. Our first goal in exploring this hypothesis was to establish a reliable model of granulation tissue apoptosis induced by an MCF.

METHODS

Design of experiments 1 and 2. After a number of preliminary studies in which experimental parameters were defined, 2 major experiments were performed. In experiment 1, 32 rats were wounded; on postwounding day (PWD) 8, 16 rats underwent MCF coverage of their wounds as shown in Fig 1,

and the other 16 had a dressing change only (control group). Eight control rats and 8 MCF rats were killed on both PWDs 10 and 12 (corresponding to 2 and 4 days, respectively, after the MCF intervention). In experiment 2, 18 rats were wounded; on PWD 5, 6 rats underwent MCF coverage of their wounds, 6 rats underwent wound edge release and dermal mobilization as shown in Fig 1, *B* (the initial maneuver of the MCF procedure), and 6 rats had a dressing change only. All rats in experiment 2 were killed on PWD 6 (24 hours after the interventional procedure).

Animal model. All animal experiments were approved by our Institutional Animal Care and Use Committee. Rats (male Wistar, 325 to 375 gm) were individually housed and fed rat chow (5001 Rodent Diet; PMI International, Inc, Brentwood, Mo) and water ad libitum. After a minimum residence of 1 week in our laboratory, isoflurane (AErrane; Fort Dodge Animal Health, Fort Dodge, Iowa) inhalation anesthesia was administered by a VMS Anesthesia Machine (Matrx Medical, Inc, Orchard Park, NY), the hair of the thorax was clipped circumferentially, the dorsum was scrubbed with 0.3% Triclosan soap (Bacti-Stat; Ecolab Inc, St Paul, Minn) and then shaved with a straight razor, and a 2 × 2 cm square (marked with a template) of dermis + panniculus carnosus was excised from the dorsum; the superior edge of the

Table. Effect of MCF coverage on granulation tissue cell population density (CPD) and wound cross-sectional area

Group	CPD (nuclei/field)	Area (mm²)
Control, t = 2	988 ± 319	8.05 ± 2.84
MCF, t = 2	494 ± 86*	6.35 ± 2.84†
Control, t = 4	752 ± 116	9.27 ± 1.66
MCF, t = 4	484 ± 124*	NA

Time (t) refers to the number of days after the MCF intervention.

NA, Wound margins too indistinct for measurement.
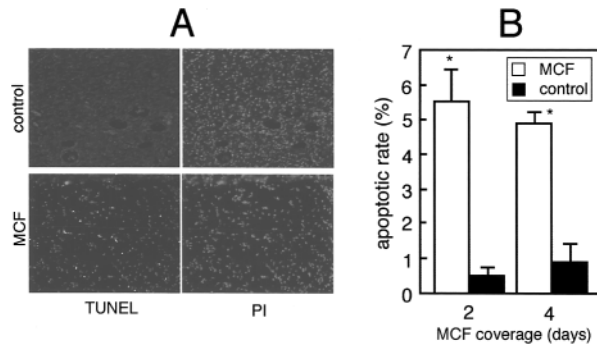
*$P < .05$ compared with control.

†$P = .055$ compared with control.

excision was 1 cm inferior to the scapular tips. The wound was covered with a polyurethane dressing (Tegaderm; 3M Health Care, St Paul, Minn), then a gauze bolster, then a circumferential wrap of elastic gauze, and finally an outer circumferential plaster wrap. An MCF (Fig 1) was performed on 5- to 8-day-old wounds (Fig 1, *A*) under general anesthesia by incising the wound margin (ie, at the junction of the dermis and the granulation tissue) circumferentially, extending the incision superiorly and inferiorly, extensively mobilizing the dermis + panniculus carnosus (as a single layer) around the wound (Fig 1, *B*), advancing this layer over the wound, and then closing with interrupted sutures of 5-0 nylon so that the final suture line was oriented vertically (Fig 1, *C*).

TUNEL assay. Tissue specimens were fixed in a ×25 volume of 3% paraformaldehyde in phosphate-buffered saline (PBS) for 24 to 48 hours (1 exchange); the tissue subsequently was dehydrated for 24 hours in 30% sucrose before embedding in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, Calif). Frozen sections (5 µm) were cut onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, Pa), soaked in 0.1% Triton X-100 (Sigma-Aldrich, St Louis, Mo) in PBS for 30 minutes at room temperature (RT), placed in 150 mL of 0.1 mol/L sodium citrate (Sigma-Aldrich), pH 6, and then cooked in a Sanyo (Chatsworth, Calif) microwave oven (model EMP-410W) for 2 minutes at 40% power (400 W), followed by a 15-minute cool-down period. Sections then were covered with 200 µL of Pronase (Sigma-Aldrich) solution, 0.25 mg/mL in 100 mmol/L Tris (Sigma-Aldrich), pH 7.5, with 1% Triton X-100, and incubated for 10 minutes at RT.[15-18] The slides were rinsed 3 times with PBS before staining with terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) by using a kit (TUNEL kit with fluorescein; Roche, Indianapolis, Ind) per the manufacturer's recommendations; 50 µL of reagent mix was used per section, and the TUNEL incubation was performed for 1 hour at 37°C in a dark, humidified chamber. To obtain a positive control, sections were covered with 200 µL of 0.5 mg/mL DNAse (Roche) in 50 mmol/L Tris, pH 7.5, with 1 mg/mL bovine serum albumin (Sigma-Aldrich), 0.1 mmol/L dithiothreitol (Sigma-Aldrich), and 1 mmol/L $MgCl_2$ (Sigma-Aldrich), incubated for 10 minutes at RT, and rinsed 3 times with PBS, all before the TUNEL reaction. Labeled sections were counterstained by covering with 100 µL of 1 µg/mL propidium iodide (PI; Sigma-Aldrich) in PBS with 20 µg/mL of DNAse-free RNAse (Roche), incubating for 15 minutes at RT in the dark, rinsing 3 times with PBS, and then coverslipping with Fluoromount G (Southern Biotechnology Associates, Inc, Birmingham, Ala). Fluorescent micrographs were captured from a Nikon (Melville, NY) Optiphot-2 fluorescent microscope with a digital camera (Spot camera; Diagnostic Instruments, Brentwood, Mo) interfaced to a personal computer. To obtain a reasonable sampling of each wound for quantification of the apoptotic rate, a specimen was sectioned in 2 locations and, after double-labeling with TUNEL and PI, a minimum of 4 randomly chosen fluoromicrographs were captured digitally from each section. The number of stained nuclei in each image was counted by using NIH Image (public domain software, http://rsb.info.nih.gov/nih-image), and the apoptotic rate for a given micrograph was equal to (No. of TUNEL-positive nuclei/No. of PI-positive nuclei) × 100%.

Statistical analysis. Rates and measurements among 3 or more groups of animals (a typical group in this report contained 6 to 8 animals) were compared by using analysis of variance (ANOVA), with statistical significance defined as $P < .05$. If a difference was found by ANOVA among the groups, then comparisons between individual groups were performed by using the unpaired *t* test, with statistical significance also
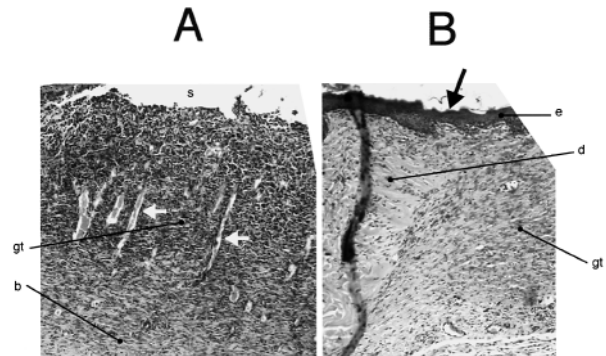
Fig 3. Effect of MCF coverage on granulation tissue apoptosis (experiment 1). **A**, TUNEL and PI double-labeling of control vs MCF-covered wounds after 2 days of flap coverage; $\times 20$ objective. **B**, Apoptotic rates in control vs MCF wounds. (*$P < .05$ compared with control.)


Fig 4. Derivation of granulation tissue blood supply. **A**, Hematoxylin-eosin section from the center of a 9-day-old control wound; *s*, wound surface; *gt*, granulation tissue; *b*, wound base. *White arrows* indicate vascular channels. **B**, Section from lateral edge of wound; *e*, epidermis; *d*, dermis. *Black arrow* indicates location of incision to perform wound edge release.

defined as $P < .05$. Data are reported as mean ± standard deviation.

RESULTS

Effect of an MCF on gross and microscopic wound morphology. Gross and microscopic study of the excisional wound model showed a linear decrease in wound area between PWDs 3 and 12 (data not shown). A sequence of whole wound hematoxylin-eosin micrographs is shown in Fig 2. On PWD 0 (immediately after excision), the wound cross-section exhibited skin edge retraction that was typical for an excision of this type (Fig 2, *A*). The skin margins are lateral, and the bridging piece of tissue is actually the deep muscles of the back lying beneath an investing fascial layer. For clarity, the deep muscles of the back were not included in the section images subsequent to PWD 0 (Fig 2, *B-I*). There was a well-developed bed of granulation tissue by PWD 5 to 6 (Fig 2, *C*), which was discoid shaped in cross-section (Fig 2, *C* and *D*). The cross-sectional shape of the granulation tissue in the control wound at PWD 16 evolved to a more ovoid appearance (Fig 2, *H*).

A striking change in gross morphology of the granulation tissue occurred immediately after MCF placement on PWD 8 (Fig 2, *E*); the cross-sectional wound shape went from discoid to ovoid. The interval from the beginning of the MCF procedure to tissue fixation in Fig 2, *E* was about 30 minutes. Wound cross-sectional area decreased in MCF-covered wounds compared with the control wounds after 2 days (Table); it was not possible to compare the cross-sectional areas after 4 days of flap coverage, because the margins of the granulation tissue in the flap-covered wounds had become too indistinct. Hematoxylin-eosin micrographs of granula-

tion tissue ± flap coverage showed that, after 2 days of coverage, the flap wounds (Fig 2, *inset G*) had markedly decreased cellularity compared with the control wounds (Fig 2, *inset F*). The cell population density (as determined by a count of PI-labeled nuclei per microscopic field; Fig 3, *A*) was decreased by approximately 50% in the MCF versus control wounds after both 2 and 4 days of flap coverage (Table). The animal weights at both 2 and 4 days after the MCF procedure were not different between the MCF and control groups ($P > .05$; data not shown).

Effect of an MCF on granulation tissue apoptosis (experiment 1). In preliminary studies an optimal regimen of granulation tissue section pretreatment with microwave radiation and proteinase digestion was defined that resulted in 90+% labeling of nuclei in DNAse-digested slides (data not shown). By using sections double-labeled with TUNEL and PI (Fig 3, *A*), it was determined that the false-positive rate of the TUNEL assay in this model was 0.1% or less (data not shown). Application of an MCF over 8-day-old granulation tissue produced a 5-fold increase in wound apoptosis compared with control wounds after both 2 and 4 days of MCF coverage (Fig 3, *B*; $P < .05$). There was no evidence of ischemic necrosis in the MCF wounds (data not shown). The blood supply to the granulation tissue in this model derives from the wound base (Fig 4, *A*) and not the lateral margin (Fig 4, *B*).

Effect of wound edge release versus flap coverage on granulation tissue apoptosis (experiment 2). A combination of wound edge release and mobilization of the surrounding dermis (the steps illus-
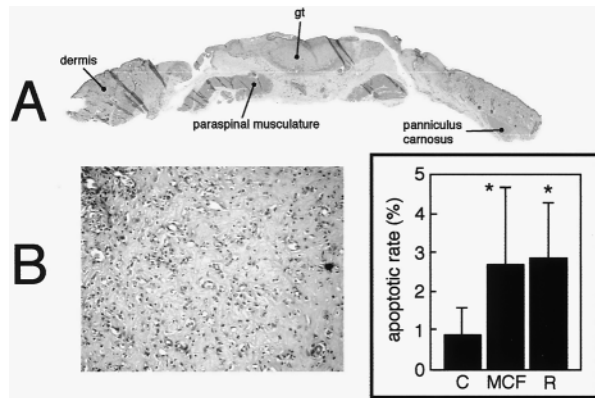
Fig 5. Effect of MCF coverage vs wound edge release on granulation tissue apoptosis (experiment 2). **A**, Whole wound micrograph at PWD 6, 24 hours after the wound edge was released; **B**, higher power view of granulation tissue from **A**. *Inset*: Apoptotic rates in control *(C)* vs MCF vs released *(R)* wounds. (*P < .05 compared with control.)

trated in Fig 1, *B*) performed on 5-day-old wounds resulted in a change in cross-sectional wound shape from discoid to ovoid (Fig 5, *A*), which was similar to the shape change seen after the MCF procedure (Fig 2, *E* and *G*). Histologic regression (ie, decreased cellularity) of the granulation tissue was evident 24 hours after wound edge release (Fig 5, *B*), which also was similar to the regression seen after the MCF procedure (Fig 2, *inset G*). The cell population density for the control, MCF, and wound edge release groups was 939 ± 147, 806 ± 166, and 789 ± 164 nuclei/field, respectively (in the last 2 groups, *P* < .05 compared to control). Wound edge release produced a 2- to 3-fold increase in granulation tissue apoptosis compared with control wounds after 24 hours of the released condition (*P* < .05; Fig 5, *inset*). This increase in apoptosis was not different from the increase seen with the MCF group (*P* > .05; Fig 5, *inset*). Hematoxylin-eosin histology of wounds at 2 to 4 days after wound edge release (data not shown) showed new formation of granulation tissue in the wound defect created by the release procedure (Fig 1, *B*).

DISCUSSION

The mechanism of MCF-induced granulation tissue regression and apoptosis observed in this study may be explained by 1 or more of the following 3 hypotheses: (1) the MCF procedure renders the granulation tissue ischemic; (2) paracrine or juxtacrine communication between the MCF and the granulation tissue signals the latter to up-regulate apoptosis; or (3) the MCF procedure decreases the mechanical loading on the granulation tissue. The MCF procedure, as illustrated in Fig 1,

involves a circumferential incision around the wound, yielding an "island" of granulation tissue. If the wound's vasculature originated from the dermal margin, then the MCF procedure would interrupt the wound's blood supply. This does not seem to be the case, however, because the wound's vasculature originates from the wound base (Fig 4). Furthermore, there is an absence of ischemic necrosis on all of the histologic sections, which would argue against the presence of ischemia. Alternatively, the overlying MCF could apply mechanical pressure to the underlying wound, which would impede capillary blood flow in the granulation tissue. Considering the laxity of the completed MCF as it lay over the wound (a subjective indicator, to be sure), this explanation also seems doubtful. Therefore, the possibility that the MCF procedure is inducing granulation tissue regression as a result of wound ischemia does not seem likely.

The next hypothesis that could explain the mechanism of MCF-induced granulation tissue regression is MCF-mediated paracrine and/or juxtacrine signaling. Interestingly, granulation tissue appears to have a graded response with respect to the thickness and type of the tissue drawn over it; the greatest wound regression occurs as a result of MCF coverage, the least regression occurs with a thin skin graft, and the result with a full-thickness skin graft is intermediate.[9,10] This dependence of the granulation tissue apoptotic response on the flap thickness would be consistent with a paracrine signaling hypothesis. The results of experiment 2 in this article, however, suggest that paracrine signaling is not important for the acute induction of granulation tissue apoptosis, because an equivalent degree of apoptosis was produced by simply releasing the wound edge (the initial step of the MCF procedure) as was produced by the full MCF procedure. Presumably, paracrine signaling was not a factor in the release-only wounds, because there was no biologic cover. Therefore, wound edge release appears to acutely inhibit granulation tissue.

The chronic inhibition of granulation tissue is a different circumstance. Although wound edge release in our model produces granulation tissue regression during the first 24 hours, the animal subsequently responds to the new wound defect created by the wound edge release (Fig 1, *B*) by generating fresh granulation tissue. In other words, there is a lag period after wound edge release during which there is an acute regression of the existing granulation tissue; this period is followed by a new proliferative phase in which the animal

attempts to fill the new wound defect. This new proliferative phase does not occur in the wounds that have an MCF pulled over the granulation tissue after wound edge release (Fig 2, *G* and *I*). This would argue that the MCF is important for chronic inhibition of the granulation tissue, which implies a role for paracrine/juxtacrine signaling. An attempt to determine the effect of an MCF without wound edge release would not be possible in this model, because creation of an MCF requires release of the wound edge and mobilization of the surrounding dermis.

Alteration of the wound's mechanical environment is the third, and hitherto unproposed, hypothesis explaining MCF-induced wound regression. Human fibroblasts in a three-dimensional collagen matrix are dependent on anchorage of the matrix to a rigid substratum for survival[14]; if the matrix anchorage to the substratum is disrupted, then the fibroblasts undergo apoptosis, even though they are still embedded in the matrix. The fact that fibroblasts in an anchored collagen matrix are mechanically loaded relative to fibroblasts in an unanchored matrix can be shown by comparing microscopic (actin cytoskeletal) and gross morphology between the 2 conditions.[14] Although we have no direct measure that the MCF procedure decreases mechanical strain in the wound matrix of our animal model, the cross-sectional morphologic change from a discoid "stretched" wound (the control) to an ovoid "relaxed" wound (post release) would suggest that the MCF procedure produces loss of wound matrix strain. We propose that MCF-induced alteration of mechanical strain is an important mechanism by which acute granulation tissue apoptosis is induced in our wound model.

REFERENCES

1. Clark RAF. Wound repair: overview and general considerations. In: The molecular and cellular biology of wound repair. New York: Plenum Press; 1996. p. 3-50.
2. Gabbiani G, Hirschel BJ, Ryan GB, Statkov PR, Majno G. Granulation tissue as a contractile organ: a study of structure and function. J Exp Med 1972;135:719-34.
3. Majno G, Gabbiani G, Hirschel BJ, Ryan GB, Statkov PR. Contraction of granulation tissue in vitro: similarity to smooth muscle. Science 1971;173:548-50.
4. Abercrombie M, Flint MH, James DW. Wound contraction in relation to collagen formation in scorbutic guinea-pigs. J Embryol Exp Morphol 1956;4:167-75.
5. Abercrombie M, Flint MH, James DW. Collagen formation and wound contraction during repair of small excised wounds in the skin of rats. J Embryol Exp Morphol 1954;2:264-74.
6. Billingham RE, Russell PS. Studies on wound healing, with special reference to the phenomenon of contracture in experimental wounds in rabbits' skin. Ann Surg 1956;144:961-81.
7. Lindquist G. The healing of skin defects: an experimental study on the white rat. Acta Chir Scand 1946;44(suppl 107):1-163.
8. Desmouliere A, Redard M, Darby I, Gabbiani G. Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. Am J Pathol 1995;146:56-66.
9. Garbin S, Pittet B, Montandon D, Gabbiani G, Desmoulière A. Covering by a flap induces apoptosis of granulation tissue myofibroblasts and vascular cells. Wound Rep Reg 1996;4:244-51.
10. Rudolph R. Inhibition of myofibroblasts by skin grafts. J Plast Reconstr Surg 1979;63:473-80.
11. Kane CD, Greenhalgh DG. Expression and localization of p53 and bcl-2 in healing wounds in diabetic and nondiabetic mice. Wound Rep Reg 2000;8:45-58.
12. Brown DL, Kao WW, Greenhalgh DG. Apoptosis down-regulates inflammation under the advancing epithelial wound edge: delayed patterns in diabetes and improvement with topical growth factors. Surgery 1997;121:372-80.
13. Carlson MA, Zhu M, Abrams JM, Grinnell F. Human fibroblasts in a mechanically stressed matrix undergo apoptosis after stress removal. Surg Forum 1998;49:643-5.
14. Grinnell F, Zhu M, Carlson MA, Abrams JM. Release of mechanical tension triggers apoptosis of human fibroblasts in a model of regressing granulation tissue. Exp Cell Res 1999;248:608-19.
15. Negoescu A, Lorimier P, Labat-Moleur F, Drouet C, Robert C, Guillermet C, et al. In situ apoptotic cell labeling by the TUNEL method: improvement and evaluation on cell preparations. J Histochem Cytochem 1996;44:959-68.
16. Cuello-Carrion FD, Ciocca DR. Improved detection of apoptotic cells using a modified in situ TUNEL technique. J Histochem Cytochem 1999;47:837-9.
17. Labat-Moleur F, Guillermet C, Lorimier P, Robert C, Lantuejoul S, Brambilla E, et al. TUNEL apoptotic cell detection in tissue sections: critical evaluation and improvement critical evaluation and improvement. J Histochem Cytochem 1998;46:327-34.
18. Strater J, Gunthert AR, Bruderlein S, Moller P. Microwave irradiation of paraffin-embedded tissue sensitizes the TUNEL method for in situ detection of apoptotic cells. Histochem Cell Biol 1995;103:157-60.