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Effect of SACCHACHITIN on Keratinocyte Proliferation and the Expressions of Type I Collagen and Tissue-Transglutaminase During Skin Wound Healing

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Abstract: SACCHACHITIN is a skin wound-healing membrane made of residual fruiting body of *Ganoderma tsugae*. Its effect on proliferating cell nuclear antigen (PCNA) expression in actively proliferating cells, type I collagen expression and tissue remodeling in the healing tissue, and the association of tissue-transglutaminase (t-TGase) with wound healing were investigated by immunohistochemical staining. The results demonstrated that PCNA expressed in keratinocytes since day 1 in the SACCHACHITIN group and persisted during entire healing process. In contrast, it was barely detectable on day 3 in the control group. At keratinocyte layer, the SACCHACHITIN group exhibited more type I collagen than did the control group since day 1. At scar tissue, type I collagen was positively stained in the SACCHACHITIN group since day 7 but not in the control group till day 12. Furthermore, t-TGase was strongly expressed on the inner wall of angiogenic vessels on day 5 of the control group but not on that of the SACCHACHITIN group until day 10. The earlier expressions of PCNA and type I collagen in the keratinocyte layer may lead to accelerated skin wound healing. In addition, the later expression of t-TGase, an indicator of apoptosis, on the inner wall of angiogenic capillaries in the SACCHACHITIN group may indicate a longer period of blood supply to the wound area, thus facilitating wound healing. These observed phenomena might underline the beneficial effects of SACCHACHITIN membrane on rapid wound healing. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 70B: 122–129, 2004

Keywords: *Ganoderma tsugae*; skin wound healing; type I collagen; proliferating cell nuclear antigen (PCNA); tissue-transglutaminase (t-TGase)

INTRODUCTION

Wound healing is a complex process, including inflammation, angiogenesis, cell proliferation, migration and differentiation, synthesis of the extracellular matrix, and remodeling of connective tissue.[1] The wound healing processes may be divided into three major steps: inflammation, tissue formation, and tissue remodeling. During the inflammation process, cytokine secretion, inflammatory cell production, and cell migration occur. The inflammation step is followed by tissue formation, such as formation of granulation tissue including lymphocytes and newly formed blood vessels. Finally, tissue remodeling takes place.

Reepithelialization of a wound occurs within hours after injury. Epithelial cells from residual epithelial structure move quickly across the wound defect. In the skin, keratinocytes of the stratified epidermal sheet or hair follicles appear to move one over the other in a leapfrog fashion.[2] One to 2 days after injury, epithelial cells at the wound margin start to proliferate.[3] Endothelial cells from the side of the venule closest to the angiogenic stimulus begin to migrate on the second day by projecting pseudopodia through fragmented basement membranes. Subsequently, these endothelial cells migrate into the perivascular space and other endothelial cells follow to form the new blood

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vessels.[4] This process of new blood vessel formation is called angiogenesis.

In addition to the proliferation and differentiation of cells around wound margin, the intercellular interaction is also essential to wound healing. The tissue consists of many kinds of cells embedded in the extracellular matrix (ECM). Nearby cells are connected closely by the ECM molecules including proteoglycan. There are two forms of ECM: interstitial matrix and basement membrane under epidermis and mesenchymal cells. The ECM molecule, such as type I collagen, is associated with cell interaction, migration, attachment, differentiation, and organization.[5] Collagens are a family of glycoproteins containing triple helices. Rigid helical collagen macromolecules aggregated into fibrillar bundles to provide the healing tissue with increasing stiffness and tensile strength.[6] The skin wound repairs through both the growth and differentiation of and the interaction between epidermal and dermal cells. Cell adhesion molecules are a family of closely related cell surface glycoproteins. They also play important roles in intercellular interactions, participate in the process of embryogenesis and development,[7] and also are involved in cell migration during wound repair.[8] Further, transglutaminase (t-TGase), a kind of cell adhesion molecules, is a calcium-dependent enzyme that cross-links the glutamate and lysine residue of two polypeptides.[9] The t-TGase has been suggested to be associated with the regulation of cell proliferation, differentiation, and wound healing.[10] It is also implicated in the modulation of cell apoptosis.[11]

The SACCHACHITIN membrane is a woven-able skin substitute made of the residual fruiting body of *Ganoderma tsugae*.[12] Previous studies in this laboratory demonstrated that the SACCHACHITIN membrane, as compared to control group, significantly accelerated the process of rat skin wound healing.[13] It has been concluded that the SACCHACHITIN membrane may have a chemotratic nature and causes an earlier inflammatory reaction at the wound area. This, in turn, results in an earlier angiogenesis, faster scar formation, and finally leads to faster wound healing.[13] The purpose of the current study is to assess the effect(s) of SACCHACHITIN on keratinocyte proliferation, type I collagen expression in the regenerating keratinocyte layer as well as the scar tissue, and the significance of t-TGase expression in the angiogenic tissue during wound healing.

MATERIALS AND METHODS

Materials

SACCHACHITIN membranes made from the residue of the fruiting body of *G. tsugae* were obtained from Dr. C. H. Su. Ketamine HCl and phenobarbital were purchased from Sigma Co. (St. Louis, MO). Wistar rats weighing from 350 to 450 g were from the Animal Center National Taiwan University. Rabbit type I collagen antibody and type I collagens as well as type II collagen were provided by Dr. W. F. Lai. Proliferating cell nuclear antigen (PCNA) monoclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, U S A). The t-TGase polyclonal antibody was purchased from Neomarkers (Fremont, CA).

Methods

Surgical and Animal Handling. Rats were anesthetized by intraperitoneal injection with ketamine (35 mg/kg) and pentobarbital (25 mg/kg) dissolved in phosphate-buffered saline (PBS). The dorsal hair of the rats was shaved with an electric razor. Two equal mirror-imaged areas (1.5×1.5 cm^2) on both sides off the dorsal central line were marked. Two pieces of full-thickness skin were excised. After cleaning off blood residues, the right sides of the lesions were covered with an autoclaved SACCHACHITIN membrane and the left sides with sterilized cotton gauze as control. Both dressings were prehydrated with 0.9% saline to assure adhesion of the dressing to the wound surface. During the entire course, the rats were housed in an air-conditioned room with 12/12 dark/light cycles. Regular rat chow and tapwater were provided.

Preparation of Histological Specimens. The rats were sacrificed with overdose pentobarbital on days 1, 3, 5, 7, 14, or 21 after skin injury. Lesions with the surrounding tissue were excised in a deep-V manner. The specimens were fixed in PBS-buffered 4% formaldehyde for 10–12 h, rinsed, and then embedded in paraffin for subsequent immunohistochemical studies. Serial sections of 5- to 8-μm thick specimens were mounted on poly-L-lysine-coated slides.

Immunohistochemical Staining. Each specimen was incubated at 60°C for 10 min to melt the wax, deparaffined twice with xylene for 15 min each, followed by sequentially immersing in 100% ethanol, 95% ethanol, 75% ethanol, and 50% ethanol for 2 min each, and then placed in dd-H_2O for 5 min. The slide was boiled in a buffer containing 0.01*M* citrate and 0.1% tween-20 for 8 min, and then cooled for 20 min. After treatment with 6% H_2O_2/methanol to remove the endogenous peroxidase, the slide was washed with dd-H_2O and rinsed with PBST (PBS containing 0.1% tween-20) for 5 min. The specimen was blocked with 10% skim milk for 30 min at room temperature (RT) followed by incubation with primary antibody in 10% skim milk either at 4°C overnight or at RT for 1 h. After washing with PBST twice, an aliquot of avidin blocking solution (Signet Laboratories, Inc.) was added for 10 min to remove the endogenous biotin, and then rinsed twice with PBST. The specimen was incubated again with biotin blocking solution (Signet) for 10 min to remove the excessive avidin, and then washed twice with PBST. After incubation with biotinylated linking reagent (DAKO LSAB® 2 System, DAKO) for 10 min at RT and washed twice with PBST, the specimen was incubated with strepavidin conjugated with peroxidase (DAKO LSAB® 2 System, DAKO) for 10 min at RT and then washed with PBST twice. Finally, an aliquot of DAKO liquid DAB^+ substrate-chromogen system (DAKO) was added for 5 min and then rinsed

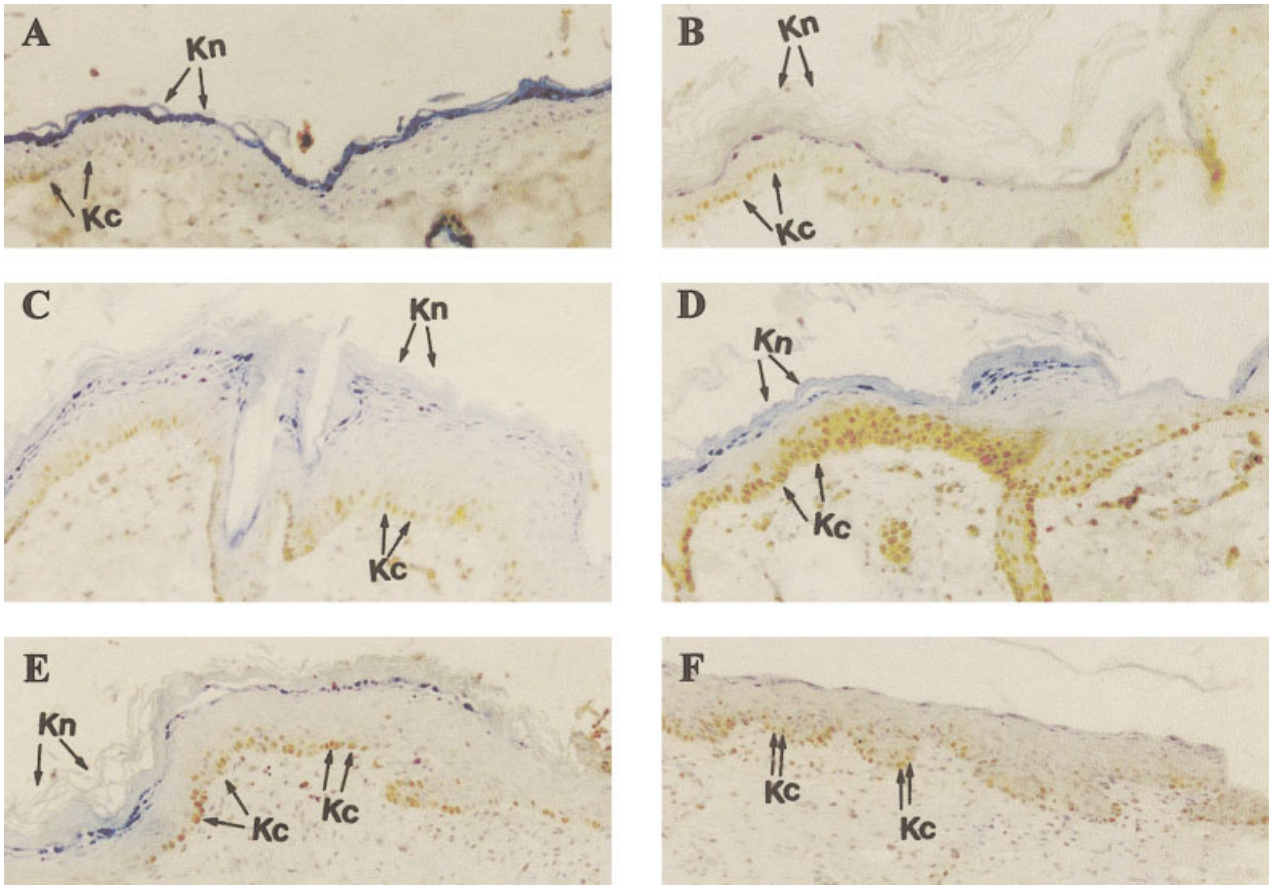

Figure 1. Immunohistological staining for the expression of PCNA at the keratinocyte layer. (A), (C), and (E) are specimens from wound margin tissues at 1, 3, and 14 days, respectively, after skin injury of the control groups; (B), (D), and (F) are those from the respective SACCHACHITIN membrane-treated groups. The brown cells are PCNA-positive cells. All specimens were observed at 100-fold magnification. Kn, keratin; Kc, keratinocyte. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

with dd-H_2O. The specimen was finally counterstained with hematoxylin for 2 min, rinsed with water, and mounted in 50% glycerol with cover glass. In the case of type I collagen staining, the protocol was similar except the use of biotin-conjugated goat antiswine immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories, Inc.) instead of biotinylated linking reagent. The swine antirabbit type I collagen primary antibody was incubated with specimen at 4°C overnight.

SDS Gel Electrophoresis and Western Blot. Collagen samples (1–20 μg) were lyophilized, redissolved in 10 μL sample buffer (100 m*M* β-mercaptoethanol and 2% SDS), and boiled for 5 min. The specimens were electrophoresed on SDS polyacrylamide gel (6%) to verify their purity. For Western blot analysis, the collagen samples were transblotted to nitrocellulose membrane at 0°C for 4 h. The membrane was blocked with 10% skim milk at RT for 1 h, followed by incubating with the primary antibody (1:400 in 10% milk) at 4°C for 24 h. After thoroughly washed, the membrane was incubated with goat antiporcine IgG-conjugated with peroxidase (1:2000, 0.2 μg/mL) at RT for 2 h. Type I collagen bands were visualized with DAB in 0.06% H_2O_2 solution for 10 min.

RESULTS AND DISCUSSION

Proliferation of Keratinocytes

PCNA is a 36-KDa protein.[14] This protein has been identified as the polymerase δ-associated protein[15] and is synthesized in early G1 and S phases of the cell cycle.[16] PCNA monoclonal antibody was used to investigate the effect of SACCHACHITIN on the proliferation of keratinocytes at wound margin during wound healing. On day 1 postinjury, the PCNA was detected in the keratinocyte layer covered with SACCHACHITIN membrane [Figure 1(A, B)] but not in the control group until day 3 [Figure 1(C, D)]. The staining in specimen from day 3 postinjury rats covered with SACCHACHITIN membrane was stronger than that from day 1 with SACCHACITIN membrane and that from day 3 control group. Keratinocyte layer of both groups displayed stronger PCNA staining on day 5 [Figure 1(E, F)] than those on day 3, respectively, and the expression persisted on days 7 and 14. The PCNA expression at the keratinocyte layer was located from the edge of the wound margin toward the direction of the wound center, where it was not healed yet. The proliferation of keratinocytes was more prominent on the wound area

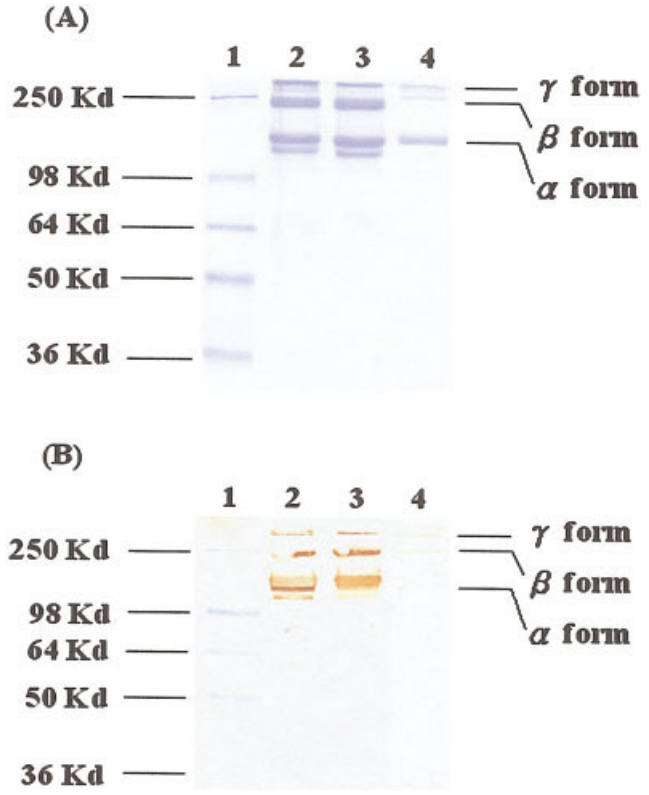

Figure 2. Interaction of porcine antirabbit type I collagen with rabbit and rat type I collagen analyzed by Western blotting. Types I and II collagens were compared on 10% SDS acrylamide gel (A) and reacted with porcine anti-rabbit type I collagen antibody after being transferred to nitrocellulose membrane (B). Lane 1 is protein marker. Lanes 2 and 3 are type I collagen of rabbit and rat, respectively. Lane 4 is rabbit type II collagen. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

covered with SACCHACHITIN membrane than that with cotton gauze [Figure 1(E, F)]. These results suggest that the onset of detectable keratinocyte proliferation in the SACCHACHITIN group was much earlier (up to 2 days) than that in the cotton gauze group. The earlier and faster the keratinocytes proliferate, the faster the wound healing proceeds.

Expression of Type I Collagen in Extracellular Matrix of the Keratinocyte

The swine antirabbit type I collagen antibody prepared by this research team was tested for its species cross-reactivity with rat type I collagen by Western blotting. As shown in Figure 2, the swine antiserum against rabbit type I collagen recognized rat-tail type I collagen as well as rabbit type I collagen. The antibody was hence used to study the expression of type I collagen in the extracellular matrix of the rat keratinocyte layer during skin wound healing.

It is of interest to note that the antibody did not stain type I collagen in the extracellular matrix of the keratinocyte of intact skin as well as that in the day 1 skin specimens of the control group [Figure 3(A)]. During the process of immunohistochemical study, the tissue specimens were boiled in citric buffer. This treatment was not as severe as boiling acid-soluble collagen samples in the loading buffer containing SDS and β-mercaptoethanol for Western blot analysis. The treatment of tissue specimens was obviously not tough enough to break the rigid structure of collagen bundles. Based on this assumption, the failure of the antibody to stain the intact keratinocyte layer may indicate that the swine antibody only interacts with the newly formed or denatured type I collagen and perhaps type I collagen fibril but not with the more matured collagen bundles under the current experimental conditions.

In the SACCHACHITIN group, the staining of type I collagen around keratinocytes was slightly positive on day 1 ($\approx$24 h) after injury as compared with that in the control group [Figure 3(A, B)]. On day 5, the staining was stronger in the SACCHACHITIN group than in the control group [Figure 3(C, D)]. The same results were observed on days 7, 10, 12, and 14 [Figure 1(E, F)]. It is interesting to note that the ECM of keratinocytes in the older (intact) tissue [to the left side of Figure 3(C, E, F)] exhibited less or no type I collagen staining. The observation further supports the assumption stated in the last sentence of the previous paragraph.

In summary, much thicker and stronger type I collagen fibril at the keratinocyte layer in the SACCHACHITIN group was observed since day 5 [Figure 3(D)]. This may suggest that there is more ECM synthesized in and secreted into the surrounding of these actively proliferating cells. It also appears that the keratinocyte in the SACCHACHITIN group are much larger in size/volume. This may indicate again a more active cellular activity in the SACCHACHITIN group. Further, richer ECM not only facilitates matrix–cell interaction but also supports cell migration and proliferation.[17] According to these results, the SACCHACHITIN membrane might induce type I collagen synthesis in keratinocytes and subsequently provide themselves with better basis for proliferation and migration, thus promoting early wound healing.

Expression of t-TGase During Wound Repair

Previous studies in this laboratory have demonstrated that the newly formed angiogenic capillary vessels were observed in wound tissue of both groups on day 3.[13] The vessel wall was much thicker in the SACCHACHITIN group than that in the control group on day 3, indicating the angiogenic vessel formation in the former was much earlier than that in the latter. The current immunohistochemical study revealed that strong t-TGase staining found on day 5 was only on the inner vessel wall of the control group but not the SACCHACHITIN group [Figure 4(A, B)]. On day 7, there is more t-TGase staining in the control group but only slightly on the inner vessel wall in the SACCHACHITIN group [Figure 4(C, D)]. On day 10, the staining of t-TGase was decreased in the control group while that in the SACCHACHITIN group turned stronger [Figure 4(E, F)]. The t-TGase staining was hardly observed on day 14 in the control group but remained obvious [Figure 4(G, H)] in the SACCHACHITIN group.

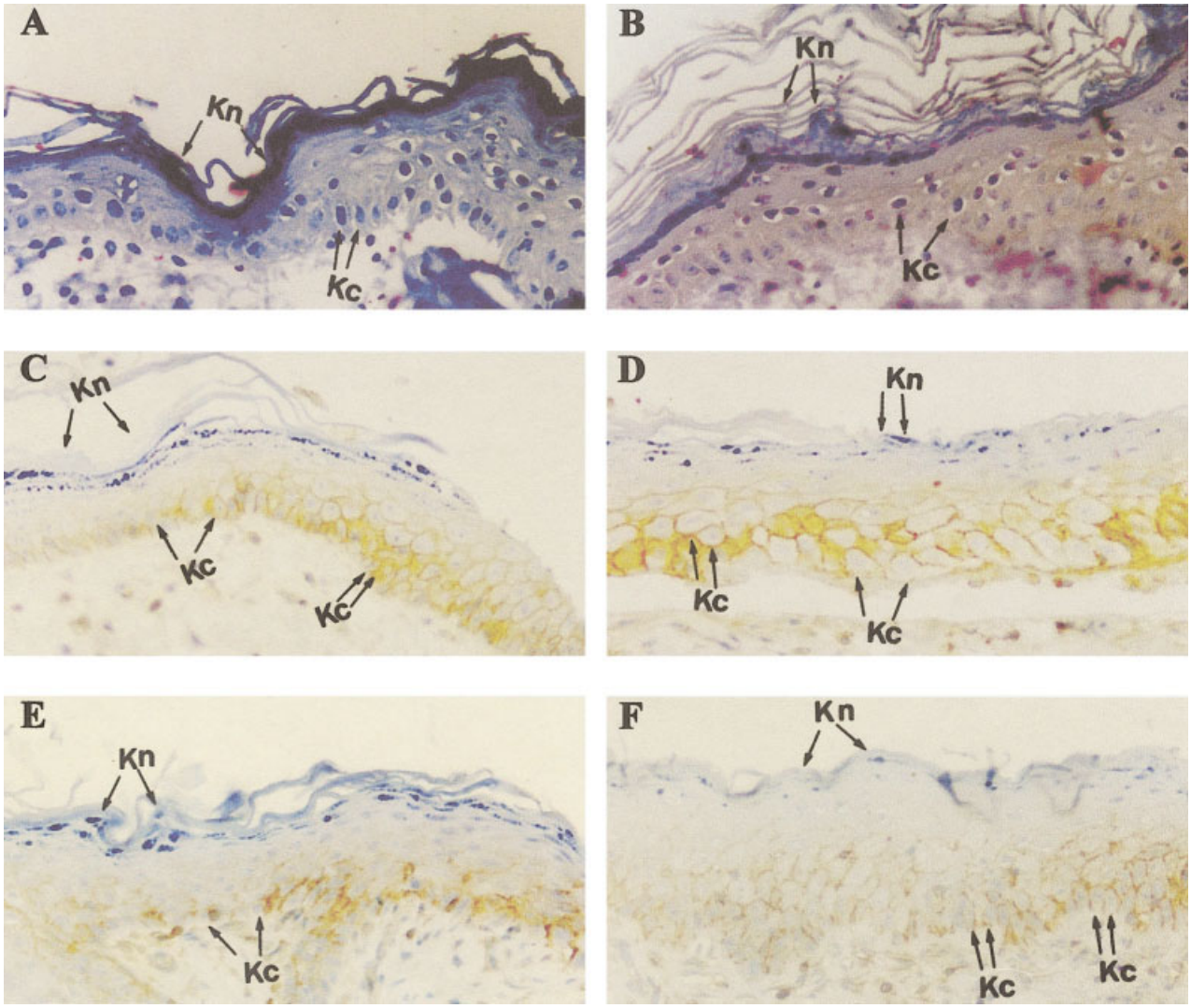

Figure 3. Immunohistological staining for the expression of type I collagen around the keratinocyte at the wound margin. (A), (C), and (E) are rat skin specimens from wound margins of the control groups 1, 5, and 14 days, respectively, after skin injury; (B), (D), and (F) are those of the respective SACCHACHITIN groups. The bright yellowish brown fibril network surrounding the keratinocyte (Kc) are freshly formed type I collagen fibrils. The slides were observed at 200× magnification. Wound margins were toward the right of the histographies. Kn, keratin; Kc, keratinocyte. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Overall, a strong t-TGase staining was observed in the control group as early as day 5, persisted, and declined since day 10. The appearance of t-TGase must be earlier than day 5. In the SACCHACHITIN group, the t-TGase staining was barely detected on day 7 and gradually increased at least up to day 14. Numerous articles reported that increased activity of t-TGase was correlated with onset of apoptosis and may serve as an indicator or modulator of apoptosis.[11] In addition, the antibody used in this study is specific to t-TGase and does not interact with factor XIII in the blood. In these regards, the late expression of t-TGase on inner vessel wall of the angiogenic capillaries in the SACCHACHITIN group may imply that there was a longer period of sufficient blood supply to the wound area. This may directly contribute to promoting accelerated wound healing.

Expression of Type I Collagen in the Scar Tissue During Skin Wound Healing

Subsequently, type I collagen antibody was used to study the expression of type I collagen in scar tissue of the healing wound. On day 7 after skin injury, there was a stronger staining of type I collagen in the scar tissue of the SACCHACHITIN group than that of the control group [Figure 5(A, B)]. The same result was observed on day 10 [Figure 5(C, D)]. On day 12, significant staining of type I collagen in the newly formed scar tissue of the control group was first observed, while that persisted since day 7 in the SACCHACHITIN group [Figure 5(E, F)]. On days 14 and 21, the relative intensities of type I collagen staining in both groups were similar; but more staining was presented in the SAC-

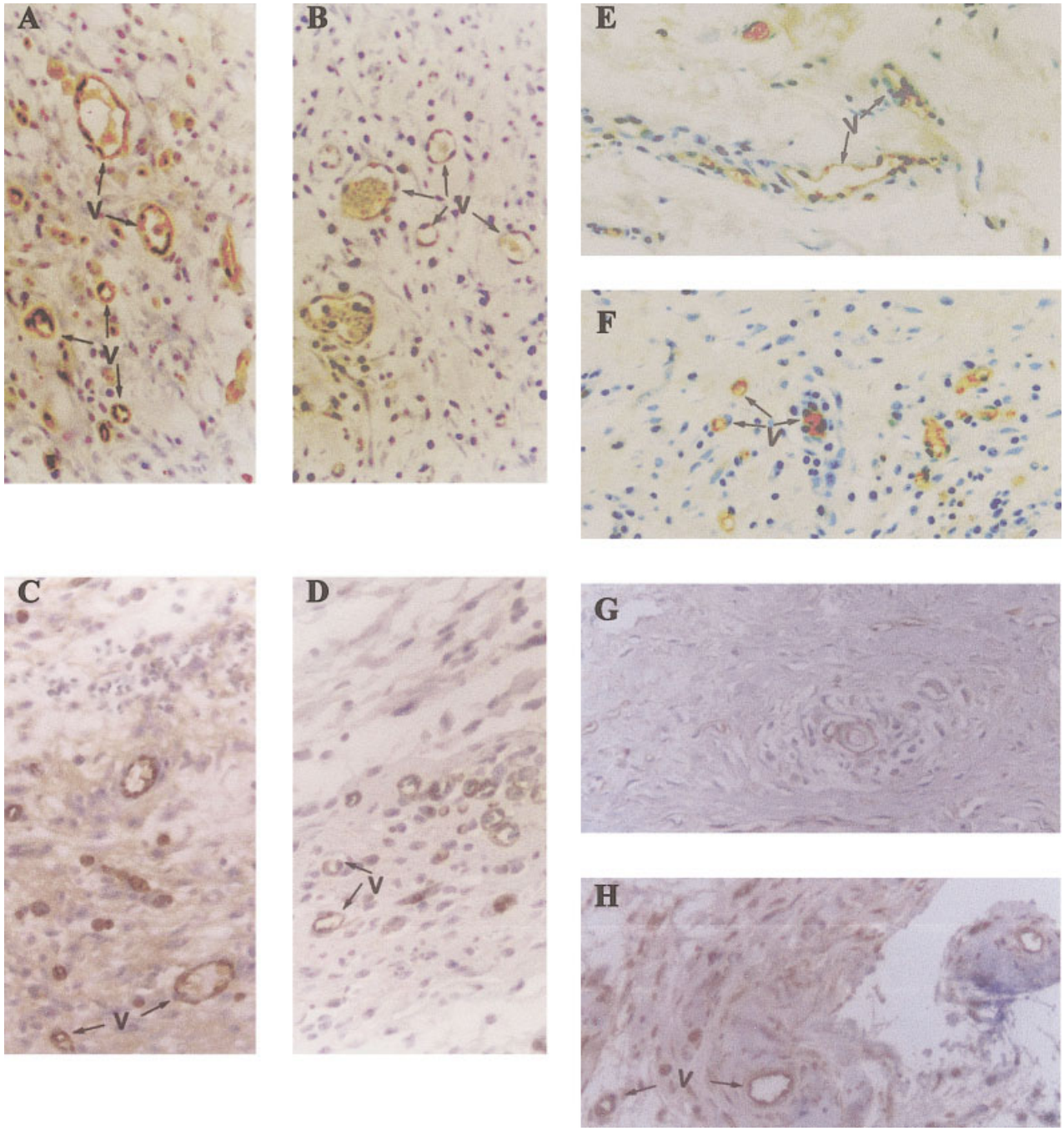

Figure 4. Immunohistological staining of angiogenic capillaries during skin wound healing with monoclonal antitissue transglutaminase. (A) and (B) are specimens of the control and SACCHACHITIN-treated tissues, respectively, from wound margins 5 days postinjury; (C) and (D), (E), and (F), or (H) and (G) are those from wound margins 7, 10, or 14 days postinjury, respectively. The brown spots are t-TGase-positive cells, which were probably in the process of apoptosis. The histographies were observed at 200× magnification. V, capillary vessels. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CHACHITIN group than in the control group [Figure 5(G, H)].

There are at least two groups of signal-modulating molecules: the soluble factors such as cytokines or growth factors and the insoluble factors including ECM. These molecules could directly or indirectly modulate cell growth, migration, and differentiation through various signaling pathways[6] and then promote tissue regeneration. According to the above data, it is deduced that significant expression of newly formed type I collagen in scar tissue occurred almost 5 days earlier in the SACCHACHITIN-treated tissue (on day 7) than that in the control group (on day 12). Collagen is one of the major

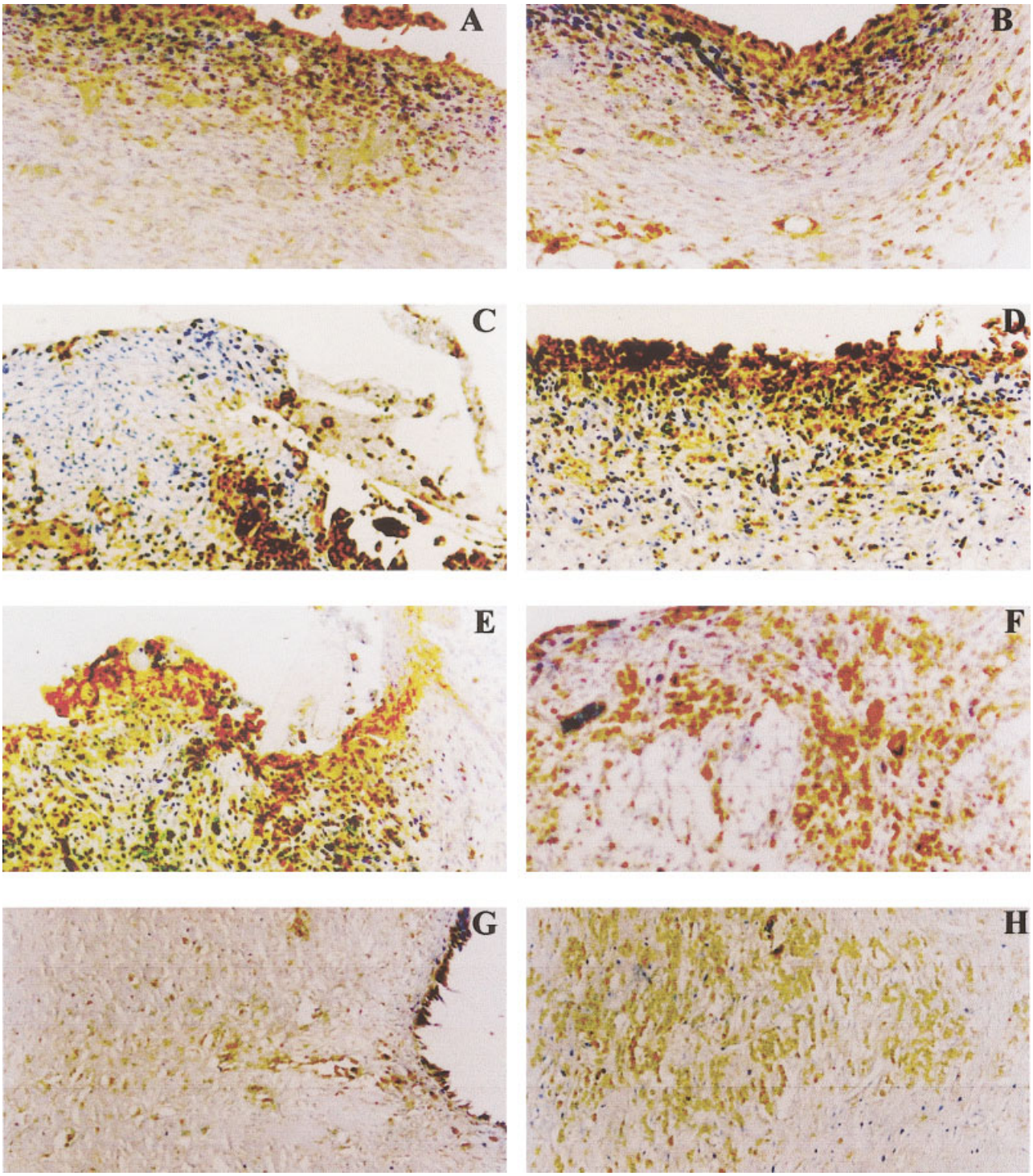

Figure 5. Immunochemical staining of type I collagen in rat skin scar tissues. Scar tissue specimens from wound margins 7 days (A and B), 10 days (C and D), 12 days (E and F), and 21 days (G and H) after skin injury were interacted with porcine antirabbit type I collagen antibody and visualized with DAB as described in the Methods section. The specimens were photographed at 100× magnifications. (A), (C), (E), and (G) are photographs of control specimens while (B), (D), (F), and (H) are those of wound margin tissues covered with SACCHACHITIN. Skin wound healing either toward the top (A–F) or the right (G and H) of the histography. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

components of ECM, which provides bases for intercellular interaction and cell migration. The earlier expression of type I collagen in the scar tissue of the experimental group may lead to an earlier regeneration of the ECM. This, in turn, results in faster tissue remodeling and wound healing.

CONCLUSION

The SACCHACHITIN membrane promotes early expression of PCNA in the keratinocyte layer and type I collagen in both keratinocyte layer and scar tissue. These would lead to accelerated wound healing. On the other hand, the later expression of t-TGase on the inner wall of the newly formed capillary vessels in the SACCHACHITIN group may imply a longer existence and later degeneration of these capillaries. A longer period of sufficient blood supply to the wound site might facilitate tissue regeneration and faster wound healing in the SACCHACHITIN group.

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