Glutaraldehyde crosslinking of collagen substrates inhibits degradation in skin substitutes grafted to athymic mice

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Collagen-based implants have been described as vehicles for transplantation of cultured skin cells for treatment of burn wounds. To optimize vascularization and repair of connective tissue, collagen solubility and glutaraldehyde crosslinking were evaluated. Cultured skin substitutes consisted of human keratinocytes and fibroblasts attached to collagen–glycosaminoglycan substrates that were prepared from acid-insoluble, or partially soluble collagen. Substrates were crosslinked with 0% or 0.25% glutaraldehyde, populated with cells, and grafted to full-thickness wounds on athymic mice (n = 6/condition). After 6 weeks, the wound area was measured by planimetry, and healed wounds were scored by histochemistry for immunoreactivity to HLA-ABC and bovine collagen. Data analysis shows that crosslinking of collagen implants with glutaraldehyde is associated ($p < 0.001$) with detection of the implant. No association was found between solubility of bovine collagen and immunodetection. Epidermis of all wounds was positive for HLA-ABC, and no differences in wound areas were found. These results suggest that glutaraldehyde crosslinking of collagen implants decreases the rate of biodegradation. Delayed degradation of crosslinked collagen may result clinically in reduced engraftment of skin substitutes.

INTRODUCTION

Efficacy of skin substitutes for permanent closure of full-thickness wounds requires restoration of anatomic structure and recovery of the physiologic functions of human skin. Requirements for skin substitutes include: rapid attachment to the wound bed; stimulation of ingrowth of fibrovascular tissue; and minimal scarring and contracture.[1] An ideal skin substitute would induce scarless dermal repair in the course of normal wound healing.[2] Scarring and wound contracture, which are associated with poor cosmetic and functional results, are common outcomes in the treatment of large skin wounds with conventional split-thickness mesh skin grafts.[3,4] Alternative materials for the treatment of skin wounds have been developed and tested with highly variable results.[5–13] Cultured epithelial autografts that are prepared by enzymatic release of keratinocyte sheets show slow formation of dermal–epidermal junction, leading to blistering, ulceration, and higher degrees of wound contracture.[8–11] Composite skin substitutes consisting of dermal and epidermal components provide better analogy to native skin and have been reported not to blister after grafting.[12–14]

Restoration of new connective tissue depends in part on collagen solubility and chemical crosslinking of the biopolymer implant. Orthotopic grafts of uncrosslinked collagen sponge applied as a dermal substitute stimulated production of dermal markers, decreased scar formation, and reduced wound contraction.[15,16] Crosslinked collagen has been shown to elicit minimal, if any, immunologic reactions.[17–20]

Previous studies describe permanent closure of full-thickness burns with cultured skin substitutes (CSS) consisting of cultured autologous keratinocytes and fibroblasts attached to collagen–glycosaminoglycan (C-GAG) substrates.[14,21] CSS become fully integrated with fibrovascular tissue and develop histiotypic properties, including pigmentation, that are comparable to native skin.[20,21] The CSS rapidly develop a continuous basement membrane, which prevents skin blistering[14,21] and promotes formation of stable epidermal tissue in a manner similar to meshed split-thickness autograft.[20]

This report describes C-GAG substrates prepared from either insoluble or partially soluble collagen and crosslinked with 0% or 0.25% glutaraldehyde. The C-GAG substrates were populated with cultured human

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skin cells and grafted to full-thickness skin wounds in athymic mice. No significant differences were found relative to collagen solubility, but only crosslinked collagen implants were detected at six weeks after grafting.

MATERIALS AND METHODS

Experimental conditions for biopolymer substrate preparation

All skin substitutes used in this study consisted of cultured human keratinocytes and fibroblasts attached to collagen–glycosaminoglycan (C-GAG) biopolymer substrates.[6] The substrates were prepared as previously described[22] with the following experimental changes in the procedure. Two different types of collagen, insoluble and partially soluble, were used to make the C-GAG co-precipitate (0.600% w/v), which was casted and lyophilized to produce acellular C-GAG matrices. The insoluble collagen was made from comminuted bovine hide collagen (USDA Eastern Regional Facility, Philadelphia, PA) partially reconstituted in 0.5*M* acetic acid and co-precipitated with chondroitin-6-sulfate (Sigma Chemical Co., St. Louis, MO). The partially soluble collagen consisted of a 50/50 mixture (% weight) of the insoluble collagen and medical grade soluble bovine collagen (Semed S, Kensey Nash Corp., Exton, PA). Two lyophilized C-GAG substrates from the same preparations of each collagen type (insoluble or partially soluble) were rehydrated using a variation of the process described in a previous report.[22] One substrate of each type was chemically crosslinked in 0.25% glutaraldehyde in 0.05*M* acetic acid (+ glut) for 24 h during the rehydration procedure, and a second substrate was incubated in 0.05*M* acetic acid without glutaraldehyde (− glut).

Preparation of cultured skin substitutes (CSS)

CSS were prepared as previously described from collagen–glycosaminoglycan substrates populated with human dermal fibroblasts and epidermal keratinocytes.[23] Briefly, dermal fibroblasts were inoculated into the porous surface of the C-GAG membrane and allowed to incubate for 1 day, at which time the membranes were inverted and keratinocytes were inoculated onto the nonporous, laminated surface. Composite grafts were incubated for 1 day submerged in MCDB 153 medium containing 0.2 m*M* calcium. On culture day 2, the calcium concentration was adjusted to 0.5 m*M*, and bovine pituitary extract was replaced with lipid supplements.[24] On culture day 3, the grafts were lifted to the air–liquid interface, and the medium was adjusted to reduce the epidermal growth factor to 1 ng/mL and to increase the calcium to 1.0 m*M*. On culture day 4, the calcium was increased to 1.5 m*M*, the epidermal growth factor was removed, and the grafts were maintained in this medium until grafting to athymic mice.

Grafting of skin substitutes to athymic mice

All animal studies were approved by the University of Cincinnati Institutional Animal Care and Use Committee. NIH guidelines for the care and use of laboratory animals have been observed. Cultured skin substitutes from the four experimental conditions (insoluble or partially soluble C-GAG ± glutaraldehyde crosslinking) were grafted onto athymic mice (BALB/c, nu/nu) on culture day 14 or 15 (n = 6/condition).[23,25] Daily irrigation of 1.0 cc of topical nutrients and antimicrobials plus recombinant human basic fibroblast growth factor (10 ng/mL) were injected into gauze dressings on days 1–13 after surgery.[23,26] On day 14 after surgery the dressings and stenting sutures were removed from all animals, and data collection was initiated as described below. The mice were rebandaged from days 14–27 with a dressing of N-Terface (Winfield Laboratories, Inc., Dallas, TX), Xeroform (Sherwood Laboratories, St. Louis, MO), cotton gauze, and Coban (3M Medical-Surgical Division, St. Paul, MN). The dressings used for days 28–41 after surgery consisted of N-Terface, cotton gauze, and Coban. Wound perimeter tracings were collected and photographs taken at 2, 3, 4, and 5 weeks after surgery. All animals had their dressings removed on day 42 after surgery. They were then photographed and the wound perimeters traced, after which the animals were sacrificed and tissue samples collected for immunohistochemistry and histology.

Morphological analysis

Graft biopsies were bisected and prepared for microscopic analysis. One half was frozen in OCT embedding compound, sectioned on a cryostat, and prepared for immunohistochemistry using standard procedures. The remaining half was fixed with 2% glutaraldehyde/2% paraformaldehyde in 0.1*M* sodium cacodylate (pH 7.4). Samples were postfixed in 1% osmium tetroxide and embedded in an epon–araldite mixture. Semithin sections (~1 μm) were stained with toluidine blue and examined using a Nikon Microphot-FXA microscope.

Antibody specificity determination

ELISA assays were performed to determine the sensitivity and specificity of the antibody system used for immunostaining of implanted collagen substrates (insoluble or partially soluble) in histological samples (see below). A nonradioactive modification of previously described methods[19] was used to detect a dilution series of both collagen solubilities. C-GAG coprecipitates were serially diluted with PBS (pH 7.6) to final concentrations of 100, 30, 10, and 3 μg/mL. Triplicate assays were performed by pipetting 100 μL of each C-GAG dilution into wells of a microwell plate (Nunc, Inc., Naperville, IL), followed by overnight incubation at 4°C to facilitate protein binding. Nonspecific antibody attachment was assessed in wells without C-GAG (0 μg/mL) by overnight incubation with bovine serum albumin (BSA) in PBS (0.5% w/v). A dilution series (1:20, 1:80, and 1:2000) of primary antibody with minimal crossreactivities (data reported by manufacturer) was reacted against the C-GAG dilutions to determine detection limits. Following C-GAG incubation and removal of excess fluid, the wells were washed three times with wash buffer (PBS/Tween 20/BSA). Rabbit anti-bovine collagen type I (Biodesign International, Kennebunkport, ME) was used as the primary antibody with 100 μL of each dilution incubated for 1.5 h at room temperature in wells coated with C-GAG or BSA. The plates were washed three times and 100 μL (0.5 μg/mL) of biotin–conjugated donkey anti-rabbit IgG, H & L (Biodesign International) was incubated for 1 h at room temperature in all wells. The wells were washed and secondary antibody was detected by reaction of all wells with 100 μL (1.5 μg/mL) of peroxidase-labeled strept-Avidin (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) for 30 min at room temperature. Bound peroxidase was detected by reaction of 100 μL/well *o*-phenylenediamine dihydrochloride solution (Sigma Chemical Co., St. Louis, MO) for 15 min at room temperature, with 50 μL/well 2*N* sulfuric acid used to stop the reaction. The chromogenic product was quantified by measuring optical density (O.D.) at 490 nm using a microplate reader (Cambridge Technologies, Inc., Cambridge, MA). Data are expressed as optical density (mean) for each collagen/antibody reaction ($n = 3$/condition).

Data collection and analysis

Wound area tracings and photographs were recorded at weekly intervals, starting 2 weeks after grafting, to monitor wound area and engraftment of human keratinocytes. Tissue samples collected at six weeks postsurgery were used to determine immunostaining for HLA-ABC antigens on keratinocytes[27] and implanted bovine collagen. Wound area was determined by direct image analysis (Image-1, Universal Imaging Corporation, Media, PA) of areas within tracings of wound perimeters. Data for wound contraction are expressed as a percentage of the original wound area (mean ± SEM).[27]

Epithelial engraftment was determined by direct immunofluorescence staining of healed epidermis with FITC-labeled monoclonal antibody (Accurate Chemical and Scientific Corporation, Westbury, NY) against a common hapten of the HLA-ABC histocompatibility antigens.[28]

Implanted C-GAG substrates were detected by indirect immunoperoxidase staining of wound biopsies with the same primary antibody against bovine collagen type I used in the ELISA procedure. Biotin conjugated donkey antibody against rabbit IgG, H & L was used as the secondary antibody. Peroxidase-labeled strept-Avidin was used to provide the enzyme that was detected using a diaminobenzidine kit (Pierce Chemical Co., Rockford, IL) to produce a chromogenic reaction product. Tissue samples used for immunohistochemical staining were prepared from cryostat sections of unfixed, fresh-frozen excised skin from wound sites. Scoring of epithelial engraftment (HLA-ABC-positive) and persistence of implanted collagen (bovine collagen type I positive) are expressed as a percentage of immunopositive wounds in each test group. Data from wound contraction and immunohistochemical staining were subjected to ANOVA testing, and groups that showed a significant difference were subjected to Fischer's Exact Test.

RESULTS

Wound closure on athymic mice

Figure 1 shows representative animals from each test condition 2 weeks after grafting. Grafts prepared from C-GAG without glutaraldehyde were fused with the surrounding murine skin and were beginning to develop focal areas of pigmentation by passenger melanocytes from epidermal cultures [Fig. 1(A,C)]. Conversely, grafts containing C-GAG that was crosslinked with glutaraldehyde had thinner, less uniform epithelium, were not completely fused, and were less pigmented [Fig. 1(B,D)]. Gross examination showed no differences among wounds treated with skin substitutes prepared from collagens of different solubility [Fig. 1(A) versus 1(C) and 1(B) versus 1(D)]. By 6 weeks after grafting, all wounds were completely closed with

hyperpigmented epithelium (not shown). There were no obvious differences at 6 weeks among those grafts prepared from collagens of different solubility or among those prepared from collagen treated with or without glutaraldehyde.

Microscopic examination of healed wounds

Figure 2 compares photomicrographs of the histologic appearance 6 weeks after grafting of regenerated skin from representative animals for each condition: (A) insoluble collagen not crosslinked; (B) insoluble collagen crosslinked; (C) partially soluble collagen not crosslinked; and (D) partially soluble collagen crosslinked. Keratinized epidermis is attached to regenerating connective tissue that is fully vascularized. Noteworthy is the absence of the implanted collagen in the newly regenerated connective tissue in wounds treated with grafts prepared from not crosslinked collagen [Fig. 2(A,C)] and the persistence of glutaraldehyde crosslinked collagen 6 weeks after grafting in healed wounds [Fig. 2(B,D)]. Reticulations of the crosslinked collagen are clearly visible in the connective tissue.

Wound area after grafting

Tracings of wound perimeters from healing CSS at 2, 3, 4, 5, and 6 weeks after grafting were converted to area measurements using computerized planimetry. Plots of wound area versus time after grafting show a time-dependent reduction in wound area during healing of CSS in athymic mice. The data indicate no significant differences in wound contraction among the four treatment groups (Fig. 3).

Primary antibody specificity

Figure 4 presents data from enzyme-linked immuno-sorbent assays (ELISA) performed using a dilution series of primary antibody to detect decreasing amounts of insoluble C-GAG [Fig. 4(A)] or partially

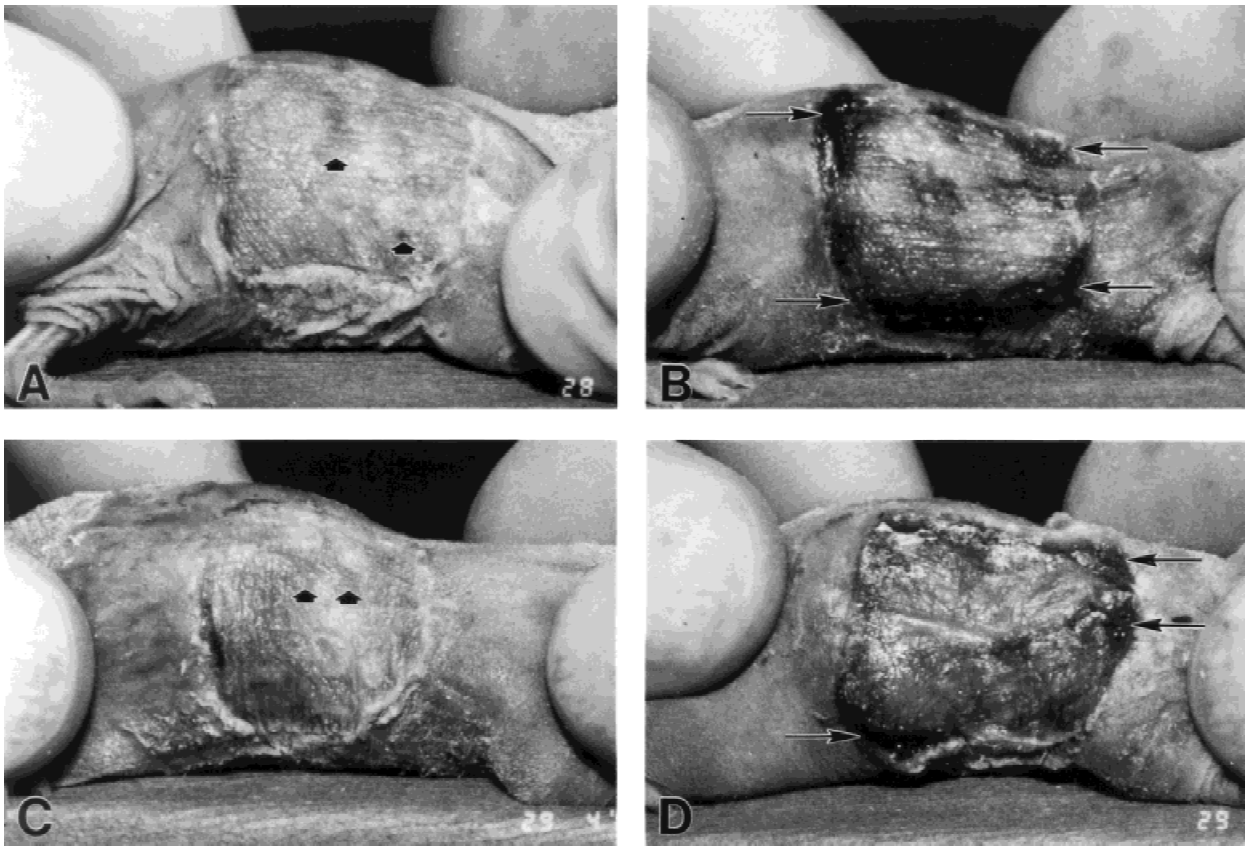

Figure 1. Appearance of athymic mice 2 weeks after grafting: **(A)** insoluble collagen not crosslinked; **(B)** insoluble collagen crosslinked; **(C)** partially soluble collagen not crosslinked; **(D)** partially soluble collagen crosslinked. Noteworthy at this time point is that wounds treated with grafts prepared with glutaraldehyde crosslinked collagen **(B, D)** are not completely fused along the wound perimeter (arrows) while those treated with not crosslinked collagen **(A, C)** are fused and exhibit focal pigmentation (arrowheads).

soluble C-GAG [Fig. 4(B)]. The data indicate a collagen concentration-dependent decrease in optical density for all concentrations of primary antibody independent of collagen solubility. The 0 mg/mL (bovine collagen) values for all three primary antibody dilutions were significantly lower ($p < 0.05$) than the respective collagen dilution series for both C-GAG types, indicating minimal nonspecific protein/antibody binding. C-GAG of both types produced significantly higher O.D. ($p < 0.05$) when the values for RαB Col I 1:80 versus 3 μg/mL collagen were compared to RαB Col I 1:20 versus 0 μg/mL collagen and RαB Col I 1:2000 versus 3 μg/mL collagen. These results demonstrate that the primary antibody chosen for this study is sensitive to and specific for both types of C-GAG used.

Immunostaining for bovine collagen

Immunostaining of healed skin for bovine collagen was performed on frozen sections (Fig. 5). Positive staining of the bovine collagen substrate prior to grafting [Fig. 5(A)] confirms specificity of the primary antibody. Collagen reticulations are stained throughout the C-GAG substrate. Specific staining of bovine collagen fragments was detected in healed wounds treated with skin substitutes prepared with glutaraldehyde crosslinked collagen [Fig. 5(B)]. Collagen reticulations were detectable in the area below the healed epidermis. No staining was detected in healed wounds treated with skin substitutes prepared from collagen that was not crosslinked [Fig. 5(C)]. Absence of nonspecific binding of secondary antibody and chromogen was confirmed by lack of reticulation staining upon omission of the primary antibody [Fig. 5(D)].

Percent immunopositive for HLA-ABC expression and bovine collagen in healed wounds

Figure 6 shows a plot of data from immunostaining of tissue sections from the four experimental groups.

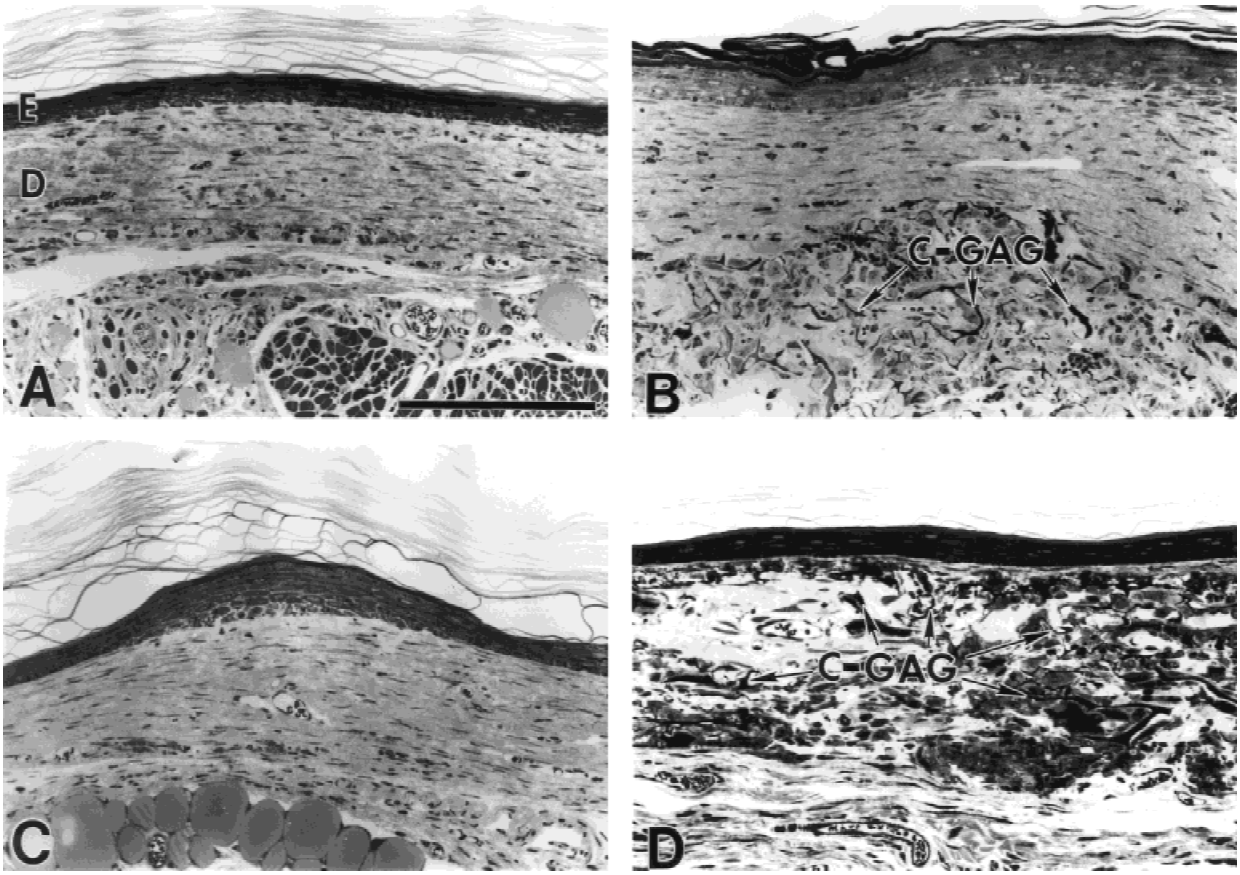

Figure 2. Histologic examination of healed skin 6 weeks after grafting to athymic mice: **(A)** insoluble collagen not crosslinked; **(B)** insoluble collagen crosslinked; **(C)** partially soluble collagen not crosslinked; **(D)** partially soluble collagen crosslinked. Histologies show hypertrophic, hyperkeratotic epidermis **(E)** attached to a well-vascularized dermis **(D).** Fragments of C-GAG implant (arrows) persist in wounds healed with grafts prepared from glutaraldehyde crosslinked collagen **(B, D).** Bar = 160 μm.

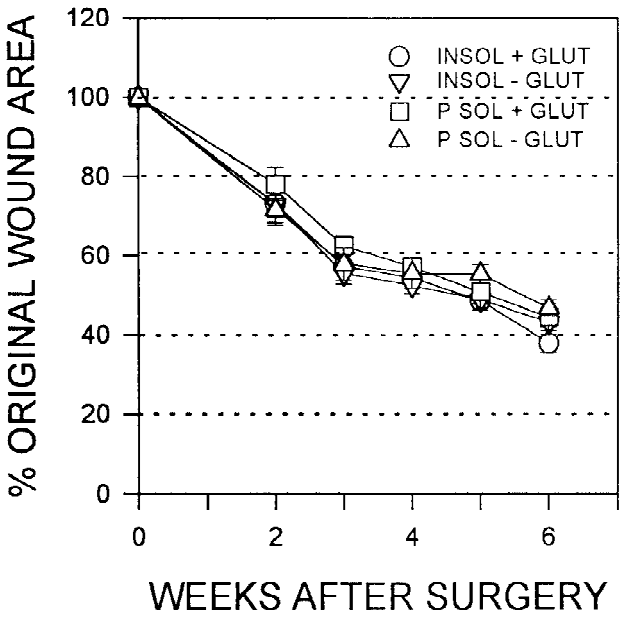

Figure 3. Plot of percent original wound area versus time after grafting of four experimental skin substitutes (CSS). CSS exhibited similar amounts of contraction during wound healing independent of collagen solubility (**insol** or **p sol**) or glutaraldehyde crosslinking (+ **glut** or − **glut**).

Skin from all animals in all groups stained positive for HLA-ABC, indicating closure of the wounds with grafted human epithelial cells. Healed skin from all animals that received collagen substrates treated with 0.25% glutaraldehyde were positive for bovine collagen independent of starting collagen solubility. No positive staining for bovine collagen at 6 weeks after grafting was found in animals that received grafts that were treated with 0% glutaraldehyde.

DISCUSSION

Data presented here suggest an increased rate of C-GAG degradation in wounds treated with grafts prepared with collagen that is not crosslinked with glutaraldehyde. No relationship was found between relative collagen solubility and its detection at 6 weeks after grafting, but glutaraldehyde treatment of both types of collagen showed significantly increased detection at 6 weeks. However, differences in preparation of the collagen substrates did not affect the healing of the wounds, which supports previous reports that treatment of full-thickness wounds with grafts of cultured cells and biopolymers provides permanent closure of skin wounds.[14,23]

Deep skin wounds heal with scar formation and contraction due to a lack of dermal regeneration,[3,4] as substantiated by the grafting of cultured epithelial autografts to treat burn wounds. Enzymatic release of these sheets immediately before grafting is associated with epithelial blistering and increases the need for regrafting.[8,9,11] Collagen has been used extensively as a medical biomaterial[29] and is one of the most biocompatible materials for repair of connective tissue in full-thickness wounds.

The culture of human epidermal keratinocytes on an implantable substrate prior to grafting constitutes a

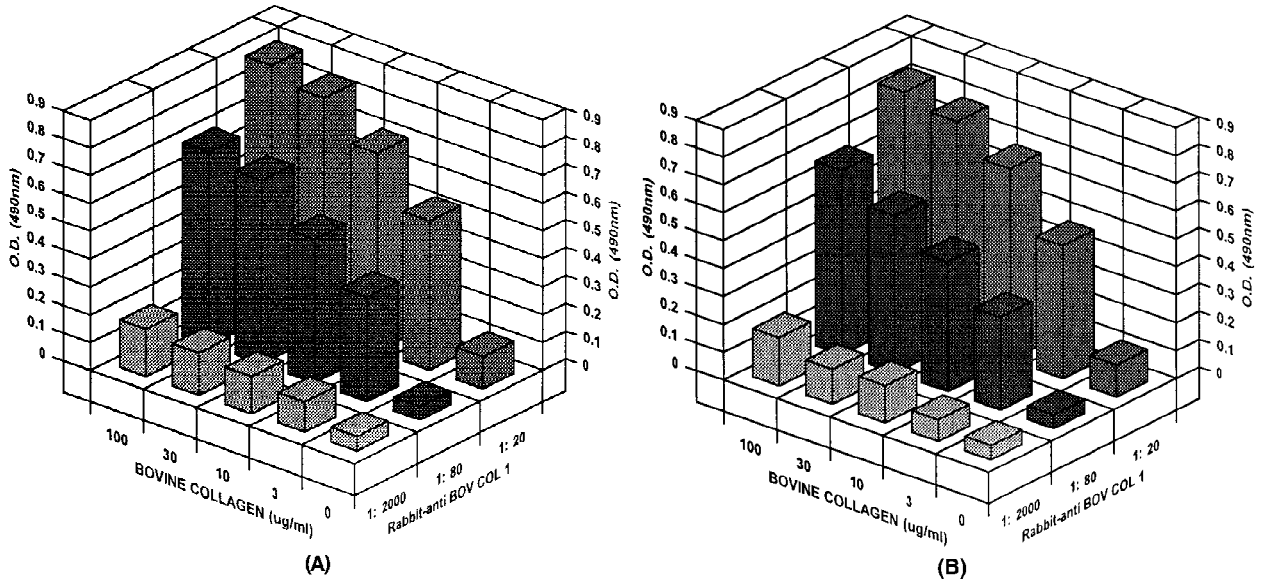

Figure 4. Plots of optical density values versus concentrations of collagen-GAG from ELISA detection with Rabbit anti-bovine collagen Type I: **(A)** insoluble C-GAG dilution series; **(B)** partially soluble C-GAG dilution series. Optical density values (mean, $n = 3$/condition) decrease based on collagen concentration for a given primary antibody concentration as well as on primary antibody concentration for a given collagen concentration. These results are independent of collagen solubility, indicating antibody specificity for both C-GAG types.

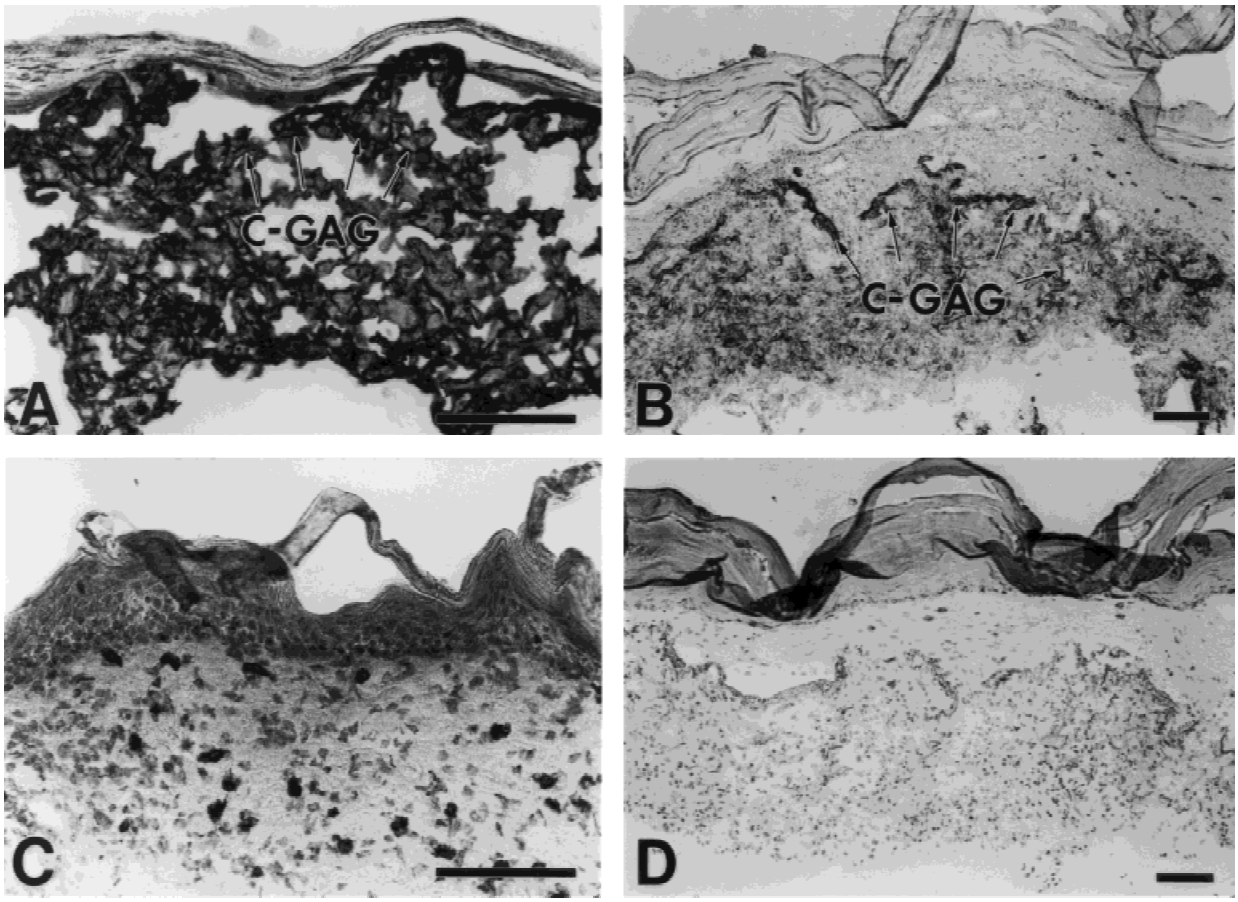

Figure 5. Immunohistochemical localization of bovine collagen in healed skin 6 weeks after grafting: **(A)** stained collagen reticulations (C-GAG) of the implant prior to grafting; **(B)** graft prepared with glutaraldehyde crosslinked collagen showing persistent collagen fragments (C-GAG); **(C)** graft prepared with not crosslinked collagen, indicating no detectable bovine collagen; **(D)** control micrograph of panel B not incubated with the primary antibody. Bars = 100 μm.

skin analog that meets surgical requirements for skin substitutes.[1] CSS, as described here, eliminate the need for enzymatic release of cultured keratinocytes and allow the deposition of basement membrane proteins *in vitro* prior to grafting.[21] The persistence of cell–substrate attachments may contribute to improved epidermal strength and account for the absence of epidermal blistering with this model.

Results from this study suggest that inhibition of degradation of collagen-based implants after treatment with glutaraldehyde may delay repair and remodeling of connective tissue. At 2 weeks after grafting, wounds treated with CSS prepared from glutaraldehyde crosslinked collagen did not have epithelium fused around the wound perimeter while those not treated with glutaraldehyde were fused. Collagen not crosslinked with glutaraldehyde may be degrading at this early time point, allowing for more rapid repair of connective tissue. This interpretation is supported by the histologic analysis at 6 weeks. Although all wounds were completely healed at this time point, fragments of the biopolymer implants were still detectable in those wounds treated with grafts prepared from crosslinked collagen. Microscopically, these wounds were as well vascularized as those treated with not crosslinked collagen.

The findings of this report provide evidence to suggest that chemical crosslinking of collagen is not necessary to prepare a biopolymer substrate for grafting full-thickness wounds with cultured cells. In addition, the absence of chemical crosslinking does not significantly alter the amount of wound contraction. However, glutaraldehyde treatment of biopolymer substrates inhibits degradation of the implant, which may lead to delayed development of connective tissue and decreased clinical engraftment. Increased biocompatibility of biopolymer substrates for cell transplantation and for optimal repair of connective tissue may contribute to improved clinical outcomes with cultured skin substitutes in the treatment of acute and chronic skin wounds.

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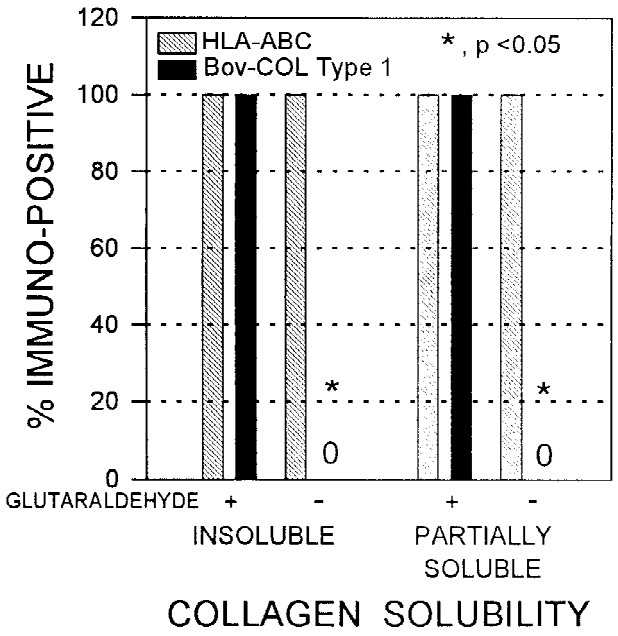

Figure 6. Plot of immunopositive reactions for HLA-ABC and bovine collagen in biopsies from healed CSS at 6 weeks after grafting. All samples stained positive for HLA-ABC expression. All glutaraldehyde crosslinked samples (+) exhibited positive staining for bovine collagen type I while staining in not crosslinked samples (−) was not detected.

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