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Wound closure with EDC cross-linked cultured skin substitutes grafted to athymic mice

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Abstract

Collagen-glycosaminoglycan (C-GAG) sponges are commonly utilized as a substitute for the extracellular matrix of dermal tissue. Cultured skin substitutes (CSS) were assessed, after fabrication using sponges cross-linked with 1-ethyl-3-3-dimethylaminopropylcarbodiimide hydrochloride (EDC) at 0, 1, 5, or 50 mM, for development of viable, stratified skin tissue anatomy in vitro, and for wound contraction and cell viability in vivo. Cross-linking the C-GAG sponges with EDC reduced in vitro contraction of the CSS from a 39% reduction in area in the 0 mM CSS to 0% in the 50 mM group. Conversely, the wounds closed with 0, 1 and 5 mM EDC groups exhibited significantly less wound contraction than the 50 mM group. Engraftment of human cells occurred in 86%, 83%, and 83% of the wounds treated with CSS fabricated using 0, 1, and 5 mM EDC cross-linked sponges, respectively, which were significantly higher engraftment rates than the 50 mM group (17%). These data suggest that low concentrations of EDC can be used to improve the biochemical stability of the C-GAG component of CSS in vitro, and promote stable wound closure.

Keywords: Skin; Wound healing; Tissue engineering; Collagen

1. Introduction

Rapid closure of full thickness wounds in severely burned patients is critically important to patient survival. Early excision of the burn eschar decreases mortality in these patients [1] but must be followed by functional wound closure with autologous dermal and epidermal cells. Split thickness skin grafts are commonly grafted to the wound to promote recovery [2,3]. However, limited donor sites and the potential advantages of reduced numbers of surgical procedures, and reduced donor site surface area promote the development of engineered skin substitutes for wound closure in critically burned patients.

Cultured epithelial autograft (CEA) sheets, acellular sponges and dermal analogs have all become available as skin replacements [4–14]. Disadvantages to CEA grafts include difficult handling, highly variable rates of engraftment on human wounds [15], blistering, ulceration [16] and excessive wound contracture [11] associated with the lack of a dermal layer. Acellular allodermis, either alone [12,13,17] or in conjunction with CEA grafts [17–20], have been utilized to promote wound closure. While these methods promote wound healing, it is commonly accepted that both the epidermis and dermis are required to achieve functional burn wound closure [21].

Cultured skin substitutes (CSS), consist of freeze-dried, lyophilized collagen-glycosaminoglycan (C-GAG) sponges populated with autologous keratinocytes and fibroblasts. CSS have been shown to effectively close full-thickness burn wounds [22–24] resulting in a number of clinically significant benefits [25,26]. Despite the clinical success of CSS, the preparation of C-GAG sponges from natural biopolymers presents a possible source of biochemical instability, and variability in rates of degradation.

Natural materials have inherent heterogeneity due to variability in source animals and processing conditions, which contributes to structural and biochemical variability in the fabricated scaffolds. Reduced stability of the biopolymer sponge combined with elevated levels of matrix

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metalloproteinase-1 production, as seen in a small subset of the burn patient population [27], can cause premature degradation of the collagen in CSS and inadequate microstructural anatomy in vitro. Cross-linking the C-GAG scaffolds via exposure to 1-ethyl-3-3-dimethyl-aminopropylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) has been shown to slow in vitro degradation rates and optimize mechanical properties of the C-GAG sponges [28]. However, CSS fabricated using EDC cross-linked C-GAG sponges have not been evaluated in vivo. Thus, it is the goal of this study to determine if CSS fabricated from EDC cross-linked C-GAG sponges not only possess the ability to form a viable, stratified skin substitute in vitro, but also to accomplish rapid and stable wound closure.

2. Materials and methods

2.1. Collagen scaffolds

C-GAG scaffolds were prepared as previously described [29] from comminuted bovine hide collagen (Kensey Nash; Exton, PA) and chondroitin-6-sulfate (GAG) (Sigma; St. Louis, MO) except without glutaraldehyde cross-linking. A co-precipitate (0.60% w/vol) of bovine collagen powder and GAG was homogenized in 0.5 M acetic acid, cast into sheets, frozen, lyophilized and physically cross-linked by vacuum dehydration at 140 °C for 24 h. The C-GAG scaffolds were then chemically cross-linked for 6 h at room temperature with a filter sterilized solution of 50 mM 2-(*N*-morpholino)ethanesulfonic (MES) acid hydrate (Sigma, St. Louis, MO) in 40% ethanol-water (pH 5.5) and 0, 1, 5 or 50 mM EDC (Sigma; St. Louis, MO) and NHS (Fluka Chemical Co.; St. Louis, MO) at a molar ratio of 1:1. The cross-linked scaffolds were then rinsed thoroughly following a procedure previously described [28] in preparation for cell inoculation.

2.2. Preparation of CSS

CSS were prepared with chemically cross-linked and control (0 mM EDC) C-GAG sponges populated with human dermal fibroblasts (HF) and epidermal keratinocytes (HK) isolated from surgical discard tissue. Skin for culture of human cells was obtained under a protocol approved by the University of Cincinnati Institutional Review Board. For inoculation of cells, the area of the C-GAG scaffold was calculated by measuring the width and length of the sponges (average width = 9 cm, average length = 9 cm). HF (passage 2) were inoculated onto rinsed control and cross-linked scaffolds at a density of 0.5×10^6 cells/cm^2 and cultured at 37 °C and 5% CO_2 in CSS medium [24]. The decrease in area of CSS in vitro from HF-mediated contraction was determined by calculating the area of the HF-collagen substrates one day after fibroblast inoculation, and was reported as percent original area ± SEM. Sponges were then inoculated with HK (passage 2) at a density of 1×10^6 cells/cm^2. One day following HK inoculation (CSS incubation day 1), the HK–HF–C-GAG substrates (i.e. CSS) were placed onto a perforated stainless steel platform covered by a cotton pad to establish an air–liquid interface and cultured up to 21 days with the CSS culture media replaced daily.

2.3. Surface electrical capacitance (SEC) measurement

Surface electrical capacitance (SEC) has been shown to be an effective, direct method for measuring skin surface hydration, which is related to barrier function [30,31]. SEC measurements were collected from the CSS grafts in vitro using the NOVA dermal phase meter (DPM 9003; NOVA Technology; Portsmouth, NH) with low SEC readings corresponding to reduced levels of surface hydration, which is an indicator of functional epidermal barrier. On culture days 6, 9 and 12, measurements were taken from four sites on each CSS (6 grafts per group, 24 measurements total) and the SEC values are expressed in DPM units as mean ± SEM.

2.4. Histology

Biopsies for histology were collected at days 7 and 14 and fixed in formalin for 1 h prior to processing and paraffin embedding. Sections were stained with hematoxylin and eosin (H&E) and imaged with light microscopy at 10× and 15× magnifications. Bright field images were collected with SPOT Advanced Imaging software (Diagnostic Instruments; Sterling Heights, MI) with a total of 8 specimens per condition per time point.

2.5. BrdU labeling

To determine the proportion of cells in the in vitro CSS, which were actively proliferating, CSS made with control (0 mM EDC) and cross-linked C-GAG sponges were exposed to medium containing 65 μg bromodeoxyuridine (BrdU) for 24 h prior to sample biopsy. Samples were taken at days 7 and 14 in vitro and processed through a standard paraffin embedment process. Slides were then baked at 60 °C for 2 h, deparaffinized with xylene and graded alcohols to rehydrate and washed with PBS. The sections were co-labeled with anti-BrdU-fluorescein isocyanate (1:10; Becton-Dickenson) and primary antibody for pan-cytokeratin (1:50; Invitrogen; Carlsbad, CA) following a protocol previously described [23]. The slides were then washed 3× in PBS, 2× in Milli-Q water, coverslipped with Vectashield hard mount media with 4′,6-diamidino-2-phenylindole (DAPI; Vector Labs, Burlington, CA), and examined microscopically. Six samples per condition were examined with a total of 10 fields of view analyzed per sample (60 total). BrdU-positive cells per field of view were recorded and data presented as the mean number of positive cells per field ± SEM.

2.6. Grafting of CSS to athymic mice

All use and care of animals in this study were approved by University of Cincinnati Institutional Animal Care and Use Committee with NIH guidelines for the care and use of laboratory animals observed. At culture day 14, CSS made with control and cross-linked sponges were cut into 2 × 2 cm squares and grafted onto full thickness wounds of equivalent size prepared surgically in athymic mice (nu/nu, Jackson Labs, Bar Harbor, ME; $n = 9$ animals per control, $n = 8$ animals per 1, 5 and 50 mM EDC group). An occlusive dressing with antibiotic ointment was applied to the graft as previously described [32]. At 2 weeks after surgery, dressings and stent sutures were removed and animals were maintained without dressings for the remainder of the assessment period.

2.7. Animal data collection and analysis

Photographs of the wound were taken from each animal at weekly intervals from 2 to 6 weeks, after which they were taken at bi-weekly intervals up to 12 weeks. Tracings of the wound area were performed at same time points along with a tracing performed at the time of the surgery ($n = 9$ (control) or 8 (1,5 and 50 mM) for $t = 2-4$ weeks, $n = 8$ or 7 for $t = 5-8$ weeks, $n = 7$ or 6 for $t = 9-12$ weeks). Wound contraction was quantified using computer planimetry and defined as wound area at specific time points divided by original wound area × 100. Data are presented as percent original area (mean ± SEM).

One animal per group was euthanized at weeks 4 and 8; the remainder of the animals ($n = 6$ for 1, 5 and 50 mM EDC groups and $n = 7$ for control) was euthanized at week 12. Two graft biopsies were taken from each animal, with one processed for paraffin embedding and another for cryomicrotomy. The paraffin sections were stained with H&E to assess cellular organization and to determine the persistence of the C-GAG

sponge. Frozen sections were used to confirm engraftment of human keratinocytes via immuno histochemical staining for HLA-ABC antigens [33,34]. The percentage of grafts on animals with HLA-ABC-positive keratinocytes at 12 weeks was reported ($n = 6$ for 1, 5 and 50 mM EDC groups and $n = 7$ for control).

2.8. Statistical analysis

For all quantitative assays, a one-way analysis of variance (ANOVA), followed by Tukey–Kramer multiple comparison analysis was performed. The data were presented as mean ± SEM, and a value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Co-culture of HK and HF on control and cross-linked collagen

As seen previously, histological samples showed dense populations of fibroblasts in the control, 1 and 5 mM EDC groups at all time points (day 14, Fig. 1). Fibroblast and keratinocyte layers were well stratified in these groups with a continuous basal cell layer present in all (arrows, Fig. 1). In contrast, the 50 mM group had no clear cell stratification with a lower cell density at all time points. A measure of graft contraction after fibroblast inoculation also indicated that the cross-linked collagen contracted less than the control (Fig. 2). Cross-linking of collagen at 50 mM completely inhibited the in vitro contraction of the CSS compared to the approximately 36% reduction in area seen in the control grafts over a 1 day period following fibroblast inoculation (Fig. 2).

Immunostaining the CSS for BrdU at day 7 revealed significantly higher numbers of viable cells in vitro in the 0 and 1 mM groups compared to that of the 50 mM group (Fig. 3). The majority of BrdU-positive cells within the control, 1 and 5 mM groups were located at the dermal–epidermal junction (DEJ). Cells which were actively proliferating were more evenly distributed throughout the dermis and epidermis in CSS cross-linked with 50 mM EDC (data not shown).

Commonly, as CSS mature at the air–liquid interface, cornified layers form causing the surface of the skin to dry and act as an epidermal barrier. The 50 mM group failed to

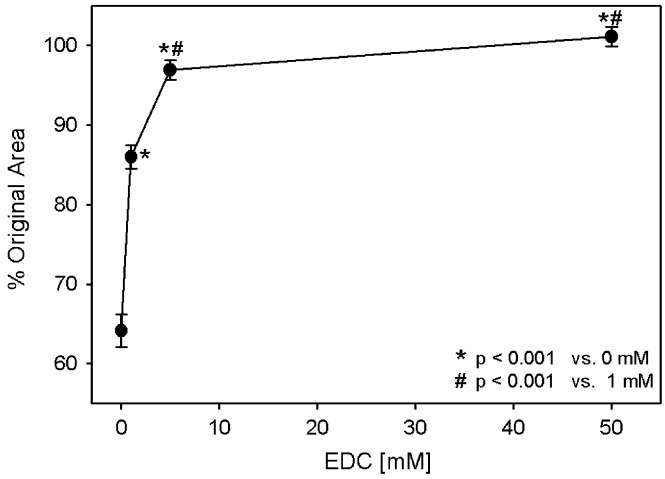

Fig. 2. Percent original graft area one day after fibroblast inoculation versus cross-linker concentration. Note shrinkage of graft is significantly reduced with 1 mM EDC.

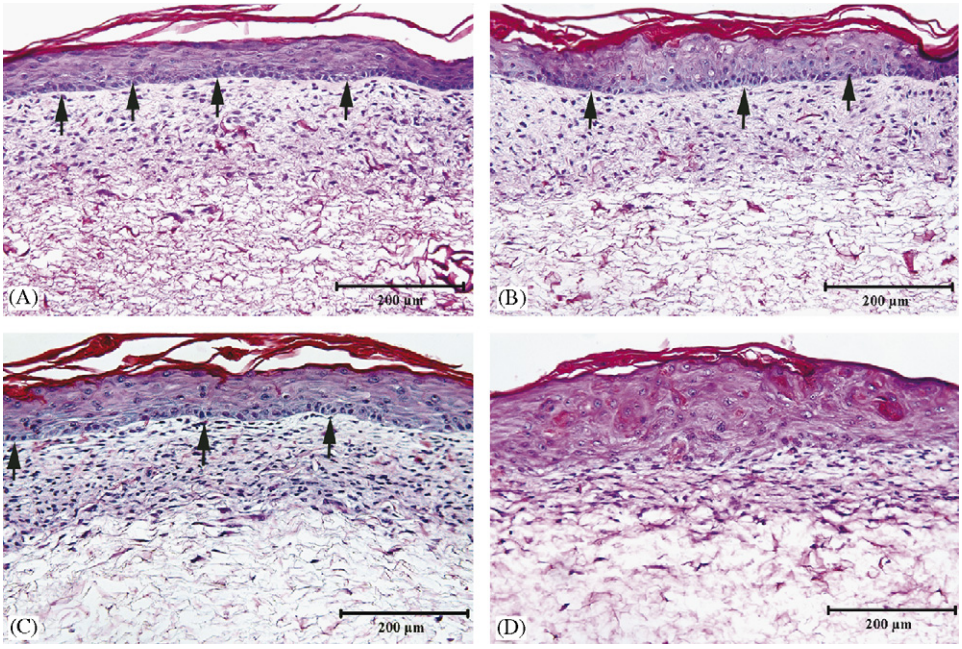

Fig. 1. H&E-stained histological sections of CSS fabricated with collagen-GAG sponges cross-linked with (A) 0 mM (control), (B) 1 mM, (C) 5 mM and (D) 50 mM EDC after 14 days in culture prior to implantation in vivo. Note continuous layer of basal layer keratinocytes present in control, 1 and 5 mM EDC CSS (black arrows), but not in the 50 mM group. Scale bar = 200 µm.

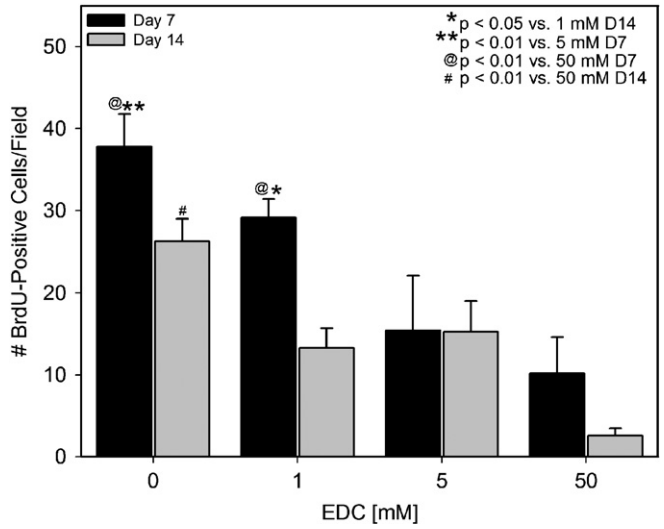

Fig. 3. Average number of BrdU-positive cells within the basal layer per field of view at days 7 and 14 in culture. BrdU incorporation decreases in proportion to time and EDC concentration. Significant differences as noted.

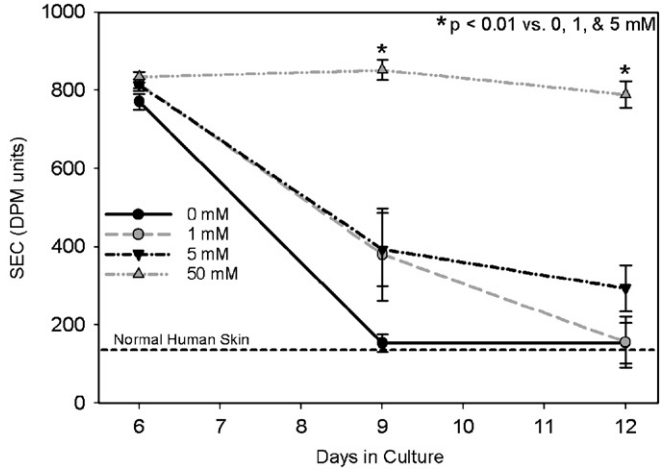

Fig. 4. Surface hydration measurements at days 6, 9 and 12 for control and experimental groups. By day 12, control, 1 and 5 mm EDC groups have approached normal human skin SEC levels, but no reduction in surface hydration is observed in the 50 mm group.

produce cornified layers as evidenced by histology and high SEC measurements at days 6, 9 and 12. The 0, 1 and 5 mm groups were significantly drier than the 50 mm group at days 9 and 12 and all approached normal human skin SEC levels by day 12 (Fig. 4).

3.2. Wound closure on athymic mice

Fig. 5 shows representative animals from each group at 2 and 12 weeks after grafting. At 2 weeks after surgery, grafts prepared with control, 1 and 5 mm EDC cross-linked CSS were well integrated into surrounding murine skin and possessed a uniformly dry epidermis (Fig. 5A, C, and E). The grafts from the 50 mm group were moist, remained distinct from adjacent murine skin and were poorly integrated into the wound (Fig. 5G). By 12 weeks, the 0, 1 and 5 mm grafts were visibly smaller but maintained their original shape (Fig. 5B, D, and F). In contrast, the 50 mm grafts had contracted into small, star-shaped wounds (Fig. 5H). There were no gross differences in surface area among the 0, 1 and 5 mm groups.

3.3. Microscopic examination of healed wounds

At 4 weeks after surgery, microscopic evaluation of a skin biopsy collected from one animal for each condition showed excellent cell infiltration into the dermis of 0, 1 and 5 mm grafts whereas the dermal component of the 50 mm graft was largely unpopulated and covered only by a thin layer of viable epidermis (Fig. 6G, black arrows). At 12 weeks after surgery, the murine wounds displayed a thick, keratinized epidermis with a non-linear DEJ in the 0, 1 and 5 mm conditions (Fig. 6B, D, and F). Conversely, a thin epidermis with a linear DEJ, characteristic of murine skin, covers the 50 mm graft (Fig. 6H). In a majority of animals from the 50 mm condition, a thick layer of undigested C-GAG sponge appears to be present at 12 weeks. This layer of sponge is densely populated with cells by 12 weeks post grafting (Fig. 6H black arrows).

3.4. Wounds area after grafting

Tracings of wound perimeters from healing CSS at 2–6, 8, 10 and 12 weeks after grafting were converted to area measurements using computerized planimetry. A plot of percent original wound area versus time shows a time-dependent reduction in wound area with the 50 mm group contracting significantly more than the other groups at each time point up to 8 weeks after surgery (Fig. 7). At 12 weeks, the 0, 1 and 5 mm groups had contracted to approximately 38% of their original area, whereas the 50 mm group had contracted to 20% original area ($p > 0.05$ at week 12).

3.5. Percent immunopositive for HLA-ABC expression

Scoring of HLA-ABC expression in CSS after grafting is shown in Fig. 8. Engraftment of human keratinocytes occurred in 86% (6/7), 83% (5/6) and 83% (5/6) of the wounds treated with CSS made from control, 1 and 5 mm EDC cross-linked sponges, respectively which were significantly higher engraftment rates than the 50 mm group (17% (1/6); $p < 0.05$).

4. Discussion

Although carbodiimides do not remain within the cross-linking bond generated between collagen and have not been associated with significant cytotoxicity in previous reports [35–39], this study showed that elevated concentrations of EDC (50 mm) resulted in decreased cell proliferation as shown by BrdU staining (Fig. 3), and poor cellular

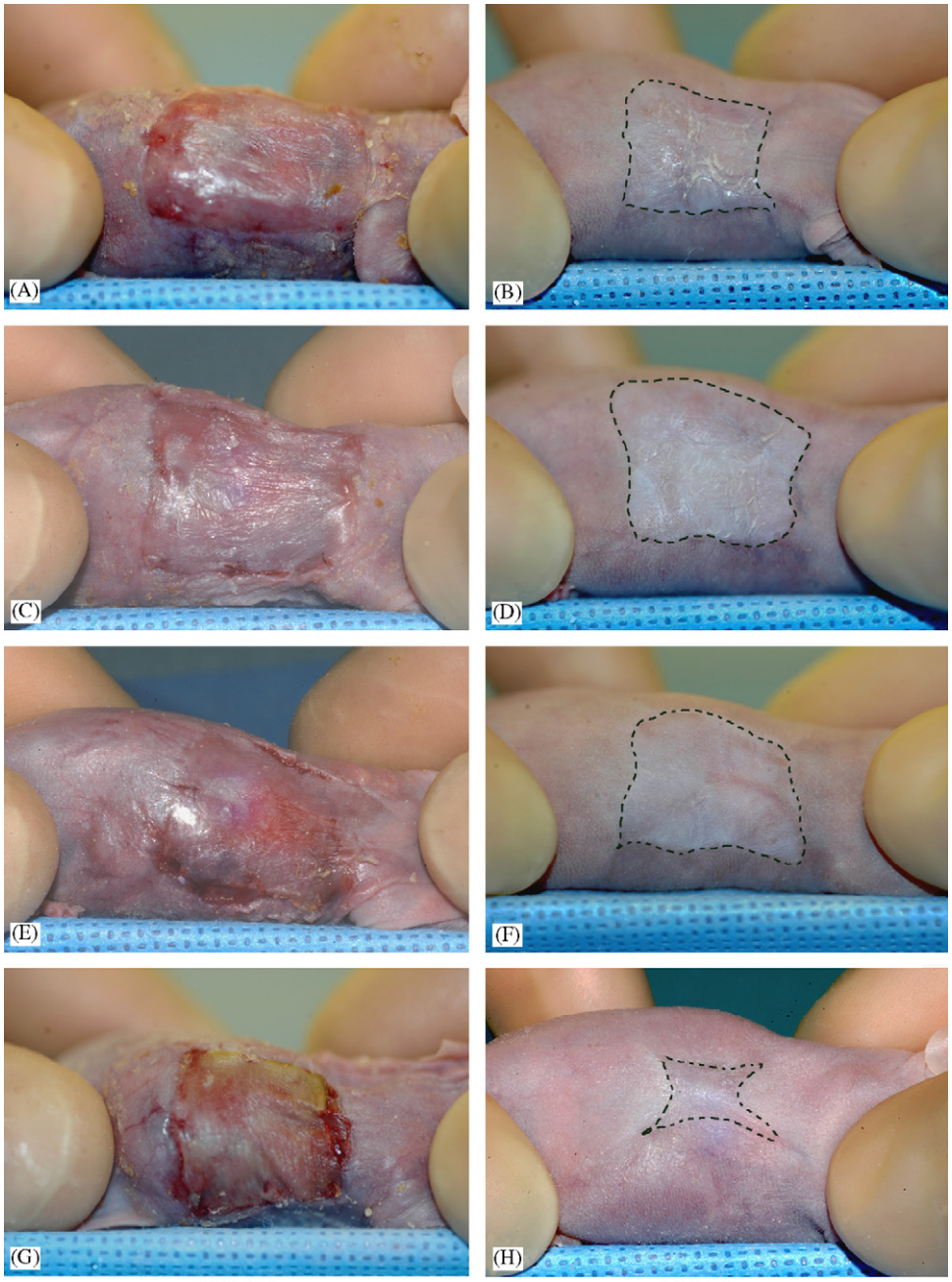

Fig. 5. Appearance of athymic mice 2 and 12 weeks after grafting. (A and B) Control CSS and CSS made using (C and D) 1 mM, (E and F) 5 mM, (G and H) 50 mM EDC cross-linked sponges. Wound areas at week 12 are traced with a dashed black line. Note the moist, yellow appearance of the 50 mM graft at 2 weeks post-grafting an indication of a poorly formed epidermis. An additional important observation is the pronounced decrease in wound area in the 50 mM group (G and H) compared to the control (A and B), 1 (C and D) and 5 mM groups (E and F).

organization (Fig. 1). Data collected presently (Fig. 1D) agree with a previous study [28] in which CSS made with 50 mM EDC cross-linked C-GAG sponges exhibit poor cell viability and organization with no stratification of the keratinocytes and fibroblasts in vitro. This anatomical disorganization coincides with a failure to form an epidermal barrier as can be seen in the high SEC values at day 12 (Fig. 4).

While cross-linking at high concentrations reduces cell viability, C-GAG sponge contraction in vitro is reduced by cross-linking with EDC. The in vitro contraction of the grafts is inversely proportional to the concentration of cross-linker with high amounts of EDC correlated with less contraction (Fig. 4). This inhibition of contraction in vitro increases the amount of total deliverable graft area for surgery compared to the control. For example, an initial total area of 1000 cm^2 of control and 5 mM cross-linked collagen sponge, which are inoculated with CF and CK and incubated for 14 days, would produce approximately 400 and 950 cm^2 of CSS, respectively. The inhibition of

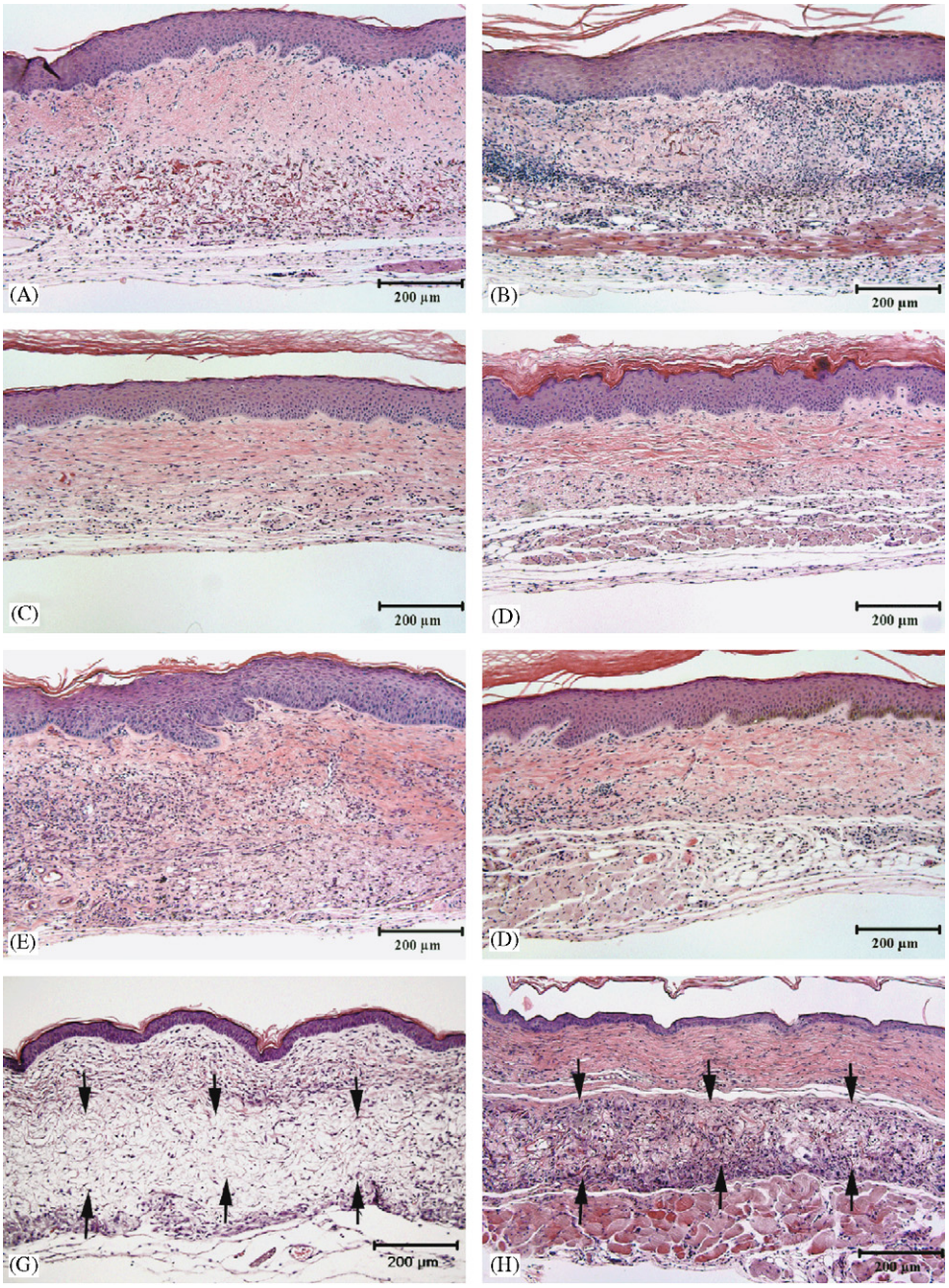

Fig. 6. Histologic examination of healed skin 4 and 12 weeks after grafting (A and B) control, (C and D) 1 mM, (E and F) 5 mM and (G & H) 50 mM EDC cross-linked CSS. A non-linear dermal–epidermal junction (DEJ) can be seen in the control, 1 and 5 mM groups whereas a thin epidermis with a linear DEJ is seen in the 50 mM group at both 4 and 12 weeks post grafting. Note the low cellular density in the dermal component of the skin healed with the 50 mM group at 4 weeks (G). Black arrows indicate the presence of undigested collagen-GAG sponge (G). By 12 weeks post grafting, cells have infiltrated the graft (H). Scale bar = 200 μm.

contraction in vitro increases the area of graft available to the patient and may significantly reduce the time frame in which a patient with massive burns can be treated.

Control and experimental CSS were grafted onto athymic mice after 12 days in culture. Two weeks after surgery, the control, 1 and 5 mM groups had good engraftment, with an intact epithelium and well-healed wound edges. Conversely, the 50 mM group did not show a keratinized epithelium and the wound was clearly moist (Fig. 5G). In addition, the wound edges were dehisced (Fig. 5G), an observation previously seen in CSS made with glutaraldehyde cross-linked C-GAG sponges [40]. As the sponges cross-linked with 50 mM EDC are highly resistant to degradation in vitro [28], it is likely that the C-GAG sponge was not significantly degraded by 12 weeks to allow integration into the wound. Histological

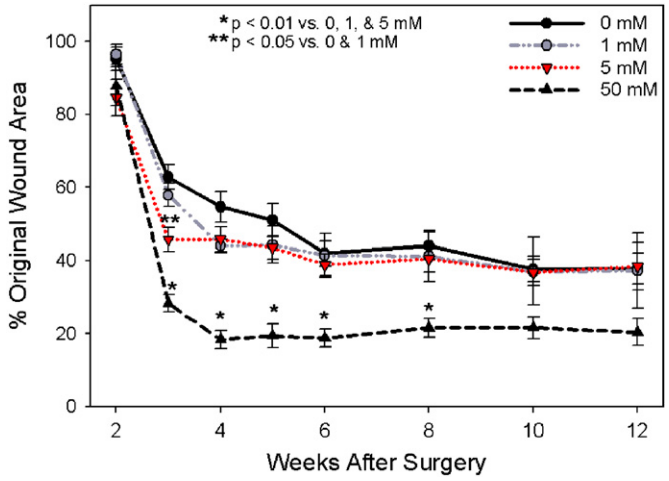

Fig. 7. Percent original wound area versus time for all experimental skin substitutes and the 0 mM control. Control CSS and CSS made with 1 and 5 mM EDC sponges exhibited similar amounts of contraction during wound healing. The 50 mM group contracted significantly more after 8 weeks in vivo.

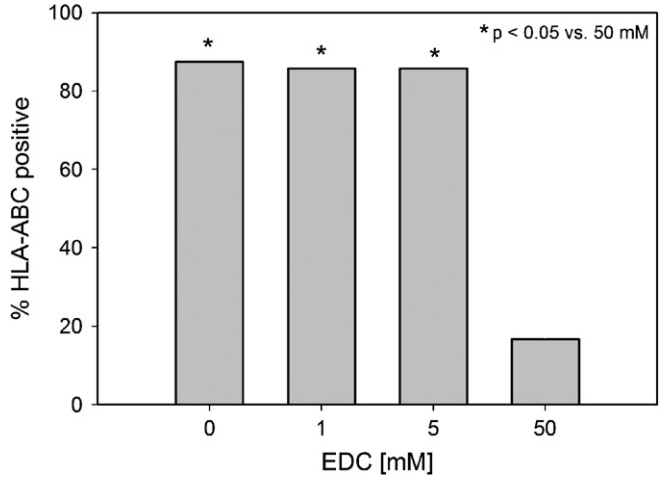

Fig. 8. Percent immunopositive reactions for HLA-ABC in biopsies from healed CSS at 12 weeks after grafting. The 50 mM group had significantly less HLA-ABC positive keratinocytes or human cell engraftment compared to the control, 1 and 5 mM groups.

evaluation of the wound at 4 weeks after grafting (Fig. 6G) indicated the C-GAG sponge was still present and not well populated with cells, possibly a result of residual toxicity of the sponge or the increased difficulty of migrating cells to degrade a path into the matrix. By 12 weeks in vivo, the 50 mM EDC C-GAG sponge was densely populated by cells and covered by a thin layer of murine epithelium (Fig. 6H).

Full-thickness wounds heal by scar formation and contraction due to the lack of dermal regeneration within the wound area [41,42] as corroborated by the considerable contracture seen in full thickness wounds closed with CEA [9]. Skin substitutes grown with and without fibroblasts indicated that substitutes fabricated with fibroblasts had a thicker epidermis, less contraction, earlier vascularization and improved epidermal formation in vivo when compared to the non-fibroblast condition [43]. CSS are comprised of both fibroblasts and keratinocytes, which satisfy the need for a dermal component to reduce wound contracture and an epidermal component to close the wound preventing infection and fluid loss. Wound contraction of the control and experimental CSS grafted to athymic mice was assessed at multiple time points. The 50 mM group contracted rapidly to only $28.3 \pm 2.3\%$ original area by 3 weeks whereas control, 1 and 5 mM groups contracted to an average of 55.5% original area. While these levels of contraction are high, it is important to note that in a murine wound model the skin contracts much more quickly and for a longer duration than human skin wounds. In addition to rapid contraction, histological evaluation of the wounds covered with 50 mM EDC CSS showed a slow infiltration of murine fibroblasts into the sponge. It is likely that any human keratinocytes within the 50 mM EDC CSS at the time of grafting were disorganized and, therefore, unable to close the wound leading to contraction of the surrounding mouse epithelium. This severe contraction of the wounds healed with the 50 mM group CSS is in contrast to its complete resistance to contraction in vitro (Fig. 2).

Although the inhibition of contraction in vitro in the 50 mM EDC groups appears to contradict the in vivo data, the increased contraction in the wounds grafted with 50 mM graft was not unexpected. With low cell viability and poor cellular organization within the 50 mM graft, the wound was closed by secondary healing or contraction, which would generate a poor long-term clinical outcome. These observations suggest that increases in acellular sponge stability and strength [28] must be balanced with high cell viability and optimal anatomic organization when healing wounds in vivo.

5. Conclusions

Cross-linking with EDC can easily regulate the biochemical stability and strength of collagen sponges used for skin substitutes [28]. C-GAG sponges evaluated presently were cross-linked at varying concentrations of EDC (0–50 mM), which include an easily degraded non cross-linked control (0 mM) and a highly resistant cross-linked sponge (50 mM) [28]. While cross-linking with EDC improves the degradation resistance of C-GAG sponge [28], and reduces contraction of the CSS in vitro, the grafts had poor human keratinocytes engraftment and increased wound contraction in vivo. CSS cross-linked at 1 and 5 mM EDC exhibited similar wound contraction as the control CSS but has greater in vitro resistance to contraction. CSS with sponges cross-linked at 50 mM were less efficacious in healing and showed rapid contraction of the wounds in contrast to the complete inhibition of contraction in vitro. These results indicate that in vitro contraction cannot accurately predict wound healing and contraction in vivo. Additionally, it can be concluded that 1–5 mM of EDC can maximize the surface area of the C-GAG sponges in vitro

without impairing human cell engraftment in vivo. This is of particular interest when total deliverable graft area is essential to the prompt closure of wounds in patients with large total surface area burns thus reducing the time and cost of treatment. Therefore, low concentrations of EDC can be used to improve the biochemical stability of C-GAG component of CSS in vitro safely without inhibiting engraftment and wound closure.

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