DE NOVO SYNTHESIS OF SKIN

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INTRODUCTION

Evidence has been steadily accumulating that polymers prepared by well-characterized methods can act as a highly specific and transient extracellular matrix (ECM) [1] in a standard animal wound healing model. These polymers are prepared by covalently crosslinking [2] purified collagen with chondroitin 6-sulfate, a glycosaminoglycan (GAG), in a process which yields a highly porous, insoluble membrane [3].

The present study is a quantitative exploration of the physicochemical features of CG polymers which impart biological activity to this class of matrices. The animal model used was the standard skin wound healing model which has been described in great detail by several groups of investigators [4-6]. In particular, we have studied the effect of mean pore diameter and, separately, of degradation rate on the effectiveness with which acellular CG polymers delay wound contraction in this model. We have also studied the effect of density of epidermal cells seeded into CG polymers on the ability of the latter to arrest wound contraction. Finally, we present new histological and ultrastructural evidence that new skin synthesized in the presence of certain CG polymers is unlike scar and similar to normal skin.

MATERIALS AND METHODS

CG polymers were prepared as described in detail elsewhere [3]. In the current series the average pore diameter was controlled in the range 0.1-850 μm by adjustment of the temperature of the freeze drying process in the range -100°C to -5°C [7]. The porous CG sheet was knife-coated with a 0.25-mm-thick layer of poly (dimethyl siloxane) prepolymer (Silastic, Medical A, Dow Corning, Midland MI) which was subsequently cured to an elastomer by immersion in 0.05 M aqueous acetic acid. This was followed by immersion into a bath of aqueous glutaraldehyde (reagent grade, Aldrich, Milwaukee WI) in 0.05 M aqueous acetic acid. Exposure to glutaraldehyde induces the CG ionic complex to undergo covalent crosslinking [3], presumably by attack on ε-amino groups of lysyl residues and formation of quaternary pyridinium residues [8,9].

Increase of the residence time in the glutaraldehyde bath from 0 to 24 h yielded polymers varying widely in their crosslink density, as reflected in values of the average molecular weight between crosslinks, Mx, which ranged from ca. 40,000 to ca. 12,000, respectively. Following rinsing in deionized water over 24 h the glutaraldehyde concentration in the rinse water was found to be considerably less than 5 ppm using the reagent PurAld(4-amino-1-hydrazino-5-mercanto 1,2,4-triazole; Aldrich, Milwaukee, MI) [10].
The average pore diameter D was determined by first embedding the porous membrane in methyl methacrylate, sectioning to a thickness of 5 μm, mounting on a glass slide and staining with 1% toluidine blue. Slides were examined under a light microscope using an image analyzer and results obtained agreed with a method [7] based on viewing in the scanning electron microscope.

The degradation rate in collagenase [11] was determined by first incubating the finely comminuted polymer for 5 h at 37°C in a stirred 0.08% w/v solution of bacterial collagenase from Clostridium histolyticum (GIBCO, Grand Island, NY) in a buffer, pH 7.4. Following filtration to separate out undigested substrate particles aliquots were treated by addition of a solution of ninhydrin and hydrindantin at pH 5.5. The resulting colored solution was incubated in a boiling water bath at 20 min and 50% w/w propanol/water was then added. The solution was allowed to stand at room temperature for 15 min and the transmittance at 600 nm was recorded. Results were expressed as enzyme units (degradation rate, R) which were calibrated using a 0.002M aqueous solution of L-leucine [12]. One enzyme unit liberates amino acids from collagen which are equivalent in ninhydrin color to 1.0 μM L-leucine in 5 h at pH 7.4 at 37°C [13].

CG polymers were seeded with purified, though uncultured, epidermal cell suspensions enriched in basal cells, as described elsewhere [14].

White female Hartley guinea pigs (Elm Hill Breeding Laboratories, Chelmsford MA) were grafted using surgical procedures which have been described [15]. The excisional protocol used followed closely the standard mammalian free skin grafting model described by Billingham and Medawar [4,5]. An anatomically well-defined wound bed [16], consisting of a layer of stripped muscle, was thereby prepared for interaction with the CG polymeric grafts. Nominal surface areas of wounds were usually 1.5 cm by 3.0 cm. In several controls similar results were obtained with 4.0 cm by 4.0 cm wounds. Animals in one control group were not grafted, but their wounds were treated simply with a Xeroform gauze [15]. The wound was photographed in the presence of a calibrated scale and its area calculated using measurements taken from the skin edges and not from the advancing edge of epithelial cell sheets [5,15]. The time required for the wound area to be reduced to 50% of its original value, t1/2 (wound half-life), was determined from the kinetics of contraction.

RESULTS

The majority of CG polymers did not change significantly the time of onset of contraction nor the wound half-life, t1/2, from the value of 8 ± 1 days which we observed with ungrafted wounds. A relatively small number of polymers was, however, capable of delaying significantly both the onset of contraction and t1/2.

Control of mean pore diameter D of CG polymers was a very effective procedure for delaying wound contraction (Fig. 1). Since the data in Fig. 1 have been obtained with polymers for which the degradation rate and the GAG content were controlled, Fig. 1 shows the effect of D on t1/2 without interference from other experimental parameters. Using the arbitrary cut-off level of t1/2 = 15 days we estimate from Fig. 1 a lower cut-off limit of 9 ± 3 μm and an upper limit of 630 ± 150 μm. However, maximum activity was displayed within a much narrower range. A maximum increase of t1/2 up to 27 ± 2 days occurred when D ranged between 20 ± 4 μm and 125 ± 35 μm.
The degradation rate $R$ of CG polymers also affected strongly the kinetics of contraction (Fig. 2). Based on the arbitrary cut-off point of $t_{1/2} = 15$ days we estimate from Fig. 2 an upper cut-off point of 140 -- 25 enzyme units. Below this limiting value, $t_{1/2}$ remained significantly unchanged down to the sensitivity limit of the assay. Maximum $t_{1/2}$ values reported in Fig. 2 are $17 ± 2$ days, considerably lower than the maximum of $27 ± 2$ days reported with a different series of polymers in Fig. 1. The mean pore diameter for the series of polymers used to obtain the data in Fig. 2 averaged $450 ± 60 \mu m$, a value which corresponds to $t_{1/2}$ values consistent with data in Fig. 1 (see arrow in Fig. 1).

Maximum biological activity in our study was displayed by CG polymers which, not only possessed mean pore diameter and enzymatic degradation rate within the limits prescribed by Figs. 1 and 2, but also were seeded with epidermal cells prior to grafting. When grafted with CG polymers which had been seeded with at least $5 \times 10^8$ cells per cm$^2$ of graft area, wounds displayed contraction kinetics which were similar to those of acellular, biologically active CG grafts over the first 40 days. After Day 50, however, the wound perimeter expanded until it asymptotically reached $72 ± 5\%$ of the original area. Neoeipidermal confluence was usually detected at about 2 weeks or less following grafting as formation of a thin, shiny surface comprising layers of keratin which could be removed and identified microscopically. Light microscopic studies also showed that formation of a new dermal layer proceeded underneath the neoeipidermis over several weeks. After about 200 days the wound perimeter, itself consisting of a thin line of scar-like tissue, eventually included an area of tissue, somewhat more than $2/3$ of the original wound area, which grossly appeared identical in color, texture and touch to intact skin outside the wound perimeter with the exception that the new skin appeared entirely hairless.

A comparative morphological study of normal guinea pig skin, scar and newly synthesized skin was conducted by light and electron microscopy one year after grafting. Newly synthesized skin was remarkably similar to normal guinea pig skin in most respects, when studied about one year following grafting [17,18]. The epidermis was variably hyperplastic, and all cell layers of the epidermis were normal in maturation sequence and in relative proportion. Langerhans cells and melanocytes, normal constituents of the epidermis, were observed. No hair follicles or other skin appendages were observed. The rete ridge pattern was well formed, and interdigitations with dermal papillae were easily identified. Within the dermal papillae, capillary loops similar to those of normal skin were found. Beneath these loops was a well-formed superficial vascular plexus composed of arterioles and venules. The plexus was in horizontal alignment to the overlying epidermis and was embedded within a dermal matrix consisting of apparently randomly-oriented interwoven collagen bundles. Small unmyelinated nerve fibers were present within dermal papillae in close approximation to the overlying epidermis in both normal and newly synthesized skin. In contrast, scar tissue showed marked atrophy of overlying epidermis, loss of rete ridges and associated dermal papillae, poorly formed microvasculature and absence of nerve fibers within dermal papillae. Furthermore, collagen fibers in the dermis of scar tissue were highly oriented in the plane parallel to the epidermis, rather than being disposed more or less randomly as in normal skin and, to a lesser extent, in newly synthesized skin.
DISCUSSION

Several investigators have stressed the absence of evidence that skin, and specifically the dermis, can regenerate from a substrate consisting of muscle tissue [5, 19]. In spite of the absence of hair follicles and other specialized skin organs, the morphological evidence is consistent with the conclusion that newly synthesized skin is distinct from scar and quite similar to intact guinea pig skin. Clearly, there is need for additional studies to identify detailed biochemical and immunological similarities and differences between newly synthesized and intact skin.

The mechanism by which CG polymers delay or arrest contraction and lead to synthesis of new skin is under study [20].

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REFERENCES

Figure 1. Variation of the wound half life, with the average pore diameter of CG polymers.

Figure 2. Variation of the wound half life with the degradation rate of CG polymers incubated in bacterial collagenase.