Porcine collagen crosslinking, degradation and its capability for fibroblast adhesion and proliferation

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Porcine dermal collagen permanently crosslinked with hexamethylene diisocyanate was investigated for its suitability as a dermal tissue engineering matrix. It was found that the chemically crosslinked collagen had far fewer free lysine groups per collagen molecule than did the uncrosslinked matrix. The ability of the matrix to support human primary fibroblast outgrowth from explants was compared for matrices that had been presoaked in various solutions, including fibroblast media, cysteine and phosphate buffered saline (PBS). It was found that superior cell outgrowth was obtained after soaking with fibroblast media and PBS. The fibroblast attachment properties of the matrix were compared against tissue culture plastic and PET. The collagen matrix showed the least amount of cell retention compared to the other to matrices, however, the general trends were similar for all three scaffolds. Longer term cultures on the collagen showed fibroblasts covering the matrix stacking up on each other and bridging natural hair follicles. However, it was also observed that the fibroblasts were not able to penetrate into the matrix structure. This was believed to result from the chemical crosslinking, as shown by the resistance of the matrix to degradation by collagenases.

1. Introduction

Many biocompatible materials have been studied for their suitability as matrices for tissue engineering. These include synthetic polymers such as poly(lactic–glycolic acid), and natural polymers, such as alginates. However, one of the most suitable materials is collagen, which comprises the main component in natural extracellular matrices (ECMs). Collagen promotes natural cell interactions such as cell migration and proliferation, it is biocompatible and can be remodelled *in vivo*. In addition to the low cross species immunogenicity, the use of collagen is perpetuated by the possession of lesser-known bioactive influences not observed with using synthetic material which can influence adhesion, spreading and morphology [1].

Collagen-based biomaterials have recently had increasing acceptance in both therapeutic and cosmetic applications. The materials can be chemically crosslinked to increase the mechanical stability [2]. Commonly used crosslinking agents include gluteraldehyde, carbodiimides and hexamethylene diisocyanate (HMDC). Chemical crosslinking can result in materials which are permanently crosslinked, and are thus unable to be remodelled *in vivo*. This may be an advantage for dermal applications such as burns treatment, where tissue contraction and scarring may be minimised. In other tissue engineering applications, the lack of ability of the matrix to be remodelled may be undesirable. Two key issues associated with the use of collagen include its degradation properties and its stability.

In vivo, the ECM of the connective tissue is remodelled continuously, allowing modification and infiltration of cells. Remodelling is upregulated dramatically when wounding occurs. The degradation and subsequent reorganisation of the ECM has important implications in cell proliferation, organisation and the creation of a suitable functioning tissue structure. Collagen degradation and remodelling results from the action of proteolytic enzymes, that are secreted primarily by fibroblasts [3, 4]. Proteases include the matrix metalloproteinases (MMPs) such as collagenase, gelatinase and stromelysin, cysteine proteinases such as cathepsin B and L, and the serine proteinases such as plasmin and plasminogen activator [5]. Although collagenase performs a crucial role in the rapid regeneration of tissue after destruction, it is unknown whether it is involved in the remodelling of soft connective tissue under steady-state conditions [5]. The exact identity of the proteases involved in cell migration and infiltration past the collagen type I barrier are still unknown [6]. When a range of proteases were tested, only MMPs-1 and -2 could penetrate collagen type I [7].

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The fibre strength and degradation rate can be influenced by combining collagen with other materials or by synthetically induced crosslinking [2]. The structure of end state collagen enables some resistance to additional protease degradation, such as by collagenases. However, this resistance varies depending upon fluctuations in environmental pH (such as at a burn wound site), temperature (due to severe inflammation) or type of collagen [8]. Bailey (2000) [9] noted that a 5 °C temperature rise can cause a 15-fold increase in the cleavage rate. Therefore, it is necessary to assess the ability of the crosslinked collagen matrix to support the following: cell adhesion and outgrowth over time, the ability of the cells to infiltrate the matrix whilst retaining some resistance to a hostile wound bed.

This study examined the potential of using a novel collagen substrate as a tissue engineered dermal replacement. This porcine collagen (Permacol, Tissue Science Laboratories) derived matrix has been chemically crosslinked to make it suitable as a permanent surgical implant. The objectives of this work were to verify that HMDC had a crosslinking effect on the collagen matrix, to characterise the extent of any crosslinking, and compare this effect to uncrosslinked collagen matrix sheet. For uses as a dermal tissue engineered device, it was also important to examine fibroblast response to the crosslinked collagen matrix.

2. Materials and methods

2.1. Collagen matrix

Crosslinked porcine dermal collagen type I sheets (Permacol™) were kindly supplied by Tissue Science Laboratories (Aldershot, UK). The collagen had been chemically crosslinked using HDMC, according to the method described fully in International Patent Number WO 85/05274 (5 December 1985). Collagen pieces with dimensions of 10×10 mm and thickness of 0.4 mm were used. The surface and transverse characteristics of some samples were modified by the inclusion of lasered pores of average pitch 500 μm and pore diameter 100 μm (Permaderm™, Tissue Science Laboratories). Other matrix samples included trypsin decellularised, uncrosslinked porcine dermal collagen type I (Tissue Science Laboratories) and milled insoluble bovine Achilles tendon collagen type I (cat. no. C9879, Sigma, Poole, UK). All collagen samples were extensively washed in phosphate buffered saline (PBS) and equilibrated in culture media containing serum prior to use.

2.2. Primary fibroblast cell isolation and culture

Human fibroblast cells were isolated from human skin biopsy samples obtained with the appropriate local ethical consent. Briefly, the epidermis was removed from the dermal tissue which was then minced into 0.5–1 mm^2 pieces. The dermis was incubated in 625 U/ml collagenase (Sigma, Poole, UK) in Dulbecco's modified Eagle's media (DMEM) (Gibco Life Technologies, Paisley, UK) for 2 h. The resulting cell suspension was resuspended in fibroblast media (DMEM supplemented with 10% fetal calf serum (Globepharm, UK), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco)). The cells were cultured at 37 °C with a 95% air, 5% CO_2 gaseous composition and 100% humidity, the culture medium was changed twice per week. Cells were used between passages 2 and 4. Fibroblast phenotype was checked using fibroblast surface monoclonal antibody staining.

2.2.1. Effect of wash solution on tissue explant outgrowth

Skin biopsy material was cut into 1 mm^3 cubes and placed onto 1 cm^2 pieces of crosslinked collagen matrix or upon tissue culture plastic. All collagen matrix samples were derived from the same batch to eliminate variations. The collagen matrix had been pre-rinsed in PBS, and allowed to soak overnight in fibroblast media; PBS; 0.1 M cysteine and completely unrinsed. Four explant tissue pieces were added onto the crosslinked collagen matrix samples in 200 μl of fibroblast media and allowed to attach for 6 h. The media volume was then increased to 0.5 ml, with standard media changes every three days. After 18 days, the tissue was removed and the remaining samples subjected to MTT assay and histological analysis.

2.2.2. Cell attachment to matrices

Previously washed collagen pieces were soaked overnight in DMEM and 10% FBS to facilitate attachment. Early passage human dermal fibroblasts ($<$ P4) were seeded at a density of 5×10^4 or 1×10^5 cells cm^{-2} directly onto either poly(ethylene terepthalate) (PET) membranes (Becton Dickinson, Heidelberg, Germany), collagen or tissue culture polystyrene and allowed to attach for hourly intervals up to 4 h. Cells were incubated and after the required time interval, unattached cells were rinsed from the surface with PBS stained with trypan blue (0.4% v/v) and counted by hemocytometer. The number of cells remaining attached was then correlated using the MTT assay.

2.3. Matrix degradation studies

The crosslinked collagen type I matrix was subjected to 2000 U/ml crude collagenase activity type IA of bacterial (*Clostridium histolyticum*) origin (cat. no. C9891, Sigma). The methods of Reddy and Enwemeka [10] and Olde Damink *et al*. [2] were used to determine the concentration of liberated hydroxyproline. Briefly, crosslinked collagen matrix samples were finely divided into pieces of $\sim 0.5\,mm^3$. Pieces were allowed to dry overnight (29 °C), and sorted into 10 mg samples. Collagen samples were rehydrated in 0.5 ml of 0.1 M Tris–HCl buffer with 0.005 M calcium chloride and 0.05 mg/ml sodium azide, by incubation at 37 °C for 1 h. Collagen samples were incubated in 100 U/ml collagenase solution (10 U/mg collagen sample) at 37 °C for up to 10 h. Enzyme action was halted by addition of 0.1 ml of 0.25 M EDTA solution. Samples were centrifuged at $600 \times g$ for 10 min (Sanyo MicroCentaur). To 40 μl of sample supernatant 60 μl of 3.3 M sodium hydroxide solution was added. Samples were then placed in an

autoclave (121 °C for 20 min). After cooling, 900 µl chloramine-T reagent (1.27 g of chloramine-T, dissolved in 20 ml of 50% n-propanol and brought to a final volume 100 ml with 0.056 M acetate–citrate buffer) was added to each sample and allowed to oxidise for 25 min at 24 °C. To develop the chromophore, 1 ml of Ehrlich's reagent (7.5 g of p-dimethylaminobenzaldehyde in 30 ml n-propanol and 15 ml perchloric acid; the volume was brought to 50 ml with n-propanol) was added and the samples were further incubated for 20 min in a water bath at 65 °C. Samples were cooled and the absorbance measured at 550 nm. A calibration curve of measured absorbance against hydroxyproline concentration was constructed.

2.4. Analytical methods

2.4.1. Analysis of extent of matrix cross-linking

The free lysine content of each matrix type was assessed by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay [11, 12]. Pieces of collagen matrix (uncrosslinked, crosslinked and perforated crosslinked) were diced into small pieces of 0.5 mm^3 and dried. The dried collagen was weighed to select pieces at intervals to 6 mg. The matrix samples were placed into a solution comprising 1 ml disodium orthophosphate (4% w/v), and 1 ml of TNBS (0.5% w/v) and incubated at 40 °C for 2 h. The reaction was terminated by addition of 3 ml of 6 M HCL, and the samples were incubated at 60 °C for 90 min to allow hydrolysis. The absorbance at 345 nm was measured. The concentration of free lysine was calculated using a molar extinction coefficient for the reaction product trinitrophenyllysine of $1.46 \times 10^4\,l\,mol^{-1}\,cm^{-1}$ at 345 nm [13].

2.4.2. Cell quantification using the MTT assay

Viable cell density was assessed by the relationship to metabolic activity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, or MTT assay [13]. MTT stock solution was dissolved in PBS pH 7.5 at a concentration of 5 mg/ml. Culture media was replaced with 1 ml of DMEM (without phenol red or serum) and an aliquot of 100 µl of MTT stock solution. Samples were incubated for 4 h. The media solution was aspirated and the remaining insoluble formazan product was solubilised with 1 ml of iso-propanol, mixed and incubated for 10 min. The optical density of the solution was measured at 550 nm. A calibration curve was constructed consisting of known fibroblast numbers (up to 4.0×10^5 cells cm^{-2}) seeded onto collagen.

2.4.3. Histology

Visualisation of matrix cultures by light microscope was achieved by standard histological techniques. Samples were mounted in OCT compound (Merck, Poole, UK) and snap frozen in liquid nitrogen before cutting 5–7 µm thick frozen sections using a cryostat. Images were obtained using a light microscope (Carl Zeiss 68040) utilising a colour video camera (JVC 3-CCD KY-F55B) fitted with an imaging system (KS 300, Imaging Associates Ltd., UK).

2.4.4. Scanning electron microscopy (SEM)

Samples analysed by low temperature SEM examination were fixed in 1.5% (v/v) glutaraldehyde, dehydrated at critical point and then gold cryo-sputter coated. Samples were observed in the field emission SEM (JSM-6210, JEOL, Japan) operated at 1.5–30 kV and maintained at a temperature of 148–163 K.

2.5. Statistical analysis

All experiments were conducted in triplicate $(n = 3)$, unless otherwise stated. Data are presented as mean ± standard deviation (SD) of the mean. Comparisons of data sets was carried out using the appropriate descriptive statistics, i.e. two-way analysis of variance (two-way ANOVA test) with individual comparisons made using a *post-hoc t*-test. Non-parametric data was assessed using the Kruskal–Wallis test (for analysis between several data sets) and Mann-Whitney U-test (for comparison between pairs).

3. Results

3.1. Evaluation of the extent of matrix crosslinking

The free lysine concentration in four different collagen matrices prior to and after HMDC crosslinking was measured to determine the extent of crosslinking. Fig. 1 shows the amount of free lysine present in each of the four matrices. The relationship between matrix sample weight and free lysine was seen to be linear ($r^2 > 0.97$ for all samples). The two crosslinked matrices gave lysine concentrations that were less than the uncrosslinked collagen matrix and the milled collagen. The milled collagen had the highest lysine availability. The number of moles of free lysine per mole of collagen (Fig. 2) was calculated from the gradients in Fig. 1. There was a significant difference $(p < 0.05)$ in the concentration of lysine liberated from the uncrosslinked compared to

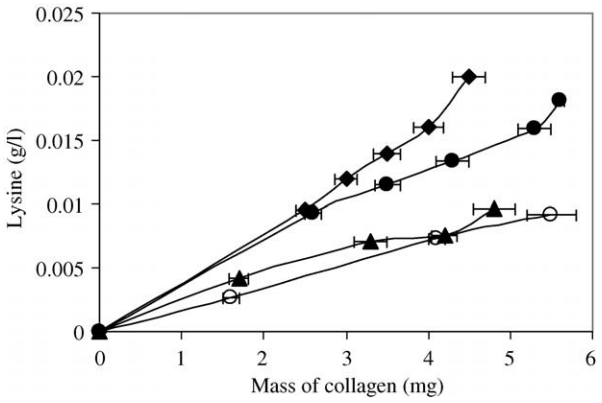

Figure 1 Free lysine concentration for a variety of collagen matrices determined using the TNBS assay. Matrices analysed: uncrosslinked, milled bovine collagen (◆); uncrosslinked, porcine collagen (●); crosslinked, porcine collagen (○); and perforated, crosslinked, porcine collagen (▲). Error bars indicate ± SD.

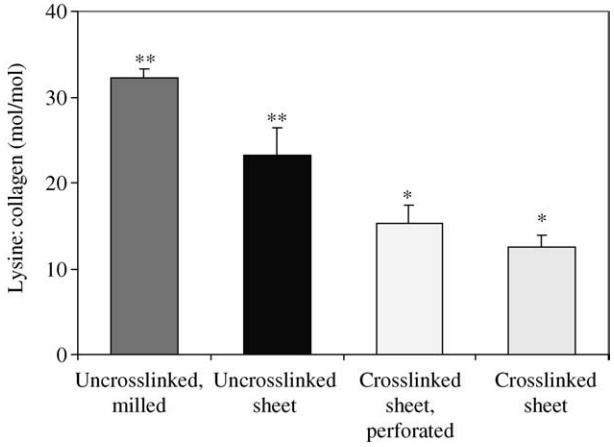

Figure 2 Difference in molar ratio of lysine : collagen for uncrosslinked and crosslinked collagen samples. Error bars indicate SD, **denotes significant difference to the other collagen samples at a 99% level, * denotes significant difference to other collagen sample at a level of at least 95%.

crosslinked collagen matrix (both samples). There was no difference between each of the crosslinked samples, or each of the uncrosslinked collagen samples.

3.2. Evaluation of fibroblast proliferation from tissue

Pre-treatment of chemically crosslinked collagen is deemed necessary for cellular proliferation to occur [14]. We examined the effect of pre-soaking the crosslinked collagen on fibroblast outgrowth from explanted dermal tissue. The matrix was soaked overnight in fibroblast media, PBS and cysteine. Positive and negative controls of tissue culture plastic and the untreated collagen matrix respectively were used. Cell outgrowth from tissue explants was achieved for all samples on the crosslinked collagen. Unwashed, crosslinked collagen (negative control) showed 18% growth of that obtained on tissue culture polystyrene (taken as 100%) (Fig. 3). The matrix samples with the highest viable cell activity were those that had been pre-soaked in fibroblast medium. However, these matrices achieved only 53% of the cell numbers of those on tissue culture plastic. Both the soaking in PBS and cysteine gave greater cell viability than the untreated matrix.

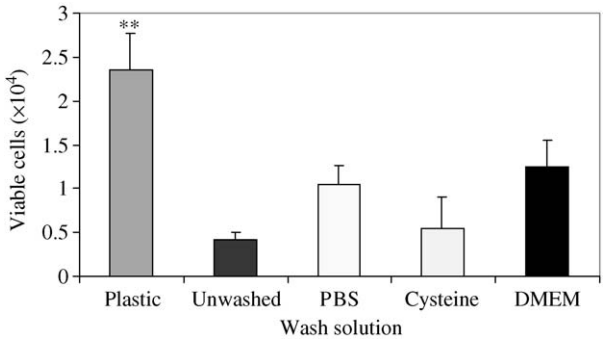

Figure 3 Effect of soaking solution on the relative cellular metabolic activity of primary fibroblasts grown from explants for 18 days on tissue culture plastic (control) and collagen matrices. Error bars indicate SD, ** represents a difference to all other samples at a 99% confidence level.

Differences were demonstrated between groups (using ANOVA) at $> 99.9\%$ confidence limit. When multiple comparisons were made within the groups using a Bonferroni *t*-test, differences could be seen between the type of soaking solution used. The growth on tissue culture plastic was significantly different ($p < 0.001$) to any of the crosslinked collagen matrix samples, independent of the soak solution. The only soak solutions that were not significantly different were the fibroblast media and PBS, and the PBS and cysteine. All soaked collagen matrix samples were significantly different to the unwashed matrix.

3.3. Cellular attachment on the matrix

Fibroblast attachment on crosslinked collagen was compared to tissue culture plastic and PET membranes. The time taken for cell attachment levels to return to the initial seeded densities was different for each matrix (Fig. 4). At a seeding density of 5.0×10^4 cells cm^2, growth on plastic resulted in cell numbers close to the inoculated levels being regained by 2 h, which then reached a plateau by 3 h. Samples on PET membranes retained numbers similar to those seeded by 3 h. The final cell attachment numbers on PET membranes were less than for tissue culture plastic until 3 h, but were always greater than the collagen matrix. Cell attachment was lowest on crosslinked collagen. A similar attachment pattern was observed at the lower seeding density of 5×10^4 cells cm^2. Analysis of the attachment data using both a Kruskal–Wallis and a Mann-Whitney test showed that there was a significant difference (95% level) between attachment on each matrix at each time point, and there was no significant difference of attachment onto any matrix when the seeding density was changed.

3.4. Microscopic observation of the cells and matrix surface

Scanning electron microscopy analysis of the collagen matrix showed the matrix as a heterogenous interwoven mesh of collagen fibres (Fig. 5). The surface varied possessing areas where fibres were dense and closely knit but also areas which had merged to form a visually smoother surface (b). Other surfaces appeared more open

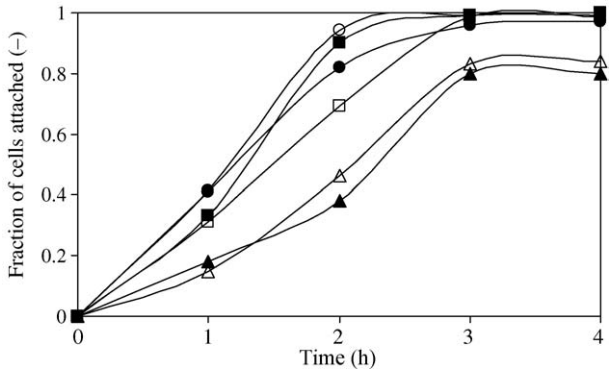

Figure 4 Fibroblast attachment onto matrices expressed as a fraction of the initial seeding density (5×10^4 cells cm^{-2}, open symbols and 1×10^5 cells cm^{-2}, closed symbols). Substrates: tissue culture plastic (●), PET membrane (■) and crosslinked collagen matrix (▲).

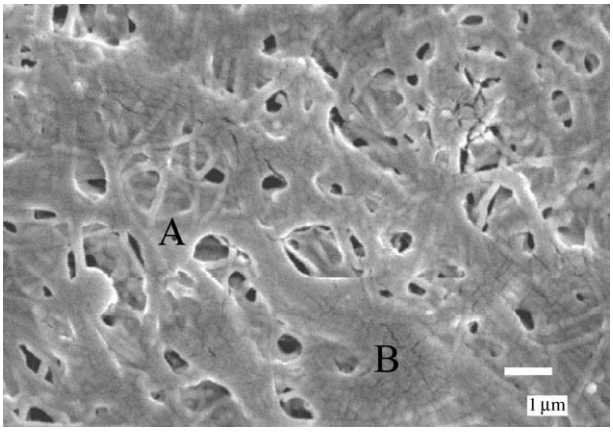

Figure 5 Micrograph of the HMDC–crosslinked collagen surface. The collagen sheet consisted of crossed fibers with spaces in between. At the surface, there were entry points into the matrix interior (a), also some areas had merged and presented a smoother amalgamated surface (b).

and presented views into the matrix interior (a). Naturally occurring hair follicles acted as channels passing from top to bottom surfaces of the collagen sheet. Human dermal fibroblasts had adhered and spread demonstrating a characteristic spindle-like morphology (Fig. 6(a)). Fig. 6(b) shows fibroblast attachment at the cell–matrix junction using pseudopodia projections from the cell membrane joining to individual collagen fibres. Fibroblasts cultured for five days were observed to bridge the open areas of the matrix, with many cells aggregating around the native hair follicles (data not shown). The staining of many nuclei at this location suggested that the cells achieved the bridging by combining to act as supports for one another without using the collagen matrix.

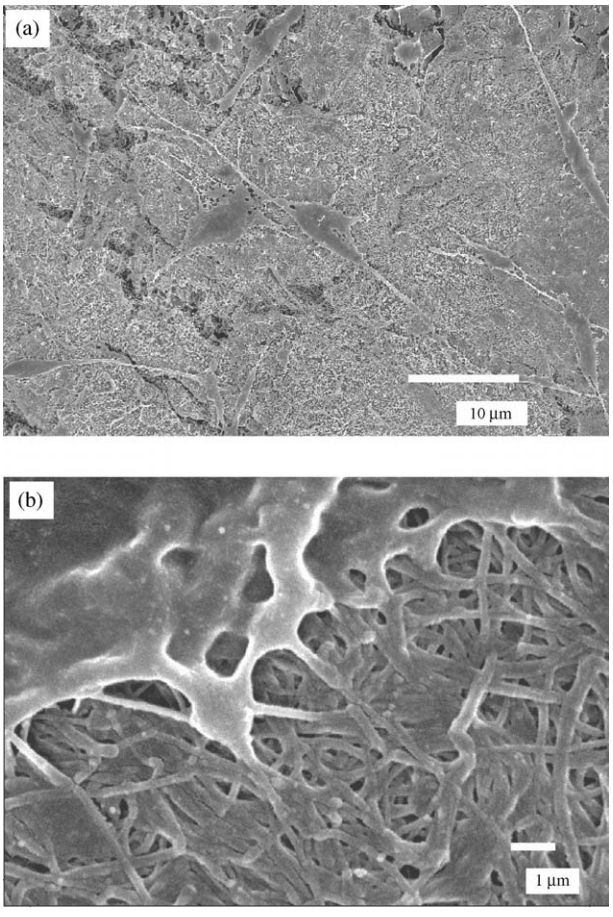

Figure 6 (a) Human dermal fibroblasts demonstrated adhesion and spreading upon the crosslinked collagen matrix surface at 48 h post-seeding. (b) Fibroblast attachment on matrix. Low temperature SEM of the attachment interface between a human dermal fibroblast and the crosslinked collagen matrix surface.

3.5. Fibroblast culture on the crosslinked collagen matrix

Fibroblasts were seeded onto the crosslinked collagen and cultured for up to three weeks. The crosslinked collagen matrix demonstrated capability of fibroblast support and maintenance over longer times by the presence of cells at five days. For longer periods up to 21 days, proliferation was demonstrated by the successive increase in numbers of hematoxylin stained fibroblast nuclei. Staining indicated the formation of confluent sheets and creation of multi-layers by three weeks as seen in Fig. 7. The matrix was also cross-sectioned to observe the depth of penetration of the fibroblasts into the collagen (Fig. 8). Transverse sections of the matrix revealed that although there were visible channels into the collagen interior, the cells remained localised to the matrix surface and followed the surface topography of the collagen.

3.6. Collagen degradation

Samples of crosslinked and uncrosslinked collagen matrix were exposed to collagenase action. Fig. 9 indicates that hydroxyproline was steadily released from both crosslinked and uncrosslinked matrix samples. The uncrosslinked matrix liberated higher levels of hydroxyproline than the crosslinked collagen samples. The rate of hydroxyproline release was also greater from the uncrosslinked collagen. After 7 h digestion the uncrosslinked collagen was visually completely digested. The crosslinked collagen matrix was not completely consumed at 10 h but had been broken down into very small, suspended fragments. Throughout the experiment, uncrosslinked samples had larger variations in the measured amounts of hydroxyproline per milligram collagen. The reduced amounts of hydroxyproline liberated by the crosslinked collagen

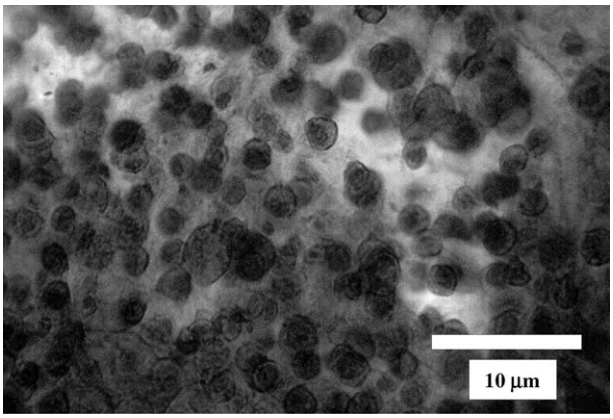

Figure 7 Longer term (3 weeks) culture of human dermal fibroblasts on crosslinked collagen. Fibroblast nuclei appeared as stacked layers when stained with hematoxylin. The image shows many cell nuclei and changing the objective revealed many nuclei at different levels below.

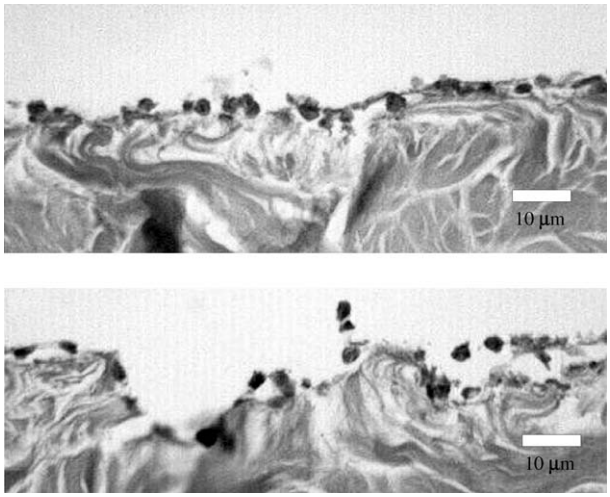

Figure 8 Transverse sections of human dermal fibroblasts cultured for 10 days on the crosslinked collagen matrix. The histological image shows that a monolayer of fibroblasts had formed which followed the contours of the collagen matrix surface. There appeared to be no infiltration of cells into the matrix interior.

samples indicated that the crosslinking procedure conferred additional resistance to collagenase degradation.

4. Discussion

In this work, we examined some of the characteristic properties of the crosslinked collagen matrix and the behaviour of fibroblasts upon it. For such matrices, issues of topography, porosity, fibre diameter, mechanical structure and shape can all have implications for the maintenance of the correct cell phenotype and morphology, associated cell expression and angiogenesis [15, 16].

The collagen matrix used here retained the same fiber orientation as when *in vivo*. Crosslinking collagen has the effect of providing changes to the chemical and mechanical strength of the matrix, possibly to the point where it cannot be biodegraded *in vivo*. In addition, crosslinking may aid in the masking of immunogenic epitopes. We tested both uncrosslinked and HMDC–crosslinked collagen by determining the number of available lysine residues which is indicative of the extent of intercollagen crosslinking [2]. HMDC–crosslinked collagen results in a reduction in the number of free amine groups (n) per mole of collagen as compared to uncrosslinked collagen. This is commonly reported as n/1000 amino acid residues in one collagen molecule [2].

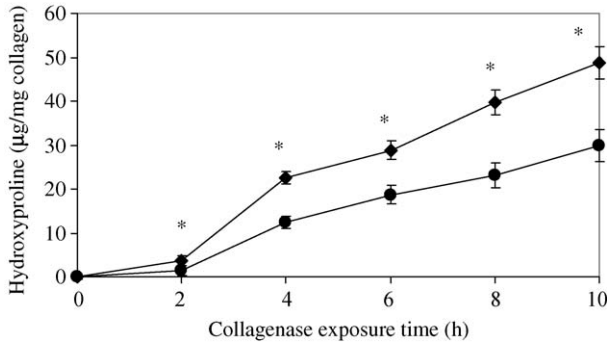

Figure 9 Effect of collagenase incubation time on release of hydroxyproline from crosslinked (●) and uncrosslinked (◆) collagen matrices. Error bars signify ± SD, *denotes significant difference between crosslinked and uncrosslinked samples at a 95% confidence level.

Our results showed that the crosslinked collagen had far fewer free lysine residues than did the uncrosslinked material. The values of 25/1000 for uncrosslinked collagen and 32/1000 for milled collagen were in line with published values for dermal ovine collagen [17]. Results for the perforated crosslinked collagen matrix (500/100) gave values of 16/1000 lysine residues per collagen molecule rather than 13/1000 for crosslinked collagen matrix. These values were slightly lower than those reported for ovine collagen [2, 18]. The concentration of the HMDC solution used for the crosslinking of the collagen matrix in this work was 0.1% (v/v) as opposed to the higher reported solution concentrations of 0.05–1.5% (v/v) [19].

The proliferation of cells from tissue explants demonstrated that all samples of the collagen matrix were capable of supporting the outgrowth of fibroblasts independent of the soaking regime. As a substrate for explant migration the non-soaked matrix demonstrated the lowest affinity for fibroblasts. This was most probably due to a residual presence of the matrix processing compounds. It was observed that equilibrating the collagen with fibroblast medium or PBS facilitated the greatest amount of cellular outgrowth on the matrix. Other studies examining explant outgrowth have also noted improvements in those samples supplemented with serum, as opposed to serum free media cultures [20]. This may have been due to the undefined adhesion factors present in serum proteins (e.g. fibronectin, vitronectin).

Crosslinking collagen with chemicals like HMDC can lead to primary and secondary cytotoxic responses. HMDC is a bifunctional molecule whose mechanism of crosslinking is via covalent bonding of the amino groups of lysine residues in the collagen. Initial reaction of the collagen with HMDC results in the formation of compounds (such as 1,6-diaminohexane), which may subsequently react with remaining isocyanate. Unreactive compounds, however, may remain as pendant molecules and these can result in toxicity to inoculated cells (primary cytotoxicity). Thus, it was critical that any residual chemicals from the HMDC treatment were washed from the collagen matrix to eliminate trace compounds. Indeed, it has been found that washing is a necessary step even for acellularised, uncrosslinked collagen [21]. Further, it has been observed that the presence of the pendant molecules may confer an additional (secondary) cytotoxicity. These molecules subsequently become exposed through cellular–scaffolding interaction, and more specifically cell secreted enzymatic digestion [2, 9, 19]. It has been observed that uncrosslinked dermal ovine collagen possessed primary cytotoxicity due to residual processing agents, but did not display the secondary toxicity exhibited by crosslinked collagen [19]. Further work from the same group isolated primary toxicity to an extractable product of the HMDC processing, whilst secondary toxicity was due to enzymatically released factors from the collagen [19, 21]. Exposure of crosslinked collagen matrix to

longer tissue culture periods (*ca.* 18 days) using human fibroblasts has been shown to reduce any *in vitro* secondary cytotoxicity [22]. Our results are in agreement with this observation, by 18 days fibroblasts were proliferating and had migrated outwards on all the matrix samples.

Other studies *in vitro* showed up to 22% inhibition of fibroblast proliferation when fibroblasts were subjected to leachable extracts from HMDC collagen sponges [23]. The effects appeared to be reduced *in vivo*, as subcutaneous implants in rats were found to be non-toxic [24]. Contrary to this, other HMDC implants still elicited immune cells 15 weeks post-implantation which suggested that there was actually some longer term background toxicity present with HMDC–crosslinked collagen [25].

The time required in order to obtain the highest fibroblast attachment numbers upon crosslinked collagen matrix was a minimum of 3–4 h. Bellincampi and Dunn [14] also found that fibroblast attachment took 4 h on HMDC–crosslinked ovine dermal collagen type I in a reconstituted fiber form. The fractional attachment was lower than the other collagen forms simultaneously tested (UV, DHT and uncrosslinked). A reason for the lower attachment may be due to the reduced water content of the HMDC–crosslinked collagen [26]. However, even uncrosslinked collagen has demonstrated initial lower attachment levels [14]. The smoother areas of the crosslinked collagen matrix surface seen during SEM analysis may indicate a surface conformational change due to the processing (cutting, handling, sterilisation) of the collagen and this may influence attachment. Extent of attachment will also vary upon cell type and cellular requirements. For example, stronger adhesion will permit fibroblast-like spreading but this may not actually be preferential with rounded cells such as chondrocytes or keratinocytes.

We observed that fibroblasts cultured on the crosslinked matrix for extended periods of time, remained on the surface of the collagen and did not penetrate into the matrix. For uses in tissue engineering it will be important for cells to be able to penetrate into the matrix structure. The matrix used here has been designed for permanence when implanted *in vivo*. This persistence also has implications for the ability of the cells to modify the matrix by secretion of collagenases, and may result in abnormalities in cellular attachment and the ability to infiltrate the matrix.

The collagenase treatment of the matrix showed that crosslinking the collagen promoted resistance to protease degradation in comparison to the uncrosslinked collagen. The collagenase activity used to challenge the matrix was much higher (10 IU/mg collagen) than the nanogram levels that occur *in vivo*. This concentration served to demonstrate whether there was a difference in the resistance of HMDC–crosslinked to uncrosslinked collagen. Hydroxyproline residues present in the collagen are normally constantly high (~ 0.13 g hydroxyproline per gram of collagen in rabbit tissue) [10] and allow a comparison of tissue samples of known mass after subjection to degradation over time at a given enzyme concentration. Thus the chemical crosslinking used to strengthen the matrix prevented the cells and their secreted proteases from disrupting the collagen structure and enabling migration.

Cysteine and serine proteases and MMP, such as gelatinase and collagenase, may be upregulated under abnormal or repair conditions [5] and an implant must be tolerant of the initial hostile wound bed, remaining long enough to fulfil its function. Implanted HMDC dermal sheep collagen in rats was degraded in six weeks, but other (photodynamically treated) crosslinked collagen has been completely resistant to degradation [27, 28].

It is important to obtain a balance between the mechanical resistance of the matrix and its ability to participate in normal cell–matrix interactions and migration. These results indicate that the matrix possessed some resistance to the collagenases, but will still degrade over time. Thus, these and other findings [7] show that collagen type I is susceptible to degradation from a wide range of collagenases. Under steady-state conditions in normal skin, fibroblasts may not be secreting collagenases and hence the matrix would be unaffected. This may also occur under normal *in vitro* conditions and explain why no infiltration was observed. However, at a wound site, degradation of implanted collagen may be more rapid due to the upregulation of collagenase synthesis by fibroblasts. Furthermore, this may be compounded by changes in enzyme reactivity due to the pH and temperature at the site.

Although the crosslinked collagen matrix can support fibroblast growth and attachment, the fibroblasts appear unable to digest a way into the interior. Infiltration has been achieved using a crosslinked bovine collagen substrate, demonstrating neovascularisation and fibroblast migration when *in vivo* [29]. Finding a compromise to the levels of matrix crosslinking that allows appropriate stability whilst permitting cells to migrate is therefore required.

Acknowledgments

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