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The influence of crosslinking agents and diamines on the pore size, morphology and the biological stability of collagen sponges and their effect on cell penetration through the sponge matrix

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Artificial skin substitutes based on autologous keratinocytes cultured on collagen substrata are being developed for treating patients with severe burns. The properties of the collagen substrate can be manipulated, for example, by crosslinking, to optimize desirable properties such as cell growth and penetration into the substrate, biological stability and mechanical strength. Collagen sponges crosslinked with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and the diamine, diaminohexane, were used to determine the effect of crosslinking on pore size and morphology, on the stability of the crosslinked sponges both in cell culture media and during incubation with collagenase, and on the penetration of keratinocytes and fibroblasts through the sponge matrix. Crosslinking of the sponges reduced the pore size, particularly at the surface, and altered sponge morphology. After crosslinking the collagen fibers were thinner, and appeared lacy and delicate. Crosslinking also influenced sponge stability. In keratinocyte serum-free medium the pore size of plain collagen sponges increased with increasing incubation time, and crosslinking appeared to prevent this, and may have stabilized sponge structure. Incubation in serum-containing Dulbecco's minimum essential medium caused a marked reduction in pore size in both plain collagen and crosslinked collagen sponges. Crosslinking did not appear to influence this cell-free contraction of collagen sponges. Treatment of sponges with EDAC markedly increased the resistance of sponges to collagenase digestion. The penetration of both keratinocytes and fibroblasts was retarded by crosslinking the sponges. Fibroblasts penetrated through the sponges to a greater extent than keratinocytes, and their proliferation rate was faster. The total number of cells populating the crosslinked sponges after 10 days culture was approximately 50% of that on untreated collagen sponges. The mechanism responsible for this effect was different with the two crosslinkers used. Diaminohexane appeared to inhibit cell growth, whereas EDAC may have caused a decrease in cell adhesion to the sponges, without an apparent inhibition of growth rate. In terms of morphology, fibroblasts were elongated to a greater extent on crosslinked sponges, and alligned themselves along the collagen fibers. Keratinocytes grew in colonies on untreated sponges, but on crosslinked sponges they grew in isolation, with minimal cell–cell interactions. It may be necessary to reach a compromise to obtain the best combination of properties for using collagen sponges as substrata for artificial skin substitutes.

1. Introduction

Victims of severe burns often become susceptible to massive fluid loss from the wound bed as well as increased risk of infection. Such burns can prove fatal if over half of the body area is damaged. Standard medical procedure for burns treatment is the introduction of an autologous split-thickness graft to the area affected. However, particularly in severely burned patients,

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sufficient donor sites may not be available, and this has led to the development of artificial skin utilizing autologous cells grown on a biocompatible matrix [1]. At the present time, collagen appears to be the most suitable substrate for such implants. This is due to such properties as low antigenicity, growth promotion, biostability, reasonable mechanical strength and ease of handling. Additionally, collagen is suitably malleable and can be produced in various forms such as films, gels, and sponges [2–7].

The structure and composition of the collagen substrate can be modified in order to manipulate the aforementioned properties, and one of the most commonly used modifications is chemically induced crosslinking [8]. In this study, we have used collagen sponges crosslinked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and the diamine, diaminohexane, to determine the effect of crosslinking treatment on pore size and morphology, on the stability of the crosslinked sponges both in cell culture media and during incubation with collagenase, and on the distribution and morphology of dermal fibroblasts and keratinocytes cultured on the sponges. Previous work by us has shown that, unlike other crosslinking agents such as glutaraldehyde which poisons cells [9, 10], incorporation of these chemical agents into collagen gels did not result in cytotoxicity, and they support the growth of both human keratinocytes and dermal fibroblasts [11, 12].

We have previously used confocal laser scanning microscopy (CLSM) to analyze the microstructure of uncrosslinked collagen sponges [13]. This proved to be a highly effective procedure offering several advantages over previous scanning electron microscopy (SEM) studies. Such advantages include the non-invasive nature of the technique; samples do not have to be dehydrated or sectioned as is necessary for conventional microscopy or SEM studies. This reduces the presence of preparation artefacts. In the present study we have utilized CLSM to investigate the effect of crosslinking agents on pore size and morphology in the sponge structure. This will greatly influence the ability of cells to penetrate and modify the sponge structures and this has been investigated by determining the penetration of human skin fibroblasts and keratinocytes through the sponge matrices.

Sponge microstructure has been shown to be altered with prolonged (up to 10 days) exposure to culture medium [13]. We have conducted a study of crosslinked collagen sponges incubated in different culture media over 10 days to find out whether or not crosslinking improves the stability of the fiber structure. Two different media were used: Keratinocyte-serum free medium (K-SFM) and Dulbecco's modified Eagle's medium (DMEM) which is commonly used for maintaining dermal fibroblasts.

Data are presented on the biological stability of the collagen sponges in terms of collagenase digestion before and after treatment with the crosslinking agents. There is some debate over the optimum biological stability of collagen substrata used for artificial skin applications. Collagen based biomaterials are rapidly degraded *in vivo*, but the degradation of the original graft material must be matched by the rate of new tissue deposition so that the physical integrity and function of the remodeled graft are maintained. Evidence from the literature is available indicating that the rate of degradation can be decreased by various crosslinking procedures including treatment with acyl azide, glutaraldehyde, cyanamide and diphenylphosphorylazide [8, 14–16].

2. Materials and methods

2.1 Collagen sponge preparation

Collatamp collagen sponge, containing 5.6 mg/ml native bovine collagen, was provided as a gift from Innocoll Co., Saal/Dona, Germany as a sample of their commercial products.

Circular discs of Collatamp sponges (15.5 mm diameter, 4 mm thickness) were rehydrated for 10 min in 70% ethanol. Sponges were washed three times in Earle's balanced salt solution (EBSS) containing 60 μg/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone for 20 min.

2.2. Addition of crosslinking agents

Solutions were prepared immediately before use. The carbodiimide, EDAC, was prepared at a 0.1 M concentration, and the diamine, 1,6,-diaminohexane, at 1 M concentration. 220 μl of EDAC and 25 μl of diaminohexane were pipetted onto the appropriate sponge. Sponges were left to crosslink for 16–18 h at room temperature. Unbound and excess chemicals were washed out 10 times with sterile distilled water, with a 30 min incubation at room temperature for each wash. After washing, growth medium was added and the sponges were incubated at 37 °C for 3, 7 and 10 days.

2.3. Cell culture

Normal human skin samples were obtained from patients admitted to the Burns Unit at Glasgow Royal Infirmary. The epidermis and dermis were separated by floating the skin on 0.5% (w/v) dispase in phosphate buffered saline (PBS) at 4 °C overnight. Keratinocytes were isolated by first mincing the epidermis, followed by digestion with 0.05% (w/v) trypsin/0.5 mm ethylene diamine tetraacetic acid (EDTA), to obtain a single cell suspension. The cells were cultured in serum-free keratinocyte medium (K-SFM) supplemented with 5 ng/ml human recombinant epidermal growth factor and 35–50 μg/ml bovine pituitary extract. Cells were used for experiments when they were three passages old and the seeding density on the sponges was 2.5×10^4 cells/sponge. Fibroblasts were isolated by mincing the dermis, and these were grown in DMEM supplemented with 10% (v/v) fetal calf serum (FCS). Cells were used for experiments when they were three passages old, and the seeding density on the sponges was 5×10^3 cells/sponge. To seed the cells, after the final wash with distilled water, sponges were equilibrated in either K-SFM or DMEM for 30 min. Sterile stainless steel seeding rings were placed on top of the sponges, cells added to the area inside the rings ($0.98\,cm^2$), and left to attach for 2 h at 37 °C before medium was added to the outside of the sponges. The seeding rings were removed

after 15–18 h. The seeded sponges were incubated at 37 °C in an atmosphere of 5% CO_2 in air, and the medium was changed after 3, 7 and 10 days.

2.4. Fixing and staining the samples

At 3, 7, and 10 days after growth medium addition, sponges were washed in PBS to remove all traces of medium. Collatamp samples were then fixed in phosphate buffered formalin (PBF) for a minimum of 24 h. PBF was washed out with several changes of distilled water for 90 min. Cells present on the sponges were visualized by staining with 0.5% (w/v) ethidium bromide for 6 min followed by washing three times with distilled water, for 5 min per wash. This was followed by a 6 min incubation with 0.1% (w/v) acriflavine to stain the collagen, after which samples were washed with distilled water to remove excess stain [13], and examined immediately by CLSM.

2.5. CLSM examination

Sponges were attached to a plastic Petri dish using cyanoacrylate adhesive and examined in a Leica CLSM using a × 25/0.75 water immersion lens and the 488 nm excitation line from an argon ion laser. The CLSM utilizes a dichroic mirror centred at 580 nm to split the fluorescence emission. Pore size samples (without cells) were examined by images collected through an additional filter set at 515 nm (rejects light below this wavelength and allows acriflavine fluorescence to pass through). When ethidium and acriflavine stains were used together in the presence of cells, an additional filter set at 590 nm was used to split up the fluorescence obtained from the two stains, allowing the images to be collected on different channels.

To measure pore sizes, the microscope was set to scan the surface of the sample and at 20 μm depths into the specimens. Pore area was measured using the random window subroutine whereby pores were outlined via a trackerball and the area calculated in arbitrary units [13]. Wherever possible, 100 pores were measured in each sponge variant at both the surface and at each 20 μm level. The microscope stage was moved randomly between measurement at each field of view to prevent any experimenter bias. For examining cell distribution, images were collected at 10 μm steps down to a depth of 120 μm. Cell nuclei were counted on each image. Ten fields of 200 × 200 μm were examined on each sponge, at each depth, and 5 sponges were examined for each cell type cultured on each variant of sponge.

2.6. Collagenase digestion

Crosslinked and uncrosslinked Collatamp samples were equilibrated in 50 mM TES (N-tris[Hydroxyethyl] methyl-2-aminoethane sulfonic acid) buffer with 0.36 mM $CaCl_2$ (pH 7.4 at 37 °C). 0.1 mg/ml of collagenase (Sigma Chemical Co., type 1A, activity = 550 units/mg solid) in TES buffer or TES buffer alone were then added and samples incubated at 37 °C for 5 h. Solution contents were then filtered through Whatman #54 filter paper and 200 μl of this filtrate mixed well with 2ml of ninhydrin color reagent (1 : 1 : 1 ratio of 4% (w/v) ninhydrin in ethylene glycol monomethyl ether [EGME], 200 mM citrate buffer with 0.16% (w/v) stannous chloride, and 50% (v/v) n-propanol). Solutions were then placed in a boiling water bath for 20 min to allow color development, after which 10 ml of 50% (v/v) propanol : water was added and the absorbance values read at 600 nm. This method has been modified from that of Weadock *et al*. [8]. Collagenase digestion was quantified as the nmoles of l-leucine liberated per mg sponge weight by a standard curve comparison.

3. Results

Fig. 1 shows the pore size distribution at the surface and at 20 and 40 μm into treated and untreated collagen sponges after 3, 7 and 10 days incubation with K-SFM. Data relating to the pore sizes at the surface, and at depths of 20 μm, and 40 μm into the sponge are presented. Pore size measurements at deeper depths could not be presented as they became too large to measure. Due to the skewed nature of the data, the median values of pore area in arbitrary units are presented. Results of Mann-Whitney tests of significance showed that, at the surface, the untreated sponges had significantly larger pore areas than all of the treated sponges at 3, 7 and 10 days (Fig 1a). None of the treated sponges differed significantly from each other with increasing incubation time in the serum free medium. However, this was not the case with the uncrosslinked sponges; there appears to be a trend towards increased pore size with increased incubation time. After 10 days, the untreated sponges showed an approximate 3-fold increase in pore size, which was significantly different from the pore sizes at 3 days.

Fig. 1b displays pore measurements at 20 μm into the sponges. After 3 days, the EDAC treated sponge and the diaminohexane treated sponge had significantly smaller pores than the untreated samples. At 7 days, the diaminohexane treated and EDAC + diaminohexane treated sponges were the only sponges that had significantly smaller pore sizes than the uncrosslinked sponge. At 10 days, the sponges crosslinked with EDAC, and with the combination of EDAC and diaminohexane, had significantly smaller pores when compared with those in the 10-day-untreated sponge. Considering the uncrosslinked sponges; the 7- and 10-day-old sponges had larger pores when compared with those after 3 days of incubation. This was also observed in the sponges treated with EDAC, and those treated with diaminohexane. However, sponges crosslinked with the combination of EDAC and diaminohexane did not show a significant increase in pore size with incubation time at this depth.

At a depth of 40 μm, and after 3 days of SFM incubation (Fig. 1c), only the EDAC-crosslinked and the diaminohexane-crosslinked sponges significantly differed from the untreated sponge, and showed a reduction in pore size. At 7- and 10- days incubation, none of the chemically crosslinked sponges had pore size measurements that differed significantly from the pore sizes of the untreated variant. There was a statistically significant increase in pore sizes of EDAC- and

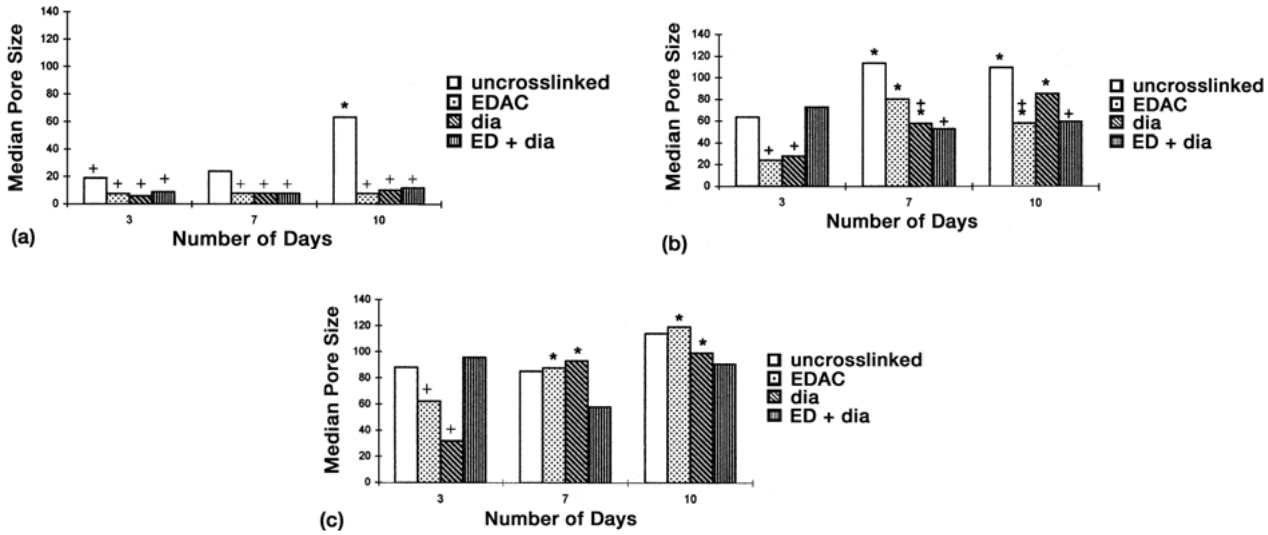

Figure 1 The change in pore size caused by incubation in K-SFM. (a) Surface, (b) 20 μm and (c) 40 μm. Results are the median of 50 measurements in arbitrary units. $^{*}P < 0.05$, by Mann-Whitney test. Significance values refer to the differences between the relevant 3 day treatment, and those of longer duration. $^{+}P < 0.05$, by Mann-Whitney test. Significance values refer to differences between uncrosslinked sponges and those treated with crosslinkers at each of the three specified days.

diaminohexane-treated sponges that have been incubated in the SFM for 7 and 10 days compared with those incubated for 3 days.

Fig. 2 shows the pore size distribution within treated and untreated collagen sponges after 3, 7 and 10 days incubation in DMEM. Again, pore measurements at deeper depths could not be presented due to pores becoming too large to measure. The median values of pore area in arbitrary units are presented. Results of the Mann Whitney tests of significance showed that, at the surface, the pore sizes of the untreated sponges at 3, 7 and 10 days were significantly larger than the pore sizes of all of the chemically crosslinked sponges incubated for the same time period (Fig. 2a). Additionally, there was a trend towards a decrease in pore size with increased incubation time in the medium. For example, in the uncrosslinked sponge, where there was an approximate 6-fold decrease in pore size when comparing the 3 day and 10 day incubations (200 units on day 3 compared with 27.5 units on day 10). The sponges treated with EDAC showed an even larger decrease in pore size with increased incubation times; an almost 10 fold reduction between 3 day and 10 day incubation times was displayed (68.5 units on day 3 compared with 7 units on day 10). This effect was less pronounced in the sponges that had been crosslinked with diaminohexane, and the combination of EDAC and diaminohexane.

At a depth of 20 μm, (Fig 2b) only the pores sizes in the EDAC treated sponge were significantly different from those in the untreated sponge after 3 days. However, after 7 days, both the diaminohexane, and the combination of EDAC and diaminohexane, had significantly larger pores than the relevant untreated sample. There was no significant difference between any of the treatments after 10 days incubation. After 10 days, only the uncrosslinked and the diaminohexane treated sponges displayed any significant decrease in pore size when compared to the 3 day incubations.

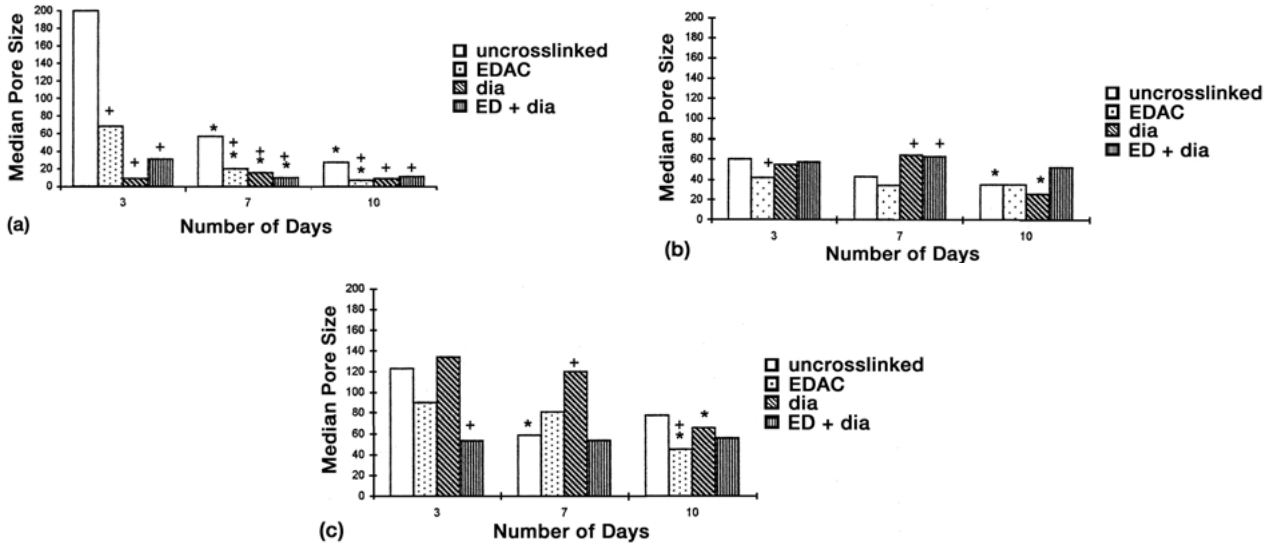

Figure 2 The change in pore size induced by incubation in DMEM. (a) Surface, (b) 20 μm and (c) 40 μm. Results are the median of 50 measurements in arbitrary units. $^{*}P < 0.05$, by Mann-Whitney test. Significance values refer to the differences between the relevant 3 day treatment, and those of longer duration. $^{+}P < 0.05$, by Mann-Whitney test. Significance values refer to differences between uncrosslinked sponges and those treated with crosslinkers at each of the three specified days.

In Fig. 2c, at a depth of 40 μm, only the pore sizes in the EDAC + diaminohexane treated sponge were significantly different from those in the untreated sponge at 3 days. However, at 7 days, the diaminohexane treated sponge had significantly larger pore sizes than those in the corresponding untreated sponge. When examining the sponges after 10 days, only the EDAC treatment had a significantly smaller pore size than the 10 day untreated sample.

At the surface of the sponges there was a significant decrease in pore size with increasing time of incubation in DMEM, and this effect was particularly evident when comparing pore sizes at 3 and 7 days in the uncrosslinked and EDAC treated sponges (Fig. 2a). This effect was not so pronounced within the interior of the sponges, at either the 20 or 40 μm depth, although the trend still exists in many of the samples. This is the inverse of the effect observed during incubation with K-SFM.

Examples of surface pore structure of the four sponge variants are shown in Fig. 3. These photographs were taken after 7 days incubation in K-SFM. The differences in pore structure between the variants can clearly be seen; the untreated sponge (Fig. 3a) had a much more open pore structure, with the collagen fibers taking on a much thicker appearance than the sponges that had been crosslinked. Fig. 3b–d show the surface pores of sponges crosslinked with EDAC, diaminohexane and the combination of the two. Here, the fibers had taken on a more delicate, lacy appearance, were much thinner and the pore size was markedly reduced. Many pores appeared to be blocked with stained material.

At a depth of 40 μm, however, pore structure was very different compared with that observed at the surface; large differences in pore size were not apparent (Fig. 4). Instead, the pores of each variant seemed to take on a more homogenous appearance. The untreated sponge (Fig. 4a) and the diaminohexane crosslinked sponge (Fig. 4c) appeared to have a similar pore geometry and also the collagen fibers appeared to be of approximately the same thickness. Although the pore sizes of the EDAC treated sponge (Fig. 4b) and the EDAC + diaminohexane treated sponge (Fig. 4d) were comparable to the other two, their fibers appeared to be slightly thinner, giving the impression that they were more delicate. These morphological observations on pore size correlate well with the quantitative data presented on Figs 1 and 2.

Fig. 5 depicts the biological stability of the different collagen sponges as a function of leucine liberated during a 5 h collagenase digestion. The lower the concentration of leucine liberated, the more stable the sponge. The

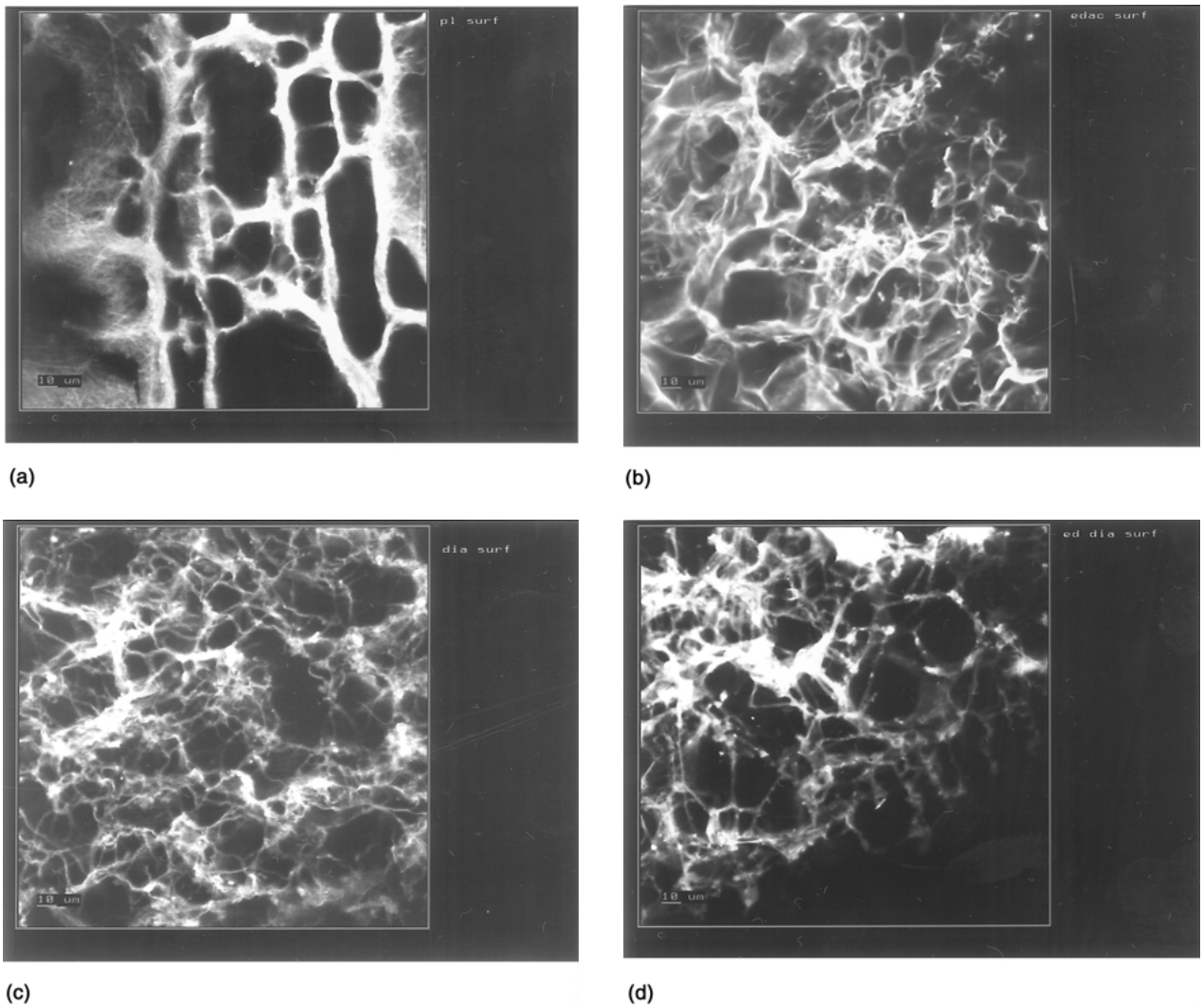

Figure 3 Confocal micrographs of the surface structure of collagen sponges. (a) Untreated, (b) EDAC crosslinked, (c) diaminohexane treated and (d) EDAC + Diaminohexane treated. Samples were incubated in K-SFM for 7 days prior to fixation and examination. Magnification is indicated by the scale bar.

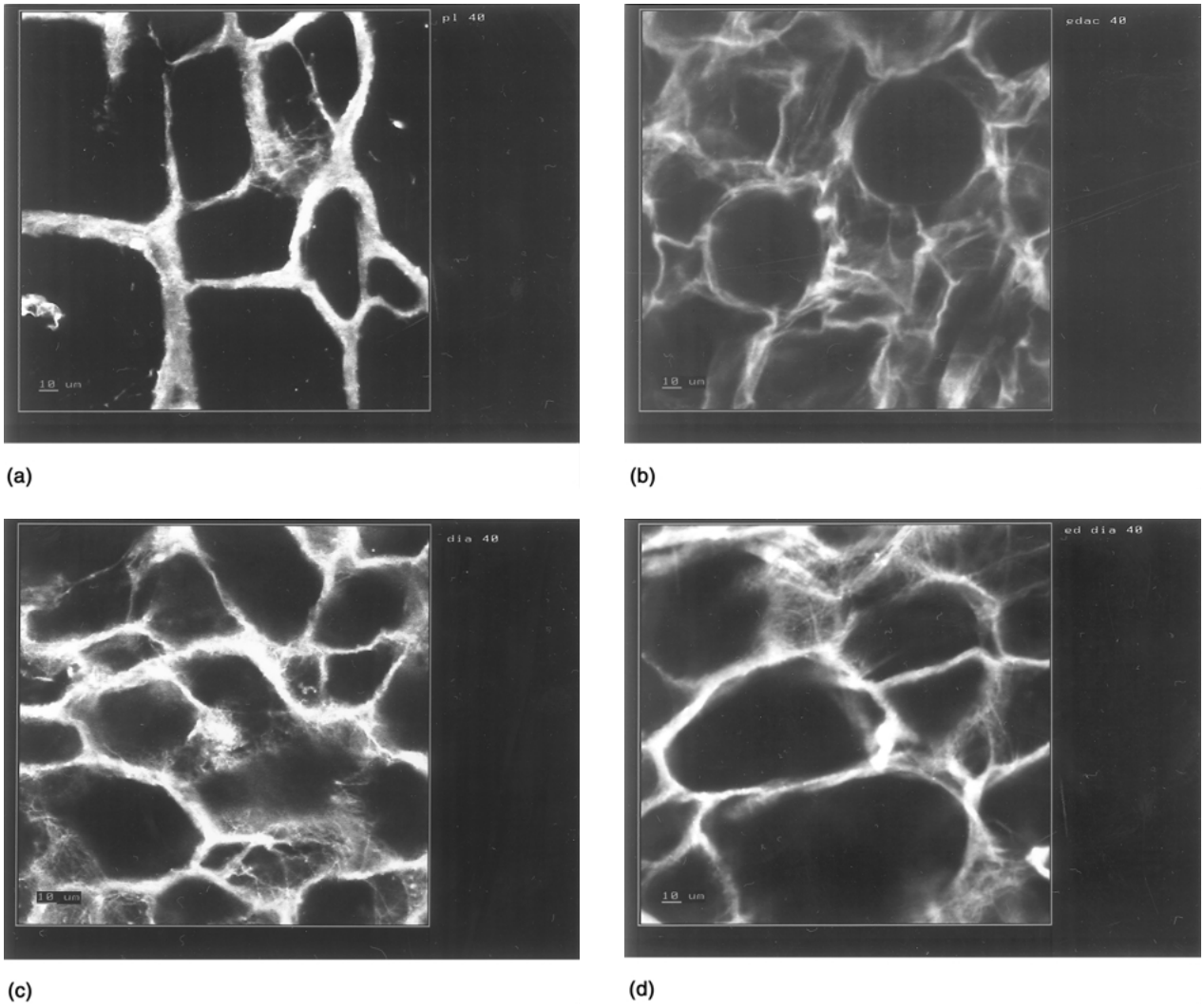

Figure 4 Confocal micrographs of the structure of collagen sponges 40 μm from the surface. (a) Untreated, (b) EDAC crosslinked, (c) diaminohexane treated and (d) EDAC + Diaminohexane treated. Samples were incubated in K-SFM for 7 days prior to fixation and examination. Magnification is indicated by the scale bar on the photograph.

biological stability of the treated sponges was compared with that of the uncrosslinked variant by ANOVA followed by Dunnett's test. Although the sponges crosslinked with diaminohexane and with the EDAC + diaminohexane combination showed a smaller concentration of leucine liberated when compared with the untreated sponge, this difference was not statistically significant. Only the sponges that had been crosslinked with EDAC displayed a significant difference in leucine liberation compared with the uncrosslinked sponges ($p < 0.05$). Results of leucine liberation indicated that the EDAC crosslinked sponges were approximately 6-fold more stable than untreated sponges.

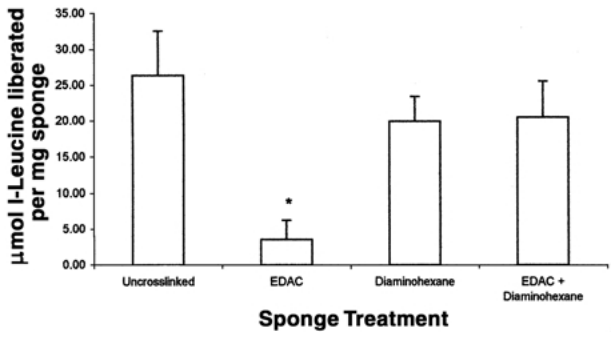

Figure 5 Collagenase digestion of the untreated and crosslinked collagen sponges. Results are mean + S.D. of $n = 4$. $^{*}P < 0.05$, compared with the uncrosslinked sponge, by ANOVA followed by Dunnett's test.

Fig. 6a–d shows the infiltration of keratinocytes into untreated, EDAC, diaminohexane and EDAC/diaminohexane crosslinked sponges. After 3 days keratinocytes had penetrated to between 50 and 60 μm into untreated, diaminohexane, and EDAC/diaminohexane treated sponges. Cells only penetrated 20 μm into EDAC treated sponges after 3 days culture. By 7 days maximum penetration was 50–70 μm into each sponge type. After 10 days penetration was greatest into untreated sponges (120 μm), followed by diaminohexane treated (90 μm), EDAC/diaminohexane (70 μm), and was least into EDAC treated sponges (50 μm). The greatest number of cells was to be found 20–30 μm into the sponges in all variants except those treated with EDAC alone. In this case maximum cell numbers were found at 10 μm depth.

Table I shows the total number of keratinocytes observed throughout the different sponge types after 3, 7 and 10 days growth. Cells growing on the untreated sponge approximately doubled in number at each time

TABLE I Total number of keratinocytes observed on each sponge variant at 3, 7 and 10 days of culture

Days in culture	Sponge treatments			
	Untreated	EDAC	Diaminohexane	EDAC + Diaminohexane
3	95	40	130	95
7	240	155	160	140
10	435	210	225	170

Values are the sum of the mean cell numbers obtained from each measured depth.

interval. By 10 days the total number of cells counted on the treated sponges was approximately 50% of that on the same area of untreated sponges. Growth rate was slower on the sponges exposed to diaminohexane, between 3 and 10 days. In contrast, initial cell reponse to EDAC treated sponges resulted in lower cell numbers at 3 days, but the overall proliferation rate between 3 and 10 days did not seem to be affected.

Fig. 7a–d shows the infiltration of dermal fibroblasts into untreated, EDAC, diaminohexane, and EDAC/diaminohexane treated sponges. After 3 days, fibroblasts had penetrated to between 30 and 40 μm into untreated, diaminohexane and EDAC/diaminohexane treated sponges. As with the keratinocytes, fibroblasts only penetrated 20 μm into EDAC treated sponges after 3 days. After 7 days cells had penetrated deepest into the untreated sponges (80 μm), and between 50 and 60 μm into each of the other sponge types. Fibroblasts had penetrated to 120 μm into untreated sponges by 10 days, 110 μm into EDAC/diaminohexane treated sponges, 90 μm into EDAC treated sponges, and 70 μm into diaminohexane treated sponges. The greatest number of fibroblasts was to be found 20 μm from the surface with all sponge types.

Table II depicts the total number of fibroblasts observed throughout the depths of each sponge variant

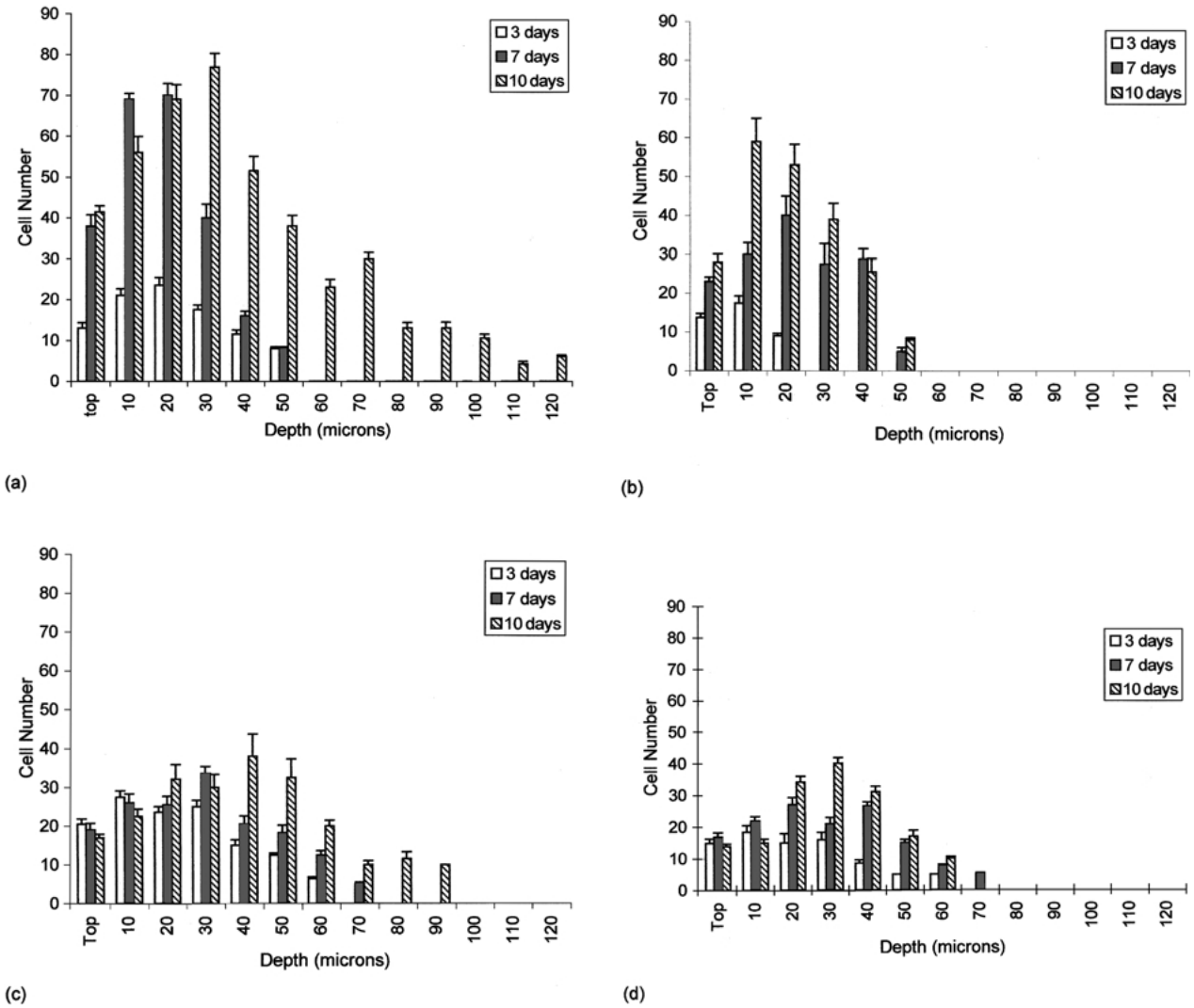

Figure 6 Penetration of keratinocytes through (a) untreated collagen sponge; (b) EDAC crosslinked collagen sponge; (c) diaminohexane crosslinked collagen sponge and (d) EDAC + diaminohexane crosslinked sponge as a function of depth after 3, 7 and 10 days culture. Results are presented as the mean ± SD of 10 fields of view on five different sponge samples.

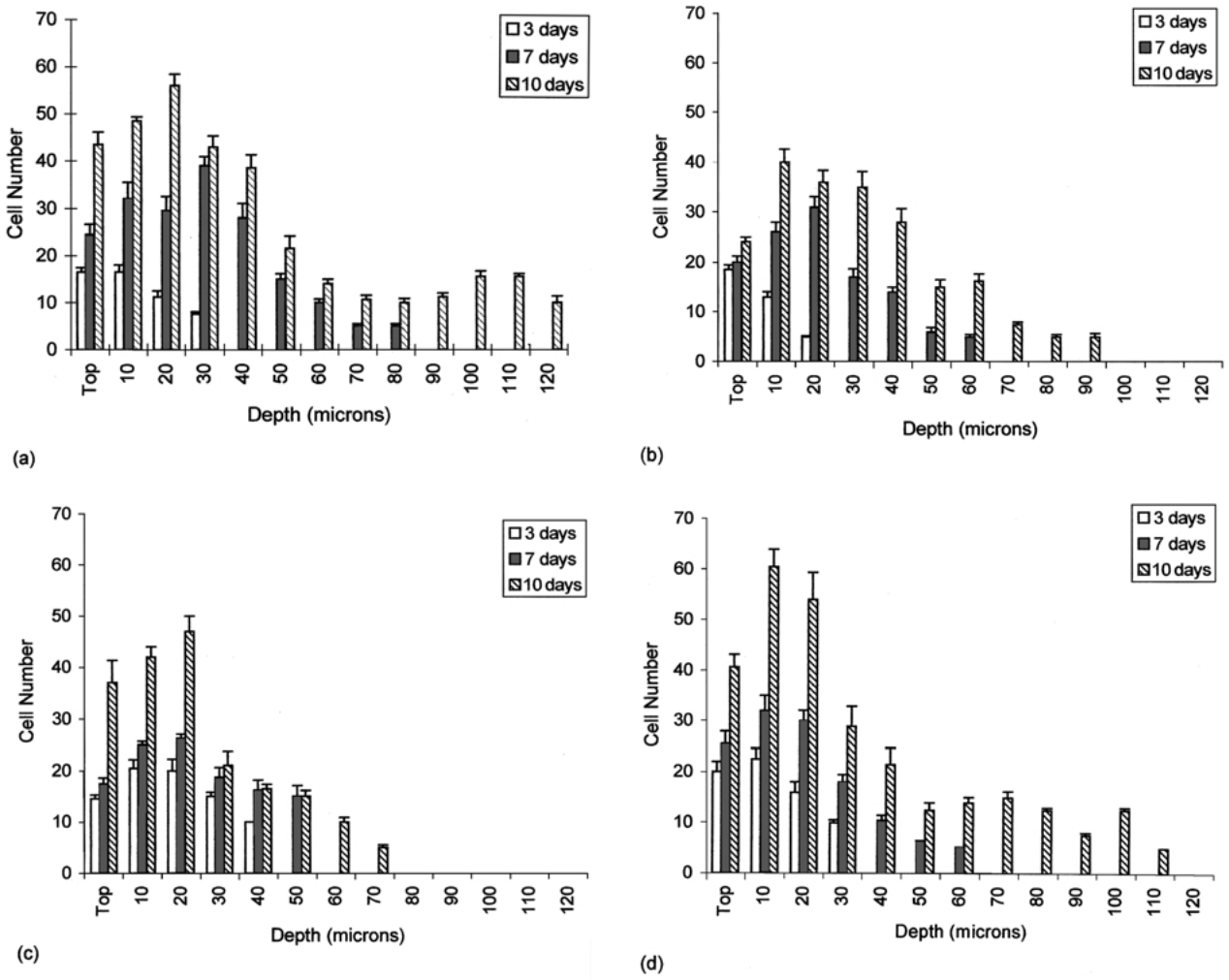

Figure 7 Penetration of fibroblasts through (a) untreated collagen sponge; (b) EDAC crosslinked collagen sponge; (c) diaminohexane crosslinked collagen sponge and (d) EDAC + diaminohexane crosslinked sponge as a function of depth after 3, 7 and 10 days culture. Results are presented as the mean ± SD of 10 fields of view on five different sponge samples.

after 3, 7 and 10 days growth. Fibroblasts proliferate more rapidly than keratinocytes on the untreated sponges; between 3 and 10 days keratinocyte numbers increased 4.6-fold whereas fibroblast numbers increased 6.8-fold during the same time. A similar pattern of response was seen with fibroblasts as shown on Table I for keratinocytes. The growth rate was decreased by exposure of sponges to diaminohexane, and the initial response to EDAC treated sponges resulted in low cell numbers at day 3, but this treatment did not affect the proliferation rate observed between 3 and 10 days.

Fig. 8 shows the morphology of keratinocytes growing on untreated sponges (a), EDAC treated (b), diaminohexane treated (c), and EDAC/diaminohexane treated sponges (d) at a depth of 10 μm. Cells had a round shape, and grew in colonies, on the untreated sponges. On all the treated sponges the keratinocytes retained their characteristic round shape, but they grew in isolation and did not form colonies. Fig. 9 shows the morphology of fibroblasts growing on untreated sponges (a), EDAC treated (b), diaminohexane treated (c) and EDAC/diaminohexane treated sponges (d) at a depth of 10 μm. The fibroblasts were elongated in the same orientation as the collagen fibers in the sponge matrix in all types of sponge. On the treated sponges the fibroblasts showed greater elongation than on the untreated collagen sponges, and this was thought to be because the pores were larger so the cells had to stretch further to adhere to the fibers forming the pore walls. There was some evidence of fiber degradation in all the sponges, as judged by the presence of blurred ''fuzzy'' fibers at the fibroblast attachment points.

TABLE II Total number of fibroblasts observed on each sponge variant at 3, 7 and 10 days of culture

Days in culture	Sponge treatments			
	Untreated	EDAC	Diaminohexane	EDAC + Diaminohexane
3	50	35	80	70
7	190	120	120	125
10	340	210	195	285

Values are the sum of the mean cell numbers obtained from each measured depth.

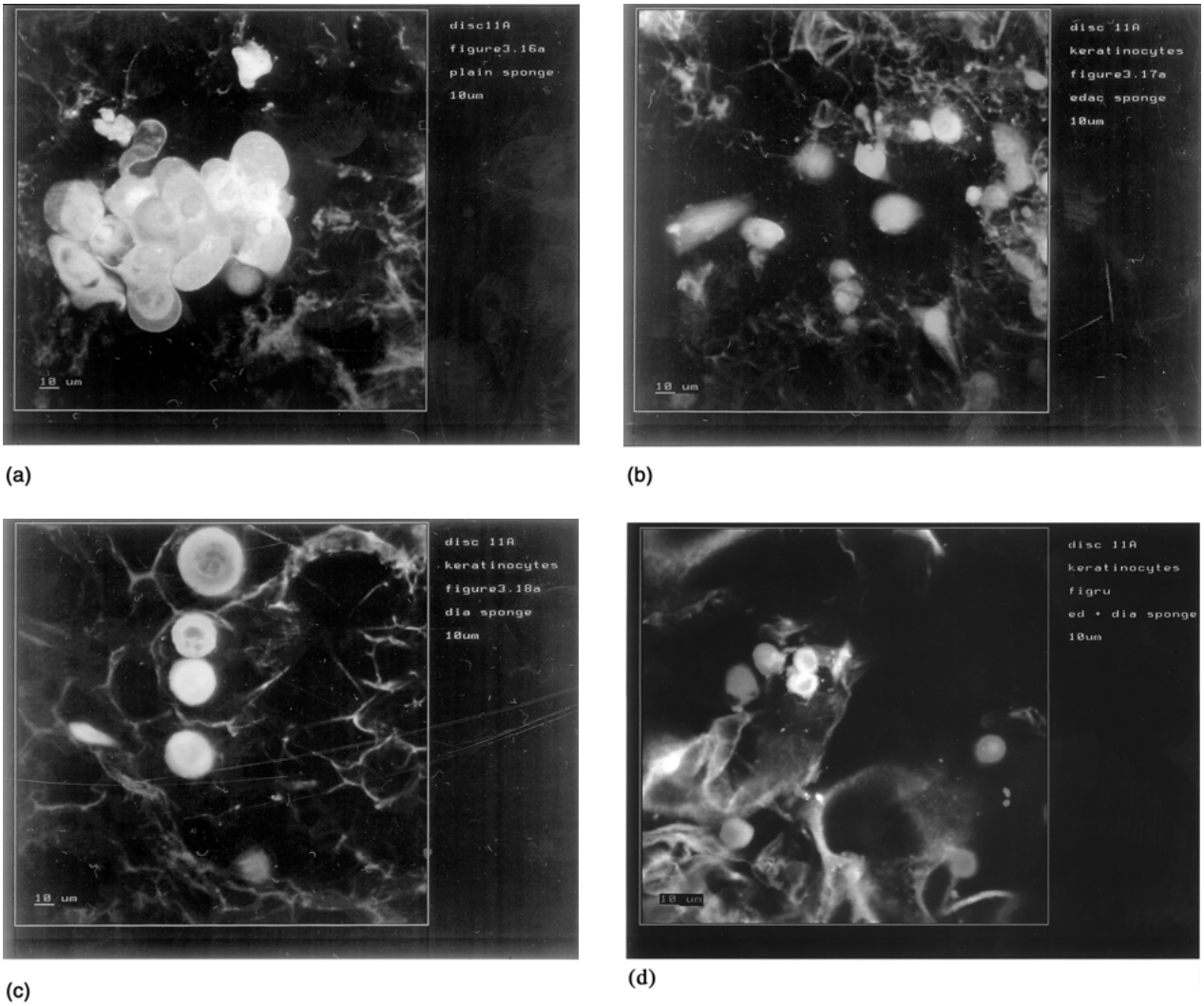

Figure 8 Confocal micrographs of keratinocytes growing on (a) untreated collagen sponge; (b) EDAC treated sponges; (c) diaminohexane treated sponges; and (d) EDAC + diaminohexane crosslinked sponges. Images were taken 10 μm into the sponges after 7 days in culture. Magnification is indicated by the scale bar on the pictures.

4. Discussion

Utilization of the CLSM permitted the structure of the crosslinked collagen sponges to be examined down to a depth of 40 μm. Although, we have previously been able to measure the pore sizes of uncrosslinked collagen sponges down to a depth of 100 μm [13], with many of the crosslinked samples measurement of pore size became somewhat difficult and more subjective, due to the fact that pores at increasing depth became increasingly larger and irregular. This was especially true with sponges that were crosslinked with EDAC and diaminohexane, where the marked physical changes in sponge structure induced by these chemical agents obstructed measurements. This was mainly due to the presence of closed and semi-closed pores that hindered objectivity.

The phenomenon of pore size increase with increasing incubation time in culture medium has been demonstrated by us previously in plain collagen sponges incubated in K-SFM [17]. This observation was confirmed here, with the uncrosslinked sponges incubated in K-SFM, and there was a statistically significant increase in pore size of plain collagen sponges at the surface and at a depth of 20 μm. The effect was most evident at the surface, diminished in the interior of the sponge, and, in fact, there was no difference in pore sizes of the uncrosslinked sponge at a depth of 40 μm when incubated in K-SFM for up to 10 days. Crosslinking appeared to prevent the surface pores from opening out in the presence of K-SFM, and may therefore have stabilized the sponge structure. However, within the crosslinked sponges, at depths of 20 and 40 μm, the pore sizes increased after exposure to K-SFM. The fact that the stabilizing influence of the crosslinking agents was confined to the surface pores may reflect the method used to crosslink the sponges, where the crosslinking chemicals were applied to the surface of preformed collagen sponges. We are currently investigating methods of adding the crosslinking agents to the collagen prior to preparing the sponges.

The mechanism(s) responsible for the increase in pore size in sponges incubated in K-SFM is/are at present unclear. We observed previously that the fibers of collagen become distorted and swollen, and appear to be partially degraded [17], and this would be consistent with biological breakdown of the collagen fibers forming the pore walls, thereby resulting in an increase in pore size. Since the medium used here is serum free, it is unlikely that there are proteases present that could account for the collagen breakdown, however, it does contain a supplement of bovine pituitary extract which

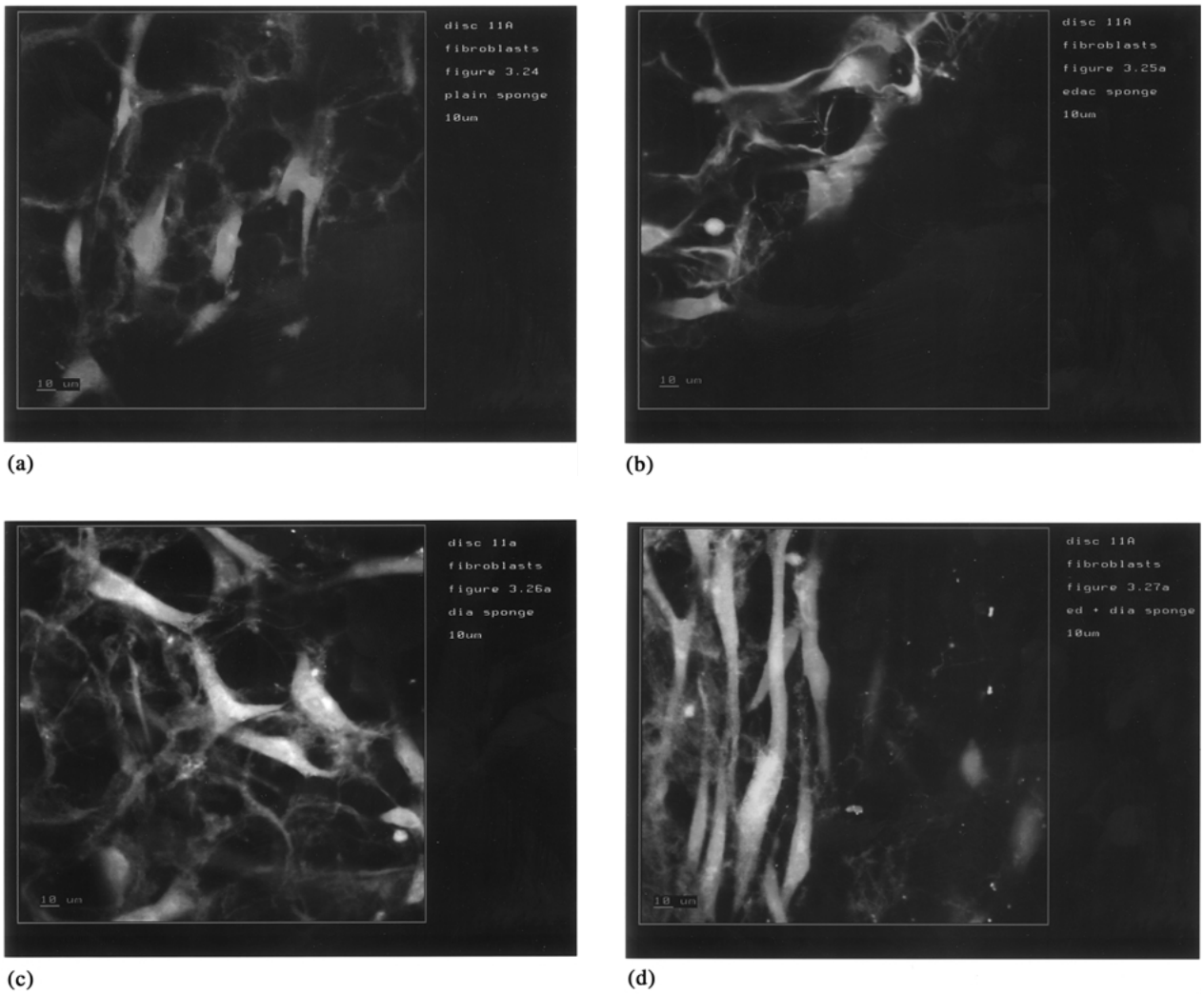

Figure 9 Confocal micrographs of fibroblasts growing on (a) untreated collagen sponge; (b) EDAC treated sponges; (c) diaminohexane treated sponges; and (D) EDAC + diaminohexane crosslinked sponges. Images were taken 10 μm into the sponges after 7 days growth. Magnification is indicated by the scale bar on the pictures.

could be the source of an enzyme capable of breaking down collagen fibers.

In contrast to the effect of incubating sponges in K-SFM, incubation in serum containing DMEM caused a marked reduction in pore size in both plain collagen sponges and in the crosslinked samples. Crosslinking did not appear to protect the collagen sponges from this effect to any extent. This effect was also most prominent at the surface of the sponges although some reduction in pore size was detectable in the interior too, particularly after 10 days exposure to the DMEM. Contraction of collagen substrates by fibroblasts *in vitro* is a well recognized phenomenon and has been used by many investigators as a model of wound healing [18, 19]. Cell-free contraction of collagen gels incubated in media has also been reported [20], and the reduction in pore size observed in our experiments may represent contraction of the collagen sponges in response to cytokines and/or growth factors present in the serum in DMEM. Contraction is known to be responsive to the presence of growth factors and cytokines in media [21]. We have previously found that cell free contraction of plain collagen gels is much more extensive in serum containing DMEM than in K-SFM [22], and this may be why a decrease in pore size was not observed in the present study with sponges incubated in K-SFM. From our results it would appear that cell free contraction of the collagen sponges is not affected by the crosslinking process, and this may be important as contraction is a vital aspect of wound healing which allows the margins of a wound to be drawn towards the center.

Crosslinking of the collagen sponges significantly reduces the average pore size of the sponges. This is most prominent at the surface of the specimens, and has been previously reported on crosslinked collagen gels, but not on sponges [11]. In contrast, internally some of the crosslinked sponges appear to have pore sizes that are greater than those of the corresponding uncrosslinked sample. This is evident at depths of 20 and 40 μm. For example, at a depth of 20 μm, and after 7 days incubation with DMEM, sponges crosslinked with EDAC, and the combination of EDAC and diaminohexane, had significantly larger pore sizes than the corresponding untreated variant. The reason for this is unclear, but it could be due to large amounts of crosslinks being formed at the surface of the sponge; this would have the physical effect of opening up the pores located in the interior of the sponge, as the surface collagen was contracted due to the crosslink formation. Such an effect would actually be advantageous for an artificial skin substitute from a cell growth/vascularisation perspective. From a practical sense, increased pore size with increasing depth is

desirable, since larger pores in the interior of an implant may help to promote vascularization of the implant when grafted onto a patient. This phenomenon was first demonstrated in 1976 by Oluwasanmi *et al*. [23], who reported that sponge pore sizes over 80 μm promoted connective tissue in-growth and increased vascular supply in rabbits. However, smaller pore sizes (like those attained by our crosslinking technique) nearer the surface of the sponges are preferable. This means that surface pores are not so large that they permit cells to fall all the way through the graft, and yet, not too small as to inhibit cellular infiltration completely.

Pretreatment with EDAC increased the biological stability of the sponges to collagenase digestion, resulting in a 5-fold decrease in the liberation of l-leucine. This effect was prevented by concomitant treatment with diaminohexane, and the reason for this is unclear but it may involve competition for binding sites on the collagen molecule. Although EDAC treatment produces a more biologically stable sponge, the CLSM photographs indicate that the collagen fibers are thinner, and appear lacy and delicate, and therefore the resulting sponge may not be as mechanically strong as plain uncrosslinked sponges. We are currently investigating the mechanical strength of crosslinked collagen sponges to establish whether or not the morphological changes occurring as a result of EDAC and diaminohexane crosslinking have an effect on mechanical strength of the crosslinked product. We have previously demonstrated that EDAC and diaminohexane treatment had little detrimental effect on the mechanical properties of collagen/chondriotin-6-sulfate gels [24].

An important aspect of the properties of the crosslinked collagen sponges is the influence of the pore size and structure on the growth and distribution of human fibroblasts and keratinocytes during culture. We found that most cells congregated at a depth of 20–30 μm into the sponges, regardless of crosslinking or cell type. This may be an optimal depth in order to surround the cells with a 3-D matrix. The importance of surrounding cells with matrix in order to maintain their differentiated phenotype is being recognized in several areas of tissue engineering [25].

The total number of cells observed on the crosslinked sponges was approximately half of that observed on the untreated collagen despite the fact that seeding density was identical on each variant. The cell numbers after 3 days in culture (both fibroblast and keratinocytes) on the EDAC sponges were lower than those on any of the other sponge types. This could be due to reduced adhesion of cells on the EDAC sponge, or to residual EDAC causing cytotoxicity. EDAC is generally regarded as a non-toxic crosslinker because it acts as a catalyst for the formation of crosslinks and is not itself incorporated into the collagen. Instead it forms a non-binding urea derivative that can be washed out preventing toxic response. In our protocol, sponges were washed out 10 times with distilled water, and this procedure was found to be adequate for rinsing out crosslinkers on collagen gels [11]. Rault *et al*. [26] also reported very low cytotoxicity of EDAC crosslinked sponges. EDAC may be acting to decrease adherence of cells to collagen, by masking receptor binding sites on the collagen molecule.

The results suggest that diaminohexane treatment may partially inhibit proliferation of both fibroblasts and keratinocytes. The fold-increase in cell numbers on diaminohexane crosslinked sponges (from 3 to 10 days) was less than in either control untreated or EDAC treated sponges. The mechanism(s) responsible for growth inhibition caused by diaminohexane is/are not known. Polyamines are generally considered to promote cell growth [27], but spermine has been shown to inhibit the growth of keratinocytes [12], and to exert toxicity in BHK fibroblast cells [28]. The mechanism may involve interaction with cellular glutathione stores and oxidative metabolism of the amines to aminoaldehydes.

Keratinocytes grew in colonies on the untreated collagen sponges, but in isolation on crosslinked sponges. This is similar to the findings of Nehrer and coworkers who were growing chondrocytes on collagen matrices of differing pore sizes [29]. They found that in smaller pore size matrices, the chondrocytes were more closely packed and cell–cell interactions were maintained to a greater extent. Cell–cell interactions play a major role not only in the expression of differentiated phenotype, but also in preventing apoptosis [30, 31]. Thus in this context, the untreated collagen sponge structure which encourages maximum interaction between cells is to be favored. Fibroblast behavior was similar in all sponge variants, with cells alligned along the fibers, and attachment and interaction being observed in all samples. Where exaggerated fibroblast elongation was observed, this was thought to be caused by cells having to stretch further to adhere to the fibers forming the walls of larger pores.

It was thought that a small pore structure on the surface of the sponge, and a larger pore size in the interior of the sponge, would suit the development of an artificial skin substitute for burns patients encouraging vascularization and integration in the interior of the sponge, while retaining keratinocytes near the surface of the skin substitute. The crosslinked sponges studied here offer the opportunity to manipulate pore size, but with concomitant undesirable affects on cell–cell interactions and cell growth. Fiber structure appears more delicate after crosslinking, but the effect of crosslinking on mechanical strength is not known yet. It may be necessary to reach a compromise to obtain the best combination of biological stability, pore size, cell growth and distribution, and mechanical properties for artificial skin substitutes based on collagen sponges.

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