JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 8 (1997) 179–184

Investigation into cell growth on collagen/chondroitin-6-sulphate gels: the effect of crosslinking agents and diamines

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Artificial skin substitutes based on cultured autologous keratinocytes need to have sufficient strength and ease of handling to be utilized successfully by surgeons in the clinic. This may be achieved by crosslinking the collagen substratum on which the cells are cultured, which in this case is a collagen gel. Increased strength must be attained without detrimental effect on cell growth. The influence of potential crosslinking agents including the glycosaminoglycan, chondroitin-6-sulphate ($Ch6SO_4$), the water soluble carbodiimide crosslinking agents 1-ethyl-3-(3-diaminopropyl) carbodiimide (EDAC), and 1,1-carbonyldiimidazole (CDI), and the polyamines putrescine, spermine and diaminohexane, on cell growth rate has been investigated. Incorporation of 20% $Ch6SO_4$ into collagen gels caused an approximately 16% increase in keratinocyte growth, but had no significant effect on that of dermal fibroblasts. Pre-formed collagen gels ($+/-$ $Ch6SO_4$) were treated with the carbodiimides. This crosslinking treatment markedly inhibited fibroblast growth (EDAC 45% inhibition, CDI 70%), without affecting that of keratinocytes. Pre-formed collagen gels ($+/-Ch6SO_4$ and carbodiimide) were treated with 0.1 M, 0.5 M or 1.0 M polyamine. Spermine inhibited the growth rate of both cell types at all concentrations tested, whereas putrescine and diaminohexane had little effect. The mechanical strength of these crosslinked gels is currently being assessed to determine the optimum composition in terms of cell growth and biocompatibility, and strength.

1. Introduction

People who suffer extensive loss of skin due to burns are in danger of succumbing to massive infection and/or severe fluid loss. Extensive burn injuries cause overwhelming psychological and physical distress to the patient and can prove fatal if over 50% of the surface of the body is affected. The standard method of healing the burn wound by surgical repair involves split thickness skin autografting. However, the lack of donor sites in patients with a high percentage of burns has led to the investigation of alternatives to this. Breakthroughs in keratinocyte culture techniques [1], and further developments in keratinocyte growth agents (for overview see [2, 3]) have increased the application of autologous cultured keratinocyte grafts in patients who have limited donor sites. Poor results regarding the application of simple keratinocyte sheets to wound beds has led to the development of skin substitutes, which usually consist of a sheet of keratinocytes attached to a biocompatible carrier which is generally collagen based. Some of these artificial skins have been used in clinical trials [4]. Good results have been obtained using cultured autologous keratinocytes and fibroblasts on a matrix composed of a collagen sponge and gel [5]. The aim of our research is the development of a skin substitute based on autologous keratinocytes cultured on a substrate suitable for grafting. Some of the desirable properties of the substitute include growth promotion, biological stability, reasonable tensile strength and ease of handling. Obviously, from a clinical point of view, some of the most important properties are the strength and ease of handling.

Chemical crosslinking has been widely used to increase the mechanical and biological stability of collagen biomaterials [6]. Crosslinking has been previously shown to be an effective means of controlling the biodegradation rate of collagen-based biomaterials, preventing rapid elution of the material into wound fluids and increasing the tensile properties to a level where they can be handled or sutured conveniently [7]. Although collagen crosslinking is well documented, information regarding the effect of the crosslinking agents on cell growth is limited. Therefore in this study, the effect of various crosslinking agents on the growth of keratinocytes and dermal fibroblasts has been investigated.

The incorporation of glycosaminoglycans (GAGs) has been shown to increase collagen gel strength [8]. When GAG is added to collagen solution in acetic acid medium (pH 3.2), a precipitate complex is seen. In order to give a homogeneous solution, the pH must be

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raised to 8–8.5. Under these conditions GAGs are not thought to be bound to the collagen, but, instead, are only trapped in the gels. Experimental studies have shown that the GAG leaches from collagen gels into the culture medium over the first 3 days in culture [9]. In this study we have used the carbodiimides, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), and 1,1-carbonyldiimidazole (CDI), in an attempt to increase retention of GAG in the collagen gels. EDAC has been previously used as a coupling reagent to bind cell adhesion and growth factors to a polymeric membrane [10]. Carbodiimides have also been used to crosslink collagen gels for spinal cord implantation [11], resulting in improved matrix mechanical properties, and also to crosslink dermal sheep collagen [12].

The carbodiimides themselves are not incorporated into the gels and, as they are water soluble, unbound and excess chemicals are easily washed away during the gel preparation. EDAC and CDI have limited crosslinking ability because of their structure and inability to polymerize [7]. For this reason the diamines, putrescine, spermine and diaminohexane, were used to increase the extent of crosslinking by increasing the number of available reactive groups resulting in formation of more diverse and longer chain links. Spermine, and its precursor spermidine, may serve as substrates for crosslinking of proteins by epidermal transglutaminase in the formation of the keratinocyte cornified envelope [13].

Polyamines are generally regarded as growth promoters [14]. The increase in polyamine concentrations found in rapidly growing tissues has stimulated many investigations into their role in cell proliferation and differentiation. They have been shown to increase the rate of both initiation and elongation of polypeptide synthesis [15] and their depletion leads to decreased protein synthesis in rat hepatoma cells [16]. Putrescine has been used as a component in a defined medium for growing keratinocytes [17]. It was hoped that, in addition to improving substrate strength by crosslinking, the diamines would have growth promoting effects on the keratinocytes and fibroblasts.

In this paper we report on the effect of the various crosslinking agents on the growth of human keratinocytes and dermal fibroblasts *in vitro*. Cell growth was evaluated using a fluorimetric assay which utilizes the non-fluorescent 5-carboxyfluorescein diacete (5-CFDA) as a probe. This probe is allowed to accumulate within cells where it is deacetylated by cytoplasmic esterases to yield carboxyfluorescein which is fluorescent. Although this readily leaches out of non-viable cells, it is retained by viable cells and allows their quantification. This assay has been shown to be an accurate, reliable and convenient method for quantifying keratinocyte growth *in vitro* [18].

2. Materials and Methods

2.1. 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 from Gibco Life Technologies BRL, Paisley, Scotland) supplemented with 5 ng/ml human recombinant epidermal growth factor (rEGF) and 35–50 μg/ml bovine pituitary extract (BPE). Cells were subcultured until they were three passages old and cell seeding density on the collagen gels was 2.5×10^4 cells/cm^2.

Fibroblasts were isolated by mincing the dermis, and these were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS). Cells were subcultured until they were three passages old and cell seeding density on the collagen gels was 5×10^3 cells/cm^2.

2.2. Collagen gel preparation

0.3% (w/v) collagen gel was formed by mixing 4.29 mg/ml collagen solution, type I, acid-extracted from rat tail tendon [19], a mixture of 10x DMEM and 0.4 M NaOH (2 : 1), and 1/1000 (v/v) acetic acid at a ratio of 7 : 1 : 2 and adjusting the pH to 8–8.5 with 1 M NaOH. In order to modify the gel with GAG, chondroitin-6-sulphate ($Ch6SO_4$) (Sigma Chemical Co.), was prepared at 3 mg/ml in 1x serum-free DMEM and incorporated into the collagen solution at 20% by replacing the two volumes of acetic acid in the above ratio. Experiments using 24-well plates were carried out by pipetting 0.4 ml of the gel-forming solution into each well. The gels were allowed to set completely for 2 h at room temperature.

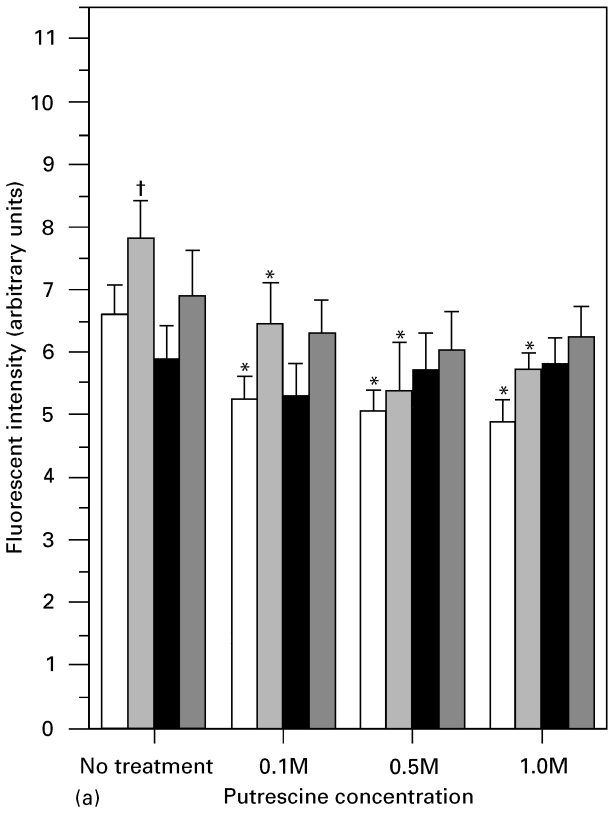
Before seeding with cells, the gels were washed twice by incubating them in Earle's Balanced Salt Solution (EBSS) (Gibco Life Technologies BRL) containing 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml fungizone for 20 min.

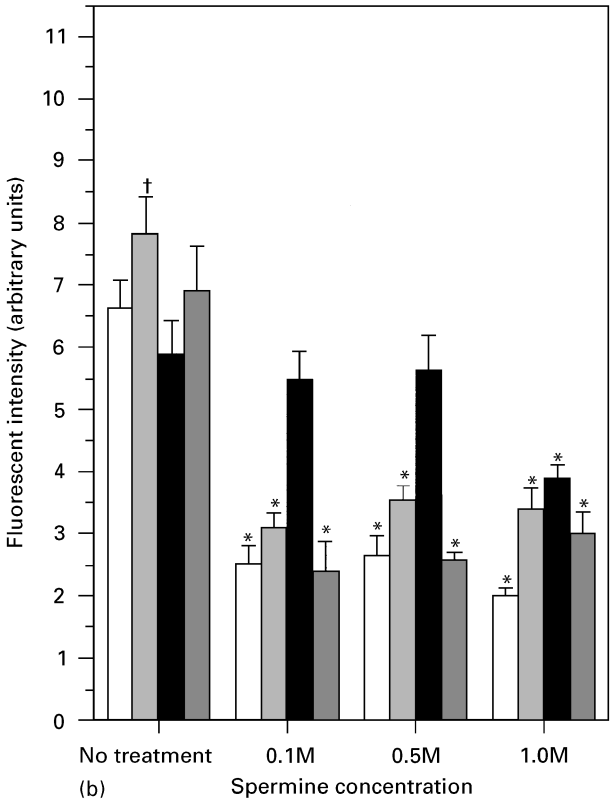
2.3. Addition of carbodiimides and diamines

Solutions were prepared immediately before use in sterile water, pH 4.0. Carbodiimide solutions, EDAC and CDI (Sigma Chemical Co.), were prepared at 0.1 M, and diamine solutions, putrescine, spermine, and diaminohexane (Sigma Chemical Co.), were prepared at 1.0 M. Carbodiimide, 450 μl, and diamine, 50 μl, were pipetted into the appropriate well containing pre-set collagen gel. Reactions were carried out for 18 h at room temperature. Unbound and excess chemicals were removed by washing the gels with sterile water. Gels were washed at least 10 times, with a 20–30 min incubation for each wash. The effect of diamine concentration on cell growth was measured by treating the gels with 0.1 M and 0.5 M solutions as described above.

2.4. Evaluation of cell growth

Cell growth was determined using the 5-CFDA assay. The medium was removed from the wells, which were





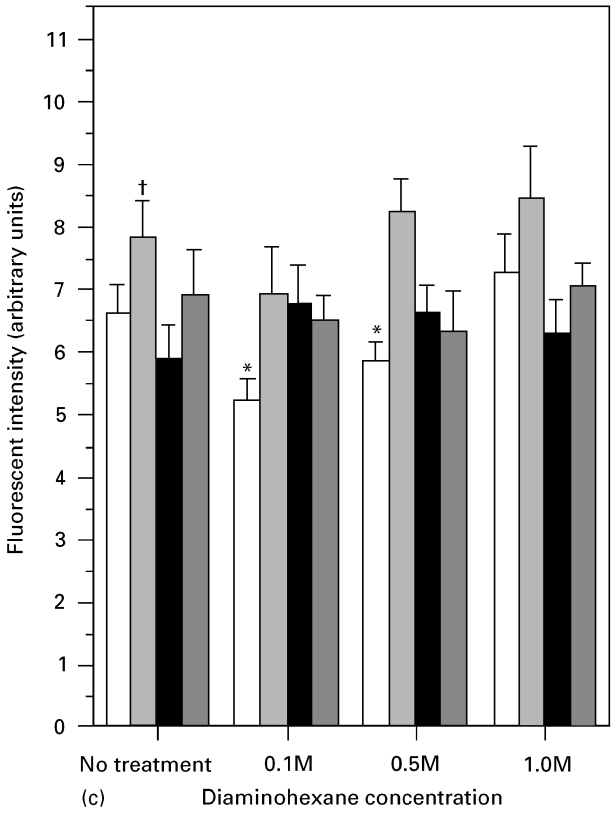


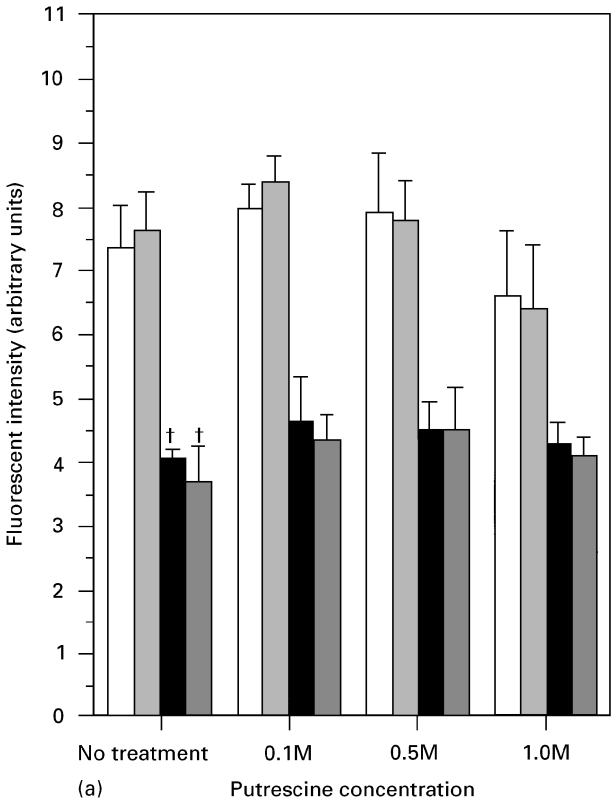
Figure 1 Effect of putrescine (a), spermine (b) and diaminohexane (c) on keratinocyte growth. Cell growth was determined using the CFDA assay and is expressed in terms of fluorescent intensity; results are means ± SD and $n = 6$. $^{\dagger}p < 0.05$, comparing growth on the gel variants in the no diamine treatment group with that on the plain collagen gel, by Anova followed by Dunnett's test. *significantly different ($p < 0.05$) from the corresponding no treatment gel variant, by Anova followed by Dunnett's test. □ plain gel; ▒ gel/GAG; ■ gel + EDAC; ▒ gel/GAG + EDAC.

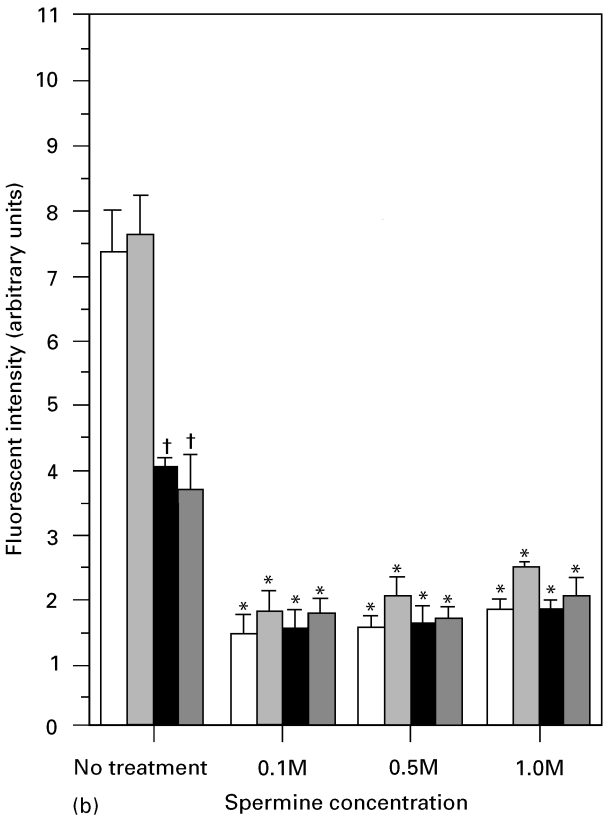
then quickly washed with PBS, pH 6.75. 1 ml of the probe solution, 25 μM 5-CFDA, was added, and this was incubated at 4 °C in the dark for 20 min. The probe solution was then aspirated and the wells quickly washed twice with PBS. 1.5 ml of Triton X-100, 0.5% (v/v) in PBS, pH 6.75, was added and this was incubated for 30 min in the dark at room temperature. Fluorescence was measured using a Shimadzu RF-5001PC spectrofluorophotometer, at an excitation wavelength of 479 nm, and an emission wavelength of 580 nm.

3. Results

Fig. 1 shows the effect of the three diamines at different concentrations on keratinocyte growth. The four gel variants used were plain collagen gel, gel/GAG, gel + EDAC, and gel/GAG + EDAC. Considering the keratinocytes and looking at the no diamine treatment variants first, there was a significant increase in cell growth rate due to the presence of GAG, and a slight inhibitory response in growth rate after EDAC treatment. Putrescine caused inhibition of cell growth, which was more evident in the absence of EDAC (Fig. 1a). It can be seen that spermine proved to be toxic to keratinocytes, markedly inhibiting cell growth at all concentrations used (Fig. 1b). Notable exceptions were that, at 0.1 M and 0.5 M spermine, EDAC seems to have prevented the toxicity of spermine. At 0.1 and 0.5 M diaminohexane growth inhibition was observed in cells grown on plain collagen gels, but there was little effect on cells grown on the other variants.

The effect of diamine concentration on dermal fibroblast growth is shown in Fig. 2. Considering the gels with no diamine treatment, the toxic growth





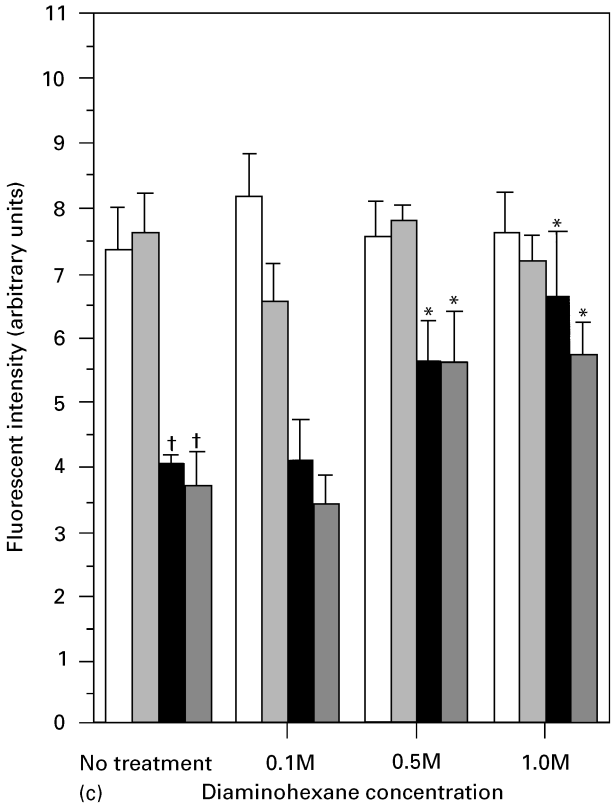


Figure 2 Effect of putrescine (a), spermine (b) and diaminohexane (c) on dermal fibroblast growth. Cell growth was determined using the CFDA assay and is expressed in terms of fluorescent intensity; results are means ± SD and $n = 6$. $^{\dagger}p < 0.05$, comparing growth on the gel variants in the no diamine treatment group with that on the plain collagen gel, by Anova followed by Dunnett's test. *significantly different ($p < 0.05$) from the corresponding no treatment gel variant, by Anova followed by Dunnett's test. □ plain gel; ▒ gel/GAG; ■ gel + EDAC; ▒ gel/GAG + EDAC.

inhibitory effect due to EDAC can be clearly seen; it was more pronounced than with keratinocytes. There was 45% inhibition of fibroblast growth compared with 12% for keratinocytes. The incorporation of GAG alone had no apparent effect on fibroblast growth. There were no significant differences in the growth rate between the corresponding fibroblast-seeded gels as the putrescine concentration increased, although there was slight inhibition of growth at 1.0 M in the absence of EDAC (Fig. 2a). With fibroblasts spermine was much more toxic at all concentrations in all gel variants than with keratinocytes (Fig. 2b). The effect of diaminohexane concentration on dermal fibroblast growth is shown in Fig. 2c. The most interesting point is that, when used at 0.5 M and 1.0 M, diaminohexane helped to overcome the toxic effect of EDAC.

Fig. 3 shows the effect of the crosslinking agent CDI on cell growth. Fig. 3a illustrates the effect of CDI treatment (1.0 M) on keratinocyte growth. No benefit in terms of cell growth was observed by the addition of CDI either alone or in combination with the diamines, putrescine or diaminohexane. As observed before with EDAC, the combination of CDI and spermine elicted a toxic response. From Fig. 3b, it can be clearly seen that CDI inhibited fibroblast growth, both alone and in combination with diamines. The CDI alone caused a growth inhibition of over 70%, which was greater than that observed with the EDAC alone (45%).

4. Discussion

We plan to crosslink the collagen gels used as substrata for hybrid artificial skin production in order to increase their strength and make the grafts produced

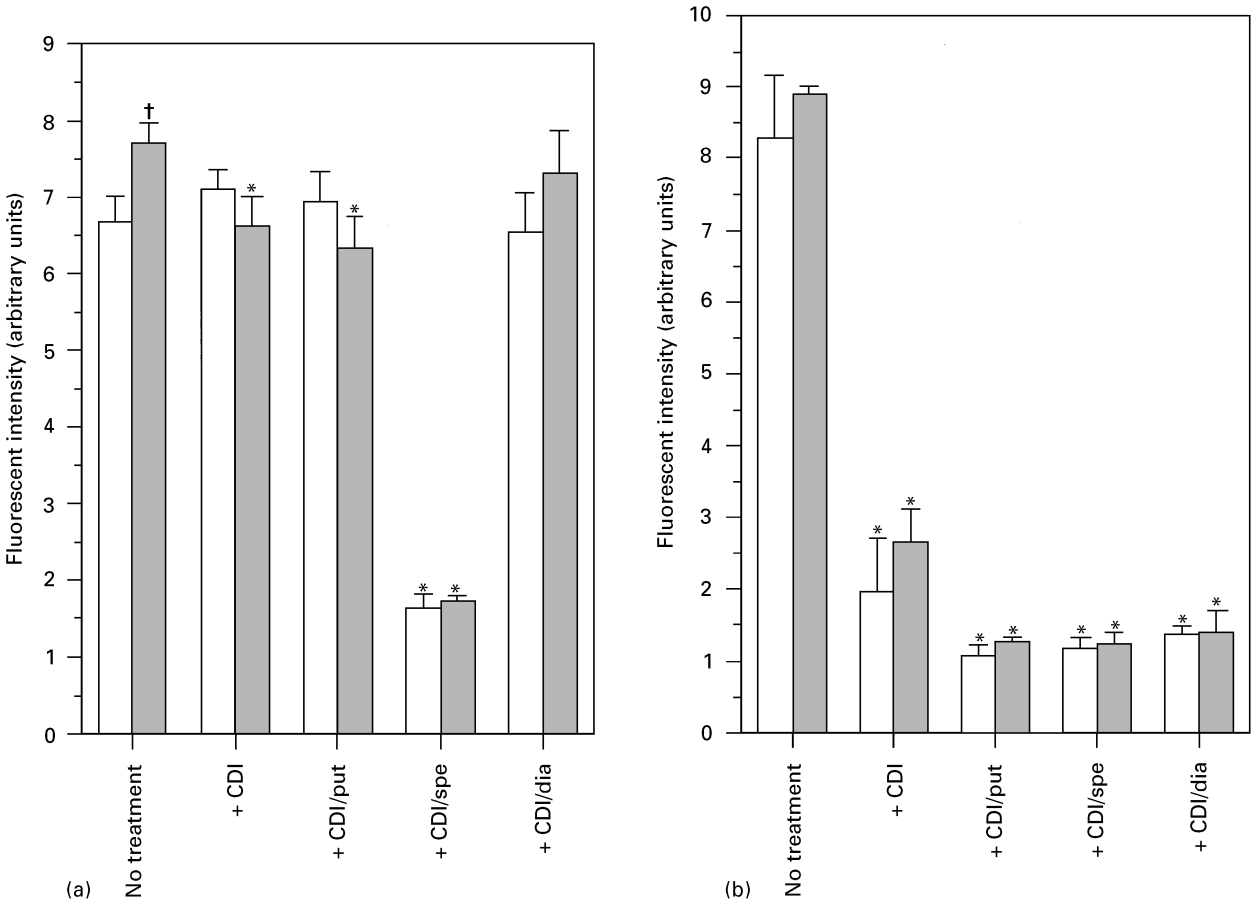


Figure 3 Effect of CDI and diamines on keratinocyte (a) and dermal fibroblast (b) growth. Cell growth was determined using the CFDA assay and is expressed in terms of fluorescent intensity; results are means ± SD and $n = 6$. $^{\dagger}p < 0.05$, comparing growth on plain collagen gel and gel/GAG without CDI treatment, by Anova followed by Dunnett's test. *significantly different ($p < 0.05$) from the corresponding no treatment gel variant, by Anova followed by Dunnett's test. □ plain gel; ▩ gel/GAG. (Put – putrescine, spe – spermine and dia – diaminohexane).

easier for surgical staff to handle. The most commonly used crosslinking agent is glutaraldehyde, but it is markedly cytotoxic. For example the cytotoxic effect of glutaraldehyde crosslinked collagen sponges has been studied [20], with residual effects of glutaraldehyde being found in animal tissues as long as six months after implantation of the collagen sponges. It is obviously of paramount importance that the crosslinking procedure used to strengthen the gel does not compromise cell viability in the graft. Our study has investigated the biocompatibility of two water-soluble carbodiimides, and three diamines, as potential crosslinking agents to strengthen collagen gels prepared in the presence and absence of GAG.

The incorporation of GAG into collagen gels resulted in an increase in the growth rate of keratinocytes, but not of fibroblasts. Despite the great interest in the incorporation of GAGs into the dermal equivalents of skin substitutes, few authors have investigated the effects of GAG on cell growth. Fibroblast growth on collagen sponges containing GAG has been investigated [8]. No stimulatory effect on growth was observed, instead the presence of $Ch6SO_4$ significantly decreased cell proliferation. Keratinocytes, in the culture conditions used here, grow over two times more slowly than fibroblasts (doubling time for fibroblasts was approximately 23 h compared with approximately 50 h for keratinocytes, data not shown). It may be the case that the fibroblasts are already growing at their maximum rate, governed by nutrient availability and/or cell-derived factors, and no treatment could increase their growth rate further. We had hoped that crosslinking by EDAC, CDI and diamines would improve the retention of GAG in the collagen gels, and thereby improve its keratinocyte growth promoting effect. However, there was no improvement in keratinocyte growth on gels containing GAG with the carbodiimines or diamines.

When a carbodiimide is added to a carboxylic acid, a highly reactive intermediate is formed which will condense with an amino group to give an amide. Therefore, in the gels, it is possible to form amide bonds between GAG and collagen, collagen and collagen, collagen and diamine, and GAG and diamine. By further manipulation of the reaction conditions we may be able to selectively increase the formation of GAG and collagen linkages, enabling more effective retention of the GAG in the gels. Putrescine is known as a growth promoter, but no discernible increase in cell growth rate was shown in our experiments. This could be due to the formation of the GAG and diamine linkages which could lead to the putrescine leaching from the gel with the GAG, so limiting its effect on cell growth.

In the experiments with EDAC and CDI, the growth inhibitory effects of both carbodiimides were much

greater in fibroblasts than keratinocytes. This is to be expected as the fibroblasts were growing more rapidly. In fact, the keratinocyte growth rate was not significantly altered by EDAC, and CDI only inhibited keratinocyte growth in the presence of GAG. In fibroblasts CDI was a more potent inhibitor of growth than EDAC (70% compared to 45%). CDI is less stable in solution than EDAC, so it is possible that the CDI has decomposed to give products that are acting as growth inhibitors. There are many potential carbodiimide crosslinking agents but there are little biocompatibility data available. Larger water-soluble carbodiimides may be able to crosslink residues with longer separation distances and therefore yield materials with a greater crosslink density and thus increased strength.

The observation that the carbodiimides inhibit fibroblast growth, without marked effect on that of keratinocytes may be of potential clinical value. A significant problem faced by many severely burned patients is the formation of hypertrophic scars, which is characterized by the accumulation, within the wound boundaries, of excessive quantities of fibrous tissue. When the wound environment is sufficiently prepared by the inflammatory cells there is an ingrowth of blood vessels into the area accompanied by fibroblasts which lay down a fibrous infill to the wound. At the same time there is stimulation of cell growth, which is known as the proliferative stage of wound repair. During the final stages of wound healing, the fibrous tissue infill is modified by the action of fibroblasts to give the final outcome of healing, the scar. Since the hypertrophic scar is marked by an excessive accumulation of collagen the most evident stage where an abnormality may develop is the proliferative stage of healing. As the carbodiimides resulted in fibroblast growth inhibition, the addition of these compounds to skin substitutes may allow selective fibroblast suppression. Obviously this is an area which requires a lot of further work, but the possible functional and cosmetic benefits should be noted.

Polyamines are regarded as growth promoters, particularly in slowly growing cells. It was thought that, in addition to strengthening the gels by improved crosslinking, polyamines may also act as growth promoters particularly for keratinocytes in our experiments. However, the polyamines, particularly spermine, inhibited growth. Spermine has been found to be toxic to other cell types, for example BHK cells, due to the formation of highly reactive oxidation states and oxidative stress within the cell [21]. Putrescine inhibited keratinocyte growth slightly and diaminohexane was the least inhibitory. An interesting point to note was that at the higher concentrations diaminohexane partially prevented the growth inhibition exerted by EDAC in fibroblasts. The mechanism for this is unclear at present. In terms of compatibility for potential use as a crosslinking agent diaminohexane is the most suitable of the three amines studied. However, if putrescine were to improve the strength of the gels markedly it could also be used as the inhibitory effects on the growth of keratinocytes were not marked, and there was no significant effect on fibroblast growth.

In conclusion, we can say that careful consideration of carbodiimide and diamine can result in crosslinked collagen substrates that have no undesired effect on cell growth. Once this is correlated with the effect of crosslinking on mechanical strength and biological stability, the optimum combination that would give the most benefit in terms of ease of handling and wound coverage, for a skin substitute can be established.

Acknowledgement

C. S. Osborne was supported by a Medical Research Council studentship.

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Received 3 June
and accepted 18 September 1996