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The attachment, spreading and growth of baby hamster kidney cells on collagen, chemically modified collagen and collagen-composite substrata

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Various replicates of collagen substrata were prepared to study the attachment, growth and spreading of baby hamster kidney (BHK) cells. Cell attachment was measured in both the presence and absence of serum. Spreading and growth did not occur in the absence of serum. Attachment to fibrous collagen was less than that found with glass, rat-tail tendon collagen or films prepared from pepsin-solubilized collagen (PS-collagen). Incorporation of hyaluronate, heparin and protamine sulphate into the fibrous collagen and the acetylation of fibrous collagen had little effect. However, incorporation of chondroitin sulphate or chemical modification of fibrous collagen by either methylation or succinylation increased BHK cell attachment. In the absence of serum, the attachment to collagen, acetylated collagen and collagen composites was reduced. The reduction in attachment was marked with fibrous collagen and gelatin films, but less so with collagen composites, acetylated collagen, rat-tail tendon and PS-collagen films. Interestingly, attachment to succinylated collagen and methylated collagen was largely unaffected by the absence of serum, and possible reasons for this are discussed. Cell shape measurements showed decreased spreading of BHK cells on chemically modified collagen films, especially on gelatin films and dried PS-collagen gels. Cell shape and spreading on PS-collagen, rat-tail tendon collagen and collagen-composite films was found to be similar to that on fibrous collagen. BHK cell growth on fibrous collagen, chemically modified collagens, collagen composites, rat-tail tendon and PS-collagen films was similar to that found on plastic tissue culture substrata. Denaturation of fibrous collagen resulted in decreased growth, and BHK cell growth was markedly reduced on PS-collagen gels and dried gels.

1. Introduction

Collagen has many properties that make it an excellent choice as a prosthetic material and as a model for natural tissue matrix. These include low antigenicity [1–3], low cytotoxicity [4], the ability to promote cellular growth and attachment [5–11] and haemostasis [1]. Reconstituted collagen has found use as a biomaterial in a variety of physical forms such as sheets, tubes, sponges, powder and fleece [12].

Collagen-based biomaterials have been widely used in clinical medicine in such diverse applications as haemostats [13–16], injectable collagen for tissue augmentation [17–23], burn and wound dressings [24–26] and for urinary tract surgery [27–29].

Despite this widespread use in medicine, little is understood about events occurring once these materials have been implanted. A better understanding of cellular interaction with collagen and modified collagen might improve collagen-based prosthetic materials, and might allow prediction of their behaviour *in vivo*.

In vitro studies have shown how collagen substrata can influence cell attachment, spreading and growth [7, 8, 10, 30]. The incorporation of glycosaminoglycans (GAGs) and fibronectin can further affect cell attachment, migration and mobility *in vitro* [7]. Chemical modification, e.g. succinylation and acetylation of the collagen, will influence cell attachment

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and growth [31]. Previous studies [32, 33] by Srivastava *et al.* showed that the incorporation of GAGs into collagen films could influence the growth and proliferation of L929 mouse fibroblasts. In this study we showed that a shape-change assay provides a quick and simple method of assessing cell behaviour on collagenous substrata.

2. Materials and methods

2.1. Baby hamster kidney (BHK) cells

BHK 21 clone 13, obtained from stocks routinely grown in the Department of Cell Biology at Glasgow University, were cultured in N-(2-hydroxyethyl)-1-piperazine-N′-2-ethylsulphonic acid (HEPES)-buffered Earle's medium (Gibco) containing 10% calf serum and tryptose phosphate broth (HECT medium). This was supplemented with bicarbonate and GPSA, a mixture containing glutamine, penicillin, streptomycin and the antimycotic fungizone amphotericin. Cells were used when the culture had attained confluency.

2.2. Preparation of acid-swollen (fibrous) collagen films

Type I bovine dermal collagen from bovine hide was supplied by Devro Ltd, UK, in the form of a dry powder. Films were prepared by homogenizing a 0.3% (w/v) suspension of the purified collagen powder dispersed in 0.05 M acetic acid (pH 3.2) in a Waring blender for 90 s at 4 °C. After degassing under vacuum for 20 min at ambient temperature, the collagen dispersions were cast on to glass coverslips or in 24-well culture dishes (Corning) to form coherent membranes.

2.3. Preparation of gelatin films

Collagen dispersions were placed in an 80 °C water bath for 60 min. The gelatin formed was allowed to cool and films were made as described above.

2.4. Preparation of rat-tail tendon collagen

Rat-tail tendon type I collagen was obtained essentially by the method of Elsdale and Bard [5]. Tendons were stripped and solubilized in 5% (v/v) acetic acid for 2 days at 4 °C. Insoluble material was removed by centrifugation at 3000 *g* for 30 min and the clear tropocollagen solution was mixed with 10% NaCl to precipitate the collagen. The collagen was pelleted by centrifugation at 3000 *g* for 45 min and was then resuspended in 3% acetic acid. The collagen suspension was dialysed using two or three changes of 3% acetic acid over 2 days. After this the collagen solution was filtered through a Nitex mesh (200 μm) and the collagen concentration was estimated using a Biuret or hydroxyproline assay, after which it was adjusted to 3 mg ml^{-1}. Films were prepared as described above.

2.5. Preparation of pepsin-solubilized collagen films, gels and dried gels [34]

Calf-skin collagen (mainly type I, containing approximately 15% type III) was solubilized by pepsin (pH 3) at room temperature. The solubilized collagen was purified by precipitation at pH 7, salt precipitation and sterile (0.2 μm) filtration. The resultant monomeric dispersed collagen was dissolved at a concentration of 3 mg ml^{-1} in 0.05 M acetic acid. 3 mg ml^{-1} films were prepared as described above. Gels were prepared by adjusting the collagen solution to physiological pH using 1 M NaOH and allowing gel formation to take place in a moist environment at 37 °C. Gels were formed on either coverslips or 24-well culture dishes and were dried in a laminar-flow cabinet for 48 h.

2.6. Preparation of chemically modified collagens

Chemically modified collagens were prepared from the fibrous collagen by succinylation [31, 35], acetylation [36] and methylation [37]. Films were prepared from each of these collagens as described above.

2.7. Collagen–hyaluronic acid composites

A series of fibrous collagen–hyaluronic acid composite materials were made essentially by the method of Yannas and Burke [26]. Hyaluronic acid (human umbilical cord, sodium salt, Sigma Chemical Co., UK) was dissolved in 0.05 M acetic acid at a concentration of 5 mg ml^{-1}. From this the appropriate volume was then added to a 3 mg ml^{-1} fibrous collagen solution to form composites containing 2.5, 5 and 10% (v/v) hyaluronic acid. This mixture was homogenized for 90 s. Following this, samples were degassed and films made as described above.

2.8. Collagen–chondroitin-6-sulphate composites

The preparation of collagen–chondroitin sulphate composites was again based on the method of Yannas and Burke [26]. A 5 mg ml^{-1} solution of chondroitin sulphate (shark cartilage, sodium salt, Sigma Chemical Co., UK) was prepared in 0.05 M acetic acid and composite films of 2.5, 5 and 10% (v/v) were made as described above.

2.9. Collagen–heparin composites

Collagen–heparin composites were prepared in the same way as the other collagen–GAG composites. That is, heparin (sodium salt, porcine intestinal mucosa, Sigma Chemical Co., UK) was added to 0.05 M acetic acid to give a concentration of 5 mg ml^{-1}. This was added to collagen (3 mg ml^{-1}) to produce composite films of 2.5, 5 and 10% (v/v) as described above.

2.10. Collagen–protamine sulphate composites

Protamine, an arginine-rich protein of molecular weight 4300, will bind to and neutralize the anticoagulent effects of heparin [38]. Protamine (protamine sulphate, sperm, Sigma Chemical Co., UK) was made up in 0.05 M acetic acid at a concentration of 5 mg ml^{-1}. This was added to collagen (3 mg ml^{-1}) to produce composites of 2.5, 5 and 10% (v/v) again as above. The final collagen concentration of all collagen composites was measured by Biuret, to ensure that they all had the same collagen concentration.

2.11. Preparation of fibronectin

Bovine plasma fibronectin was purified from calf serum by affinity chromatography on gelatin-sepharose (Engvall and Ruoslahti [39]), and was used to coat collagen films by dilution (from 10 to 200 μg ml^{-1}) directly into medium from solution (1 mg ml^{-1}) in 8 M urea.

2.12. BHK cell-spreading assay

Collagen, modified collagen and collagen-composite films were prepared by adding 200 μl 3 mg ml^{-1} collagen in 0.05 M acetic acid to 13 mm-diameter glass coverslips. These were dried overnight in a laminar-flow cabinet at room temperature. Dried films were then washed with HEPES saline to neutralize any remaining acid, and a suspension of BHK cells was added at a concentration of 5×10^4 cells ml^{-1}. Cells were incubated for 2 h at 37 °C and were then fixed in buffered formaldehyde for 20 min. Coverslips were then stained with 0.5% basic fuchsin (Gurr) for 5 min, washed with deionized water and dehydrated with a series of alcohol concentrations and cleared in xylene.

Cell shape changes and spread areas were measured using a × 50 objective on a Leitz Ortholux microscope equipped with a Hamamatsu Vidicon C1000 camera, with M1438 gain expansion and zero-offset module. The cell images were digitized for input to the screen memory of a BBC Archimedes microcomputer using a Data Harvest video interface. The shapes and spread areas for 100–200 cells sample^{-1} were measured [40]. For a detailed description of cell shape measurements, see [41].

2.13. BHK cell attachment assay

Collagen, modified collagen and collagen-composite films were prepared as described above. BHK cells in culture were labelled overnight at 37 °C with 0.5 mC sodium chromate (Amersham), 500 mC mg^{-1} chromium: 0.6 μg sodium chromate ml^{-1} culture medium. Cells were then trypsinized to remove cells from the culture flask and were resuspended in growth medium. Following this, cells were washed five times with HEPES saline to remove excess label and any remaining trypsin. Cells were resuspended in growth medium or Hanks' HEPES buffer and 5×10^4 (mean d.p.m. 75 000) added to each coverslip (efficiency of $^{51}Cr = 30\%$). After incubation at 37 °C for 2 h the coverslips were washed three times with HEPES saline and then counted on a gamma-counter. The percentage cell attachment was obtained by comparing cell attachment to the collagen substrata with cell attachment to the washed glass.

2.14. BHK cell-growth assay

Collagen, modified collagen and collagen-composite films were formed in Corning 24-well plates. Plates were then washed three times with HEPES saline containing 10% GPSA. This was to remove any excess acid and to maintain sterility. BHK cells (5×10^4) were added to each well and cells were incubated for 3–4 days at 37 °C. After this time cells were removed from each well by adding trypsin-versene (0.5 ml) for 15 min, then HECT growth medium was added to make a total volume of 2 ml well^{-1}. Cells were resuspended and added to 18 ml 0.9% NaCl and counted using a Coulter counter (model ZB). It should be noted that each well was checked microscopically to ensure that there were no remaining cells which would lead to erroneous cell counts. The percentage cell growth was obtained by comparing the growth on collagen substrata with that on culture plates alone.

3. Results

3.1. BHK attachment

In the presence of serum, BHK cell attachment to films prepared from acid-swollen (fibrous) collagen was only 73% of the attachment to washed glass (Table I). Incorporation of heparin, hyaluronate and protamine had little effect on the attachment to fibrous collagen films, whereas incorporation of chondroitin sulphate increased the attachment to levels found with washed glass (Table I). In the absence of serum, attachment to fibrous collagen was greatly reduced to 20% of that of the glass control. This reduction in attachment was also found with all collagen composites, but to a much lesser extent, especially with hyaluronate at concentrations of more than 5%.

Chemical modification by acetylation had little effect on cell attachment compared with fibrous collagen alone. However, succinylation and methylation of fibrous collagen resulted in increased cell attachment, and interestingly this attachment appeared to be largely serum-independent (Table II). Denaturation of collagen to form gelatin resulted in a drastic reduction of cell attachment, both in the presence and in the absence of serum.

BHK cell attachment to PS-collagen and to rat-tail tendon collagen films was increased compared with fibrous collagen, both in the presence and in the absence of serum (Table III). However, as with the collagen composites, cell attachment was considerably reduced in the absence of serum in all cases.

TABLE I BHK cell attachment to collagen-composite films

Collagen composite	Adhesion, serum present (%)[a]	Adhesion, no serum present (%)[a]	Number of coverslips measured
Acid-swollen (fibrous) collagen	73 ± 14	20 ± 6	100
+ Hyaluronic acid 2.5%	71 ± 14	59 ± 14[b]	50
+ Hyaluronic acid 5%	76 ± 18	77 ± 17[b]	50
+ Hyaluronic acid 10%	83 ± 20	68 ± 19[b]	50
+ Chondroitin sulphate 2.5%	100 ± 17[b]	44 ± 14[b]	50
+ Chondroitin sulphate 5%	106 ± 16[b]	56 ± 19[b]	50
+ Chondroitin sulphate 10%	96 ± 17[b]	65 ± 16[b]	50
+ Heparin 2.5%	74 ± 12	41 ± 16[b]	50
+ Heparin 5%	67 ± 15	39 ± 13[b]	50
+ Heparin 10%	70 ± 18	56 ± 10[b]	50
+ Protamine sulphate 2.5%	65 ± 21	34 ± 5[b]	50
+ Protamine sulphate 5%	80 ± 20	40 ± 5[b]	50
+ Protamine sulphate 10%	70 ± 13	43 ± 6[b]	50

[a] Means ± s.d.
[b] Statistically significant difference ($p < 0.05$) from fibrous collagen as determined by Mann–Whitney U-test.

TABLE II BHK cell attachment to modified collagen films

Collagen modification	Adhesion, serum present (%)[a]	Adhesion, no serum present (%)[a]	Number of coverslips measured
Fibrous collagen	73 ± 14	20 ± 6	100
Methylated collagen	106 ± 19	91 ± 17[b]	100
Acetylated collagen	78 ± 14	40 ± 17[b]	100
Succinylated collagen	102 ± 16	77 ± 14[b]	100
Gelatin	27 ± 11[b]	10 ± 2[b]	100

[a] Means ± s.d.
[b] Statistically significant difference ($p < 0.05$) from fibrous collagen as determined by Mann–Whitney U-test.

TABLE III BHK cell attachment to collagen

Type of collagen	Adhesion, serum present (%)[a]	Adhesion, no serum present (%)[a]	Number of coverslips measured
Fibrous collagen	73 ± 14	20 ± 6	100
Pepsin-solubilized collagen	107 ± 21[b]	35 ± 9[b]	50
Rat-tail tendon collagen	98 ± 20[b]	40 ± 20[b]	100

[a] Means ± s.d.
[b] Statistically significant difference ($p < 0.05$) from fibrous collagen as determined by Mann–Whitney U-test.

3.2. Cell shape

Table IV and Fig. 1b show that cells are slightly more elongated and less well spread on fibrous collagen films than on washed glass surfaces (Fig. 1a). Incorporation of hyaluronic acid, heparin, protamine sulphate and chondroitin sulphate had little effect on BHK cell shape compared with fibrous collagen films, although on chondroitin sulphate films the BHK cells tended to be slightly rounder in shape (Fig. 1c).

The effects of collagen modification on BHK cell shape can be seen in Table V. Methylation and succinylation markedly reduced the spread area, whereas acetylation made little difference compared with collagen alone. Denaturation of the collagen had a marked effect on the shape of BHK cells, which remained round and spread little.

BHK cell spreading on rat-tail tendon collagen (Table VI) was similar to that found on fibrous collagen. However, dried gels from PS-collagen had a marked effect on the BHK cell shape. As can be seen in Fig. 1d, cells remained round and did not spread. This effect was observed only with dried gels, as spreading on PS-collagen films was similar to that on fibrous collagen films.

Table IV shows the serum requirement for BHK cell spreading and elongation on fibrous collagen films; both are much reduced in the absence of serum. The effect of coating collagen films with different concentrations of fibronectin can be seen in Figs 2 and 3. The addition of fibronectin increased the spread area and elongation, with peak values at $50\,\mu g\,ml^{-1}$. The decrease in spreading and elongation obtained with fibronectin levels greater than $50\,\mu g\,ml^{-1}$ was thought to be due to increased amounts of urea present in the preparation (see Section 2) having an inhibitory effect. This increase in the spread area and elongation on fibronectin-coated collagen films compared with on non-coated films is illustrated in Fig. 1e–h.

3.3. BHK cell growth

The results obtained show that the growth of BHK cells on plastic was similar to that on fibrous collagen-coated surfaces. Incorporation of hyaluronic acid, chondroitin sulphate, protamine sulphate and heparin made no difference to cell growth (Table VII). Similarly, modification of collagen had no effect (Table VIII) and growth was the same from a different source of collagen, i.e. growth on rat tail was similar to that on fibrous collagen substrata (Table IX). However, growth was found to be slightly reduced on gelatin

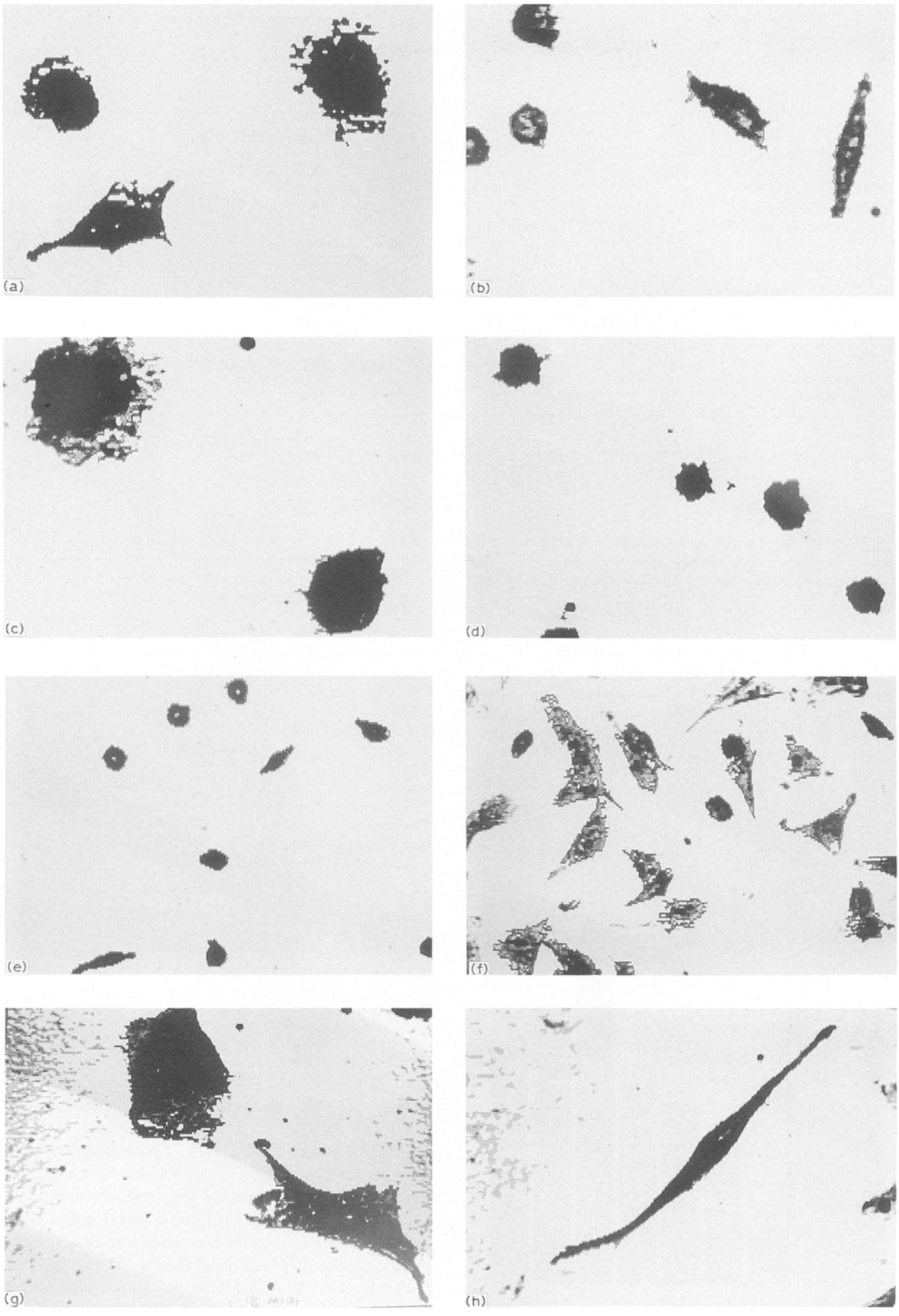

Figure 1 Digitized images of BHK cells on collagen substrata: (a) spreading on glass, (b) spreading on fibrous collagen; (c) on fibrous collagen–chondroitin-6-sulphate (5%) composite films; (d) on dried PS-collagen gels; (e) on fibrous collagen; (f) on fibrous collagen coated with 50 $\mu g\,ml^{-1}$ fibronectin (note the increased number of cells per field compared with fibrous collagen); (g) on fibronectin-coated fibrous collagen films, showing large, very well spread cells, (h) a large, elongated BHK cell on fibrous collagen coated with fibronectin. (a–d, g, h) $\times 370$ and (e, f) $\times 250$.

TABLE IV BHK cell shape measurements on collagen composites

Collagen composite	Spread area (μm^2)[a]	Elongation[a]	Number of samples, 100 cells sample^{-1}
Fibrous collagen	218 ± 16	0 99 ± 0.08	10
Fibrous collagen (no serum)	107 ± 29[b]	0.70 ± 0 11	10
+ Hyaluronic acid 2.5%	256 ± 48	0.74 ± 0.24	10
+ Hyaluronic acid 5%	210 ± 12	0.79 ± 0.20	10
+ Hyaluronic acid 10%	205 ± 18	0.86 ± 0.20	10
+ Chondroitin sulphate 2.5%	226 ± 22	0.68 ± 0.12	10
+ Chondroitin sulphate 5%	226 ± 15	0.59 ± 0.12	10
+ Chondroitin sulphate 10%	218 ± 22	0.60 ± 0.07	10
+ Heparin 2.5%	206 ± 38	0.86 ± 0.14	10
+ Heparin 5%	200 ± 26	0.94 ± 0.10	10
+ Heparin 10%	186 ± 35	0.88 ± 0.10	10
+ Protamine sulphate 2 5%	197 ± 26	1.07 ± 0.14	10
+ Protamine sulphate 5%	193 ± 33	0.99 ± 0.12	10
+ Protamine sulphate 10%	197 ± 33	1 12 ± 0.12	10
Washed glass (control)	293 ± 43[b]	0.76 ± 0.11	10

[a] Means ± s d.
[b] Statistically significant difference ($p < 0.05$) from fibrous collagen as determined by Mann–Whitney U-test.

TABLE V BHK cell shape measurements on modified collagen

Collagen modification	Spread area (μm^2)[a]	Elongation[a]	Number of samples, 100 cells sample^{-1}
Fibrous collagen	218 ± 16	0.99 ± 0.08	10
Methylated collagen	136 ± 16[b]	0.89 ± 0.08	10
Acetylated collagen	179 ± 18[b]	1.06 ± 0.12	10
Succinylated collagen	140 ± 18[b]	0.96 ± 0.10	10
Gelatin	88 ± 18[b]	0.48 ± 0.14	10

[a] Means ± s.d.
[b] Statistically significant difference ($p < 0.05$) from fibrous collagen as determined by Mann–Whitney U-test.

TABLE VI BHK cell shape measurements on collagen

Collagen type	Spread area (μm^2)[a]	Elongation[a]	Number of samples, 100 cells sample^{-1}
Fibrous collagen	218 ± 16	0.99 ± 0.08	10
Pepsin-solubilized collagen	191 ± 32	1.03 ± 0.10	10
Rat-tail tendon collagen	183 ± 34	0.96 ± 0.25	10
Pepsin-solubilized (dried gel)	89 ± 13[b]	0.34 ± 0.08	10

[a] Means ± s.d.
[b] Statistically significant difference ($p < 0.05$) from fibrous collagen as determined by Mann–Whitney U-test.

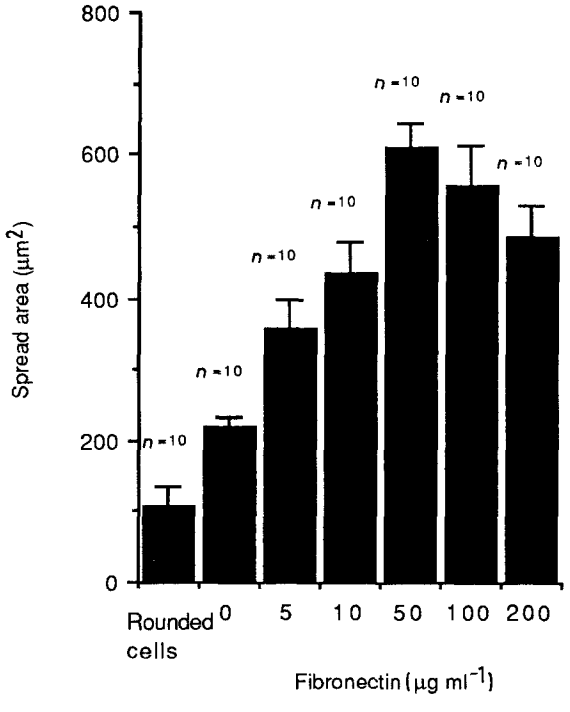

Figure 2 Spread area of BHK cells on collagen films coated with fibronectin

films and markedly reduced on collagen gels and dried gels, although growth on films prepared from PS-collagen was the same as on fibrous collagen films.

4. Discussion

The results of this study, like those reported previously [42, 43], showed that the attachment and spreading of BHK cells on dried collagen films required the presence of serum. Those earlier studies also demonstrated differences in the attachment of BHK cells on two-dimensional surfaces compared with three-dimensional hydrated collagen gels. Differences in cell behaviour on two- and three-dimensional surfaces have also been described by Tomasek *et al.* [44], who used the cold soluble globulin component of serum for BHK attachment. These and other studies [39, 45–47] have shown that the factor responsible for this is fibronectin.

The role of fibronectin can be seen clearly from the results presented. First, serum was required for cell

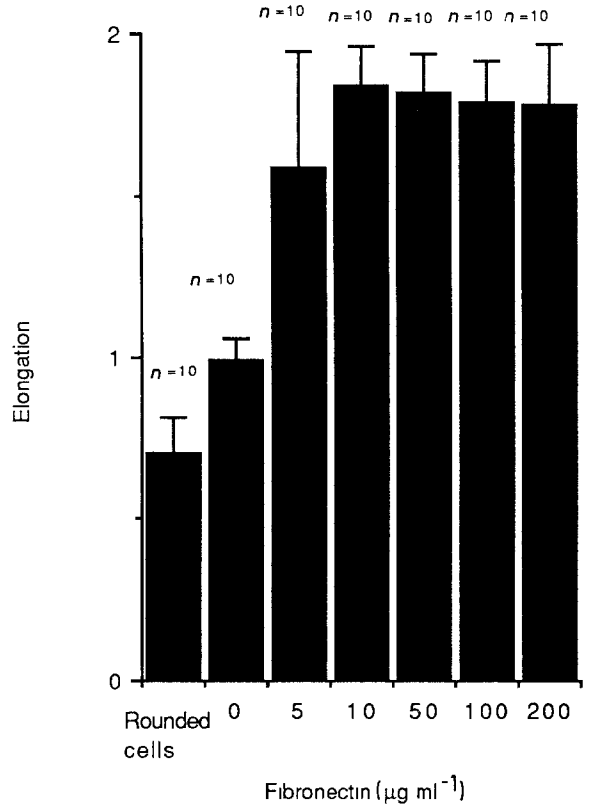

Figure 3 Elongation of BHK cells on collagen films coated with fibronectin.

TABLE VIII BHK growth on modified collagen

Collagen modification	BHK growth (%)[a]	Number of samples
Fibrous collagen	104 ± 25	97
Methylated collagen	92 ± 19	36
Acetylated collagen	107 ± 18	41
Succinylated collagen	104 ± 16	38
Gelatin	70 ± 29[b]	51

[a] Means ± s.d.
[b] Statistically significant difference ($p < 0.05$) from fibrous collagen as determined by Mann–Whitney U-test.

TABLE IX BHK growth on collagen

Collagen	BHK growth (%)[a]	Number of samples
Fibrous collagen	104 ± 25	97
Pepsin-solubilized collagen	122 ± 28	38
Rat-tail tendon collagen	107 ± 19	52
Pepsin-solubilized collagen (dried gel)	19.0 ± 10[b]	32
Pepsin-solubilized collagen gel	31 ± 10[b]	32

[a] Means ± s.d
[b] Statistically significant difference ($p < 0.05$) from fibrous collagen as determined by Mann–Whitney U-test

TABLE VII BHK cell growth on collagen-composite films[a]

Collagen composite	BHK growth (%)[b]	Number of samples
Fibrous collagen	104 ± 25	97
+ Hyaluronic acid 2.5%	97 ± 23	47
+ Hyaluronic acid 5%	94 ± 20	48
+ Hyaluronic acid 10%	96 ± 18	47
+ Chondroitin sulphate 2.5%	107 ± 19	49
+ Chondroitin sulphate 5%	109 ± 19	47
+ Chondroitin sulphate 10%	103 ± 14	48
+ Heparin 2.5%	123 ± 38	39
+ Heparin 5%	111 ± 26	40
+ Heparin 10%	102 ± 14	41
+ Protamine sulphate 2.5%	118 ± 38	39
+ Protamine sulphate 5%	111 ± 28	41
+ Protamine sulphate 10%	105 ± 20	39

[a] Cell growth on collagen-composite substrata was not found to be statistically significantly different ($p < 0.05$) from fibrous collagen substrata as determined by Mann–Whitney U-test.
[b] Means ± s.d

spreading and, secondly, increased spreading was obtained by the addition of exogenous fibronectin. In addition, serum was shown to increase the attachment to collagen three-fold. The results in this study also show that attachment and spreading on gelatin films was markedly reduced even in the presence of serum. This contradicts the findings of other workers [42, 48], who found that serum increased cell attachment to levels near those of native collagen. The reasons for this difference are unknown, but it may be due to the type or source of collagen used to prepare the gelatin. The gelatin used here may have different fibronectin binding properties from commercially prepared gelatin.

Certainly this study shows differences in cell attachment to collagen from different sources. Both rat-tail tendon and PS-collagen were more adhesive than the fibrous collagen. An interesting finding was the failure of cells to spread after 2 h on dried gels made from PS-collagen, despite the presence of serum. These were made from PS-collagen in which fibrillogenesis has been allowed to take place before drying. Coating dried gels with fibronectin before adding cells did not result in cell spreading (data not shown). It would appear that, as with films coated with gelatin, fibronectin binding properties are different from those of fibrous collagen. The cells did, however, spread and grow on PS-collagen films at the same rate as on fibrous and rat-tail tendon collagen films. It has been suggested by Grinnell *et al.* [49] that fibroblasts use different recognition sequences for attachment on collagen gels compared with collagen-coated surfaces. Therefore, it is possible that on dried gels some of these sequences, i.e. Gly–Arg–Gly–Glu–Ser–Pro (GRGESP), compete with Arg–Gly–Asp (RGD) sequences for cell attachment. The result of this is that cells still attach to the substrate, but RGD-mediated spreading is decreased.

Incorporation of hyaluronic acid, heparin, chondroitin sulphate and protamine sulphate showed a similar pattern of cell attachment, with increased attachment in the presence of serum. However, composite films also showed an increased attachment of cells in the absence of serum compared with collagen films alone. This could be due to a charge effect; e.g.

chondroitin sulphate and heparin increase the negative charge, whereas protamine will show an increased positive charge. Increasing or decreasing the charge of substrata can affect attachment, and this is described below. A more likely explanation in the case of GAG composites is the presence of specific receptors for extracellular matrix (ECM) on the cell surface. A study by Harper *et al.* [50] demonstrated a fibronectin-independent mechanism of cell attachment to ECM components. The components mediating this were found to be produced by fibroblasts and were non-collagenous glycoproteins. Serum-independent cell attachment to collagen–GAG composites was also shown by Brown and Akeson [51]. They found that neuroblastoma and melanoma cell lines adhered better to collagen–GAG substrates than to collagen alone. The maximum attachment at 2 h was to collagen–hyaluronic acid and attachment appeared to be fibronectin-independent.

Chemical modification of collagen had a marked effect on the attachment of BHK cells. Attachment to methylated, acetylated and succinylated collagen substrata was found to be increased, especially in the absence of serum. This agrees with a study by Kasai *et al.* [31], who found increased attachment of L929 cells to methylated and succinylated collagen substrata. It was proposed that attachment of these cells was fibronectin-independent and due to the increased positive charge from methylation and the increased negative charge from succinylation. This would also be consistent with the results described here.

The lack of spreading on succinylated and methylated collagen may be due to a non-physiological attachment mechanism in which cells attach so tightly that spreading is inhibited. A more likely explanation is a lack of integrin binding by these substrates preventing spreading. The failure of cells to spread on gelatin may also be due to a lack of integrin binding but, unlike methylated and succinylated collagen, it was certainly not due to increased cell attachment.

BHK cells grew equally well over 4 days on most substrates, despite differences in attachment and shape. Exceptions were gels and dried gels made from PS-collagen. A previous study [52] found that human fibroblasts, when grown on collagen gels, proliferated at a slower rate. It was proposed that this was due to a decreased response to platelet-derived growth factor by cells on collagen gels. It is possible that the thin elongated shape of reduced surface area adopted by fibroblasts on gels makes access by growth factors much more difficult than the flattened shape on collagen films. The rounded shape adopted by BHK cells on dried collagen gels also resulted in poor growth. This could be caused by the rounded cells having reduced responsiveness to platelet-derived growth factor, again due to reduced surface area.

Cells on gelatin also appeared to have nearly normal growth despite poor initial attachment and spreading, and this agrees with results found in other studies [6, 48]. The reason for poor initial attachment and spreading on gelatin films remains unclear. It could be due to altered surface properties caused by the denaturation procedure or the source of native collagen. The growth of cells on modified collagen substrata was similar to that on native collagen. It would appear that initial differences in adhesion and shape on these substrates has no effect on cell proliferation.

Similarly, the proliferation of BHK cells on collagen composites was similar to that on plastic culture substrata, again despite attachment and shape-change differences. The inclusion of fibronectin, hyaluronic acid (and less so dermatan sulphate) in to collagen sponges has been shown to increase fibroblast infiltration and growth [9–11]. It was also shown by these workers that chondroitin-6-sulphate increased cell attachment but not infiltration or growth, a similar finding to the results reported in this study. It seems that there are differences in attachment and proliferation of cells on collagen–GAG substrates, and these depend on which cell type is being used.

Differences in cell proliferation were also found by Srivastava *et al.* [32, 33], who found decreased proliferation of L929 cells on collagen films compared with on plastic culture dishes. These studies also show differences in growth with different collagen–GAG composites. For example, hyaluronic acid at the 2.5% level increased cellular attachment and growth. Moreover, when the concentration of this polysaccharide was increased, an inhibitory effect was observed. Conversely, increasing concentrations of chondroitin sulphate at 5% and 10% gave enhanced cellular attachment and growth. Both cellular attachment and growth were also increased by the addition of fibronectin.

Other workers have demonstrated that cell growth is increased on collagen substrata. Rucker *et al.* [53] found increased growth of rabbit fibroblasts on microcrystalline collagen films and Gospodarowicz *et al.* [54] found that corneal epithelial cells grew to a greater density on collagen-coated dishes.

In the case of BHK cells it appears that the initial attachment and spreading can be affected by the nature of the substrata, whereas the proliferation of BHK cells is dependent on fibronectin coating of substrata. It still remains unclear why some substrata show a delay in attachment and spreading before proliferating as normal. Perhaps this is due to differences in the binding of fibronectin to different collagen substrata.

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