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Quantitative evaluation of the growth of established cell lines on the surface of collagen, collagen composite and reconstituted basement membrane

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Summary. As a step in the development of a system for assessing growth of human urothelium and transitional cell carcinoma, the growth of two established cell lines on collagen-based membranes has been evaluated. HT1080 (metastatic human fibrosarcoma) and WI38 VA13 (virus-transformed human fibroblasts) were grown on substrates of collagen, collagen/hyaluronic acid or chondroitin sulphate and reconstituted basement membrane (Matrigel). Cell growth was quantified using a new fluorimetric assay utilizing carboxyfluorescein diacetate. There were differences in morphology between cells grown on collagen and those grown on polystyrene. There were, however, no differences in growth of the WI38 VA13 cells on collagen compared with polystyrene, but growth of the HT1080 cells was increased on membranes of collagen/2.5% hyaluronic acid and collagen/5% chondroitin sulphate, and decreased on Matrigel. Adequate growth on collagen substrates is dependent on cell line. The fluorimetric assay used was suitable for quantifying cell growth on such substrates.

Key words: Collagen – Cell culture – Urothelium

Previous work in our department [8] has shown that it is possible to grow human urothelium and transitional cell carcinoma on tissue-culture-treated polystyrene and collagen substrates. There is evidence that growth and replication of transitional cell epithelium from explants of ureteric and bladder mucosa are promoted by establishing these tissues on a layer of collagen [12]. Srivastava et al. [17] demonstrated that incorporation of glycosaminoglycans (hyaluronic acid, 2.5% and 5%, and chondroitin sulphate, 5%) into a collagen matrix produced a substrate which allowed better cellular growth and attachment than native, type-I collagen alone.

In addition, type IV collagen in the form of a reconstituted basement membrane (Matrigel), which also contains laminin and heparan sulphate, has been used as a substrate for cell growth and to assess tumour cell invasion [3–5, 7, 11, 18, 20]. Cells derived from metastatic tumours have been found to pass through a layer of this substrate more rapidly than non-metastatic cells [11]. In order to define an optimal substrate for culturing human urothelium and transitional cell carcinoma, we have initially evaluated the growth characteristics of two established cell lines on membranes of native type-I collagen, composite collagen materials with added glycosaminoglycans (hyaluronic acid, 2.5% and 5% and chondroitin sulphate, 5%) and reconstituted basement membrane (Matrigel).

The response of the cells to the substrates was evaluated by morphological examination and by determination of the number of viable cells growing on the substrate layer. There is a need for a simple and reproducible technique for determining numbers of cells growing on collagen membranes. Electronic counting of cells detached from the substrates by enzymic or chemical means has been used [11, 13, 17]. However, electronic Coulter counters are unable to distinguish between living and dead cells or between single cells and aggregates. Reznikoff et al. [13] also reported that only 20% of cells were removed from the surface of a collagen membrane even after prolonged exposure to trypsin.

Incorporation of tritiated thymidine as a measure of DNA synthesis has also been used to quantify cell growth on collagen substrates [12]. This is a complex technique requiring handling of radio-isotopes.

An alternative method of obtaining accurate cell numbers is to use fluorescent probes. Fluorescein diacetate (FDA) is a non-fluorescent and non-polar molecule, which readily permeates viable cell membranes. Once inside a cell, it is deacetylated by non-specific cytoplasmic esterases to yield the polar, fluorescent compound fluorescein. The viable cell membrane is relatively impermeable to fluorescein, which therefore accumulates within the cell as the reaction proceeds [14, 15].

The rate of leakage of fluorescein is sufficiently high, however, to render fluorimetric analysis inaccurate [7, 16].

The recently developed derivatives of fluorescein, caboxyfluorescein (CF) and 2′7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) are retained to a greater

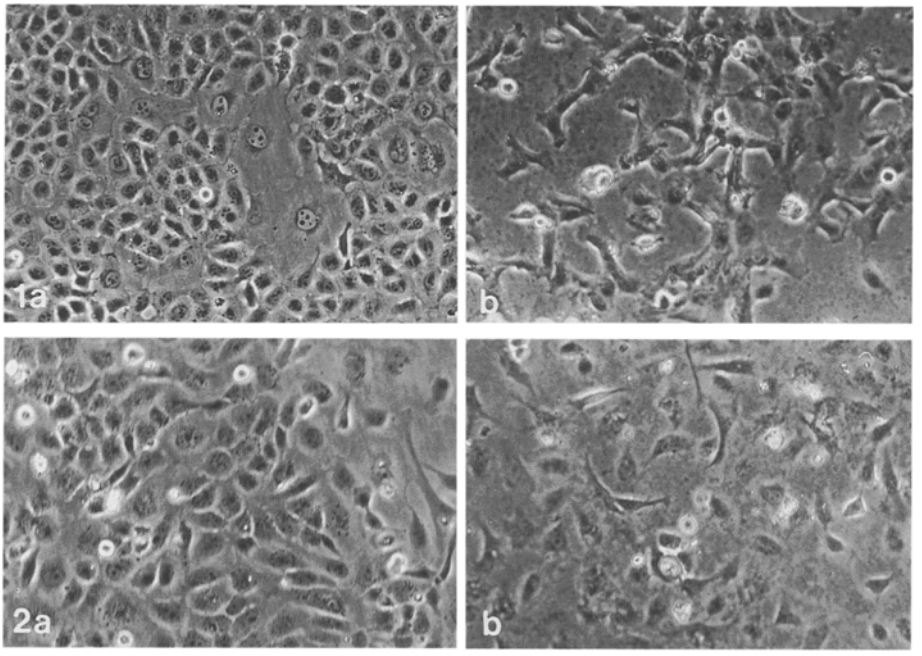

Fig. 1. Phase-contrast micrographs of HT1080 cells growing on **a** tissue-culture-treated polystyrene and **b** a membrane of Matrigel

Fig. 2. Phase-contrast micrographs of WI38 VA13 cells growing on **a** tissue-culture-treated polystyrene and **b** a membrane of Matrigel

extent by viable cell membranes [6, 21]. Esters of these molecules have been successfully used in fluorimetric assays of cell viability. The accuracy of these assays has been shown to match or exceed that of tests based on radiolabelling, and they are simpler and quicker to perform [6, 21].

A modified fluorimetric assay is described utilizing 5′-carboxyfluorescein diacetate [16] for the determination of the growth of cells on collagen-based substrates. This novel method is compared with that of enzymic detachment and particle (Coulter) counting.

Materials and methods

Cell lines

Two cell lines were used: HT1080 (European Collection of Animal Cell Cultures), derived from a malignant and metastatic human fibrosarcoma [10], and WI38 VA13 (Flow Laboratories, Irvine, Scotland), which is an SV40-transformed human fetal lung fibroblast line.

Both cell lines were maintained in Eagle's modifed minimum essential medium supplemented with 10% foetal bovine serum and 1% non-essential amino acids.

Substrates

Native type I collagen prepared from pancreatin-treated fresh bovine dermis (Devro, Moodiesburn, UK) was made into a 0.3% (w/v) suspension in 0.05 M acetic acid. The resultant slurry was homogenized and degassed in a vacuum oven. Composite collagen/hyaluronic acid (2.5% and 5%) and collagen/chodroitin sulphate (5%) suspensions were prepared as previously described [17].

Films were formed by adding 0.5 ml of suspension to each well of a 24-well tissue-culture plate and allowed to dry for 24–48 h in a laminar-air-flow cabinet at 37°C.

Matrigel (Flow Laboratories) was reconstituted on ice and diluted 1:8 with sterile water. The mixture was thoroughly vortexed in an ice-cold glass tube using a mechanical mixer. A 0.5 ml aliquot of the suspension was then rapidly dispensed into the wells of a 24-well microplate and allowed to air dry as above.

Stock solutions

For the fluorimetric assay, 5′-carboxyfluorescein diacetate (CFDA) (Sigma, Poole, England) was prepared as a 2.5 m*M* stock solution in dimethylsulphoxide (DMSO) and stored at −20′C. Sterile phosphate-buffered saline (PBS) was prepared at pH 6.75. Triton X-100 (Sigma) was dissolved in PBS at 0.5% and stored at 4′C.

Fluorimetric growth assay

The collagen films were allowed to dry and inspected by light microscopy to confirm that they were intact. All films were then washed repeatedly with PBS at pH 7.2 until the buffer remained neutral. Each well was inoculated with 0.6 ml of a freshly trypsinized cell suspension containing 2×10^4 cells/ml. The cell density was confirmed by staining a sample of the suspension with Trypan blue and counting in a haemocytometer. Uncoated tissue-culture-treated polystyrene wells were used as the control substrate. The plates were incubated at 37°C in an atmosphere of 95% air and 5% CO_2 for 72 h to allow cells to attach and divide.

At the end of this period, the medium was aspirated and the cells washed with PBS. The CFDA stock solution was then diluted 100-fold in PBS at pH 6.75 and 1.0 ml of this probe solution added to each well. The plates were then left undisturbed for 15 min to allow the probe to accumulate within the cells. The supernatants were aspirated and each well washed twice with PBS to remove any extracellular fluorochrome. Triton X-100 solution (1.5 ml) was then added to each well. The plates were gently shaken over a 10-min period to let the fluorochrome leach from the cells.

A 1.0 ml sample was then withdrawn from each well, taking care not to abrade the films, and transferred to a minicuvette. The fluorescence of each sample was then measured as a 10 s integrated value in a Perkin Elmer spectrofluorimeter with monochromators set at an excitation wavelength of 479 nm and an emission wavelength of 580 nm.

Coulter counting of cells

Preparation of the plates was as described above. At the end of the growth period the medium was aspirated from the wells and the cells were washed with sterile Earle's balanced salt solution. The cells

Table 1. Results of fluorimetric assay of HT1080 and WI38 VA13 cells growing on various substrates. Values are expressed as the fluorescence intensity (mean and standard error of mean, SEM) of at least four replicate samples. Statistically significant differences are noted

Substrate	WI38 VA13 cells		Significant difference from control	Significant difference from collagen	HT1080 cells		Significant difference from control	Significant difference from collagen
	Mean	SEM			Mean	SEM		
Control	263	8	–	–	611	10	–	–
Collagen	261	33	–	–	624	31	–	–
Collagen + 2.5% HA	227	45	–	–	702	47	–	–
Collagen + 5% HA	252	52	–	–	751	43	$P=0.007$	$P=0.031$
Collagen + 5% CHS	313	35	–	–	738	48	$P=0.022$	–
Matrigel	263	7	–	–	394	9	$P=0.000$	$P=0.000$

Ha, hyaluronic acid; CHS, chondroitin sulphate

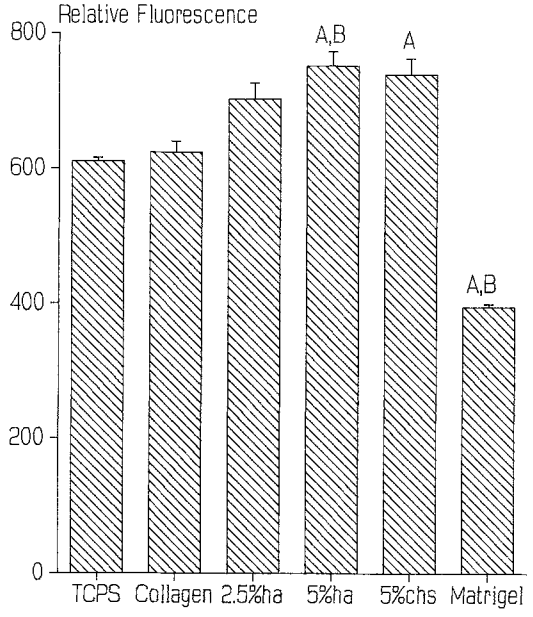

Fig. 3. Histogram of the relative fluorescence of HT1080 cells on each substrate. Fluorescence intensities are mean values of at least four replicate samples. *Extension bars* = standard error of mean. *TCPS,* tissue-cultured-treated polystyrene; *2.5%ha,* collagen + 2.5% hyaluroninc acid; *5%ha,* collagen + 5% hyaluronic acid; *5%chs,* collagen + 5% chondroitin sulphate. *A,* fluorescence significantly different from TCPS; *B,* fluorescence significantly different from collagen

were then detached from the substrates using 1 ml of a solution containing 0.25% (w/v) trypsin and 0.2 g/l EDTA (ethylene diamine tetraacetic acid) (Gibco, Paisley, Scotland). The plates were incubated at 37°C for a period of 10–20 min. Excess medium containing 10% serum was then added to inhibit the trypsin. Samples from each well were then diluted with Isoton and analyzed using a Coulter counter (model TA II).

Statistical methods

One-way analysis of variance (AOVONEWAY, Minitab) was used to compare mean values between substrates at the 95% confidence level ($P<0.05$:$F\gg 4$).

Results

The morphology of the two cell types grown on polystyrene and Matrigel is recorded in Figs. 1 and 2. On tissue-culture-treated polystyrene, the majority of the HT1080 cells (Fig. 1a) appeared oval and granular with a large nucleus. The cells tended to grow in confluent groups and there was only a small number with an elongated shape and filopodial formation.

In contrast, when grown on Matrigel, the same cells tended to be separate, a higher proportion were elongated and the majority of cells exhibited filopodial formation (Fig. 1b). The appearance of the cells grown on the other collagen membranes was similar.

The WI38 VA13 cells displayed similar differences in morphology between substrates (Fig. 2a, b). When growing on polystyrene there was less uniformity of shape compared with the HT1080 cells, but on the Matrigel more cells became elongated with filopodia. These features were common to all the collagen substrates and may reflect growth down into the collagen layer.

The results of the fluorimetric assay are given in Table 1, and summarized for each cell type in Fig. 3 (HT1080 cells) and Fig. 4 (WI38 VA13 cells).

The growth of HT 1080 cells was significantly greater on substrates of collagen + 5% hyaluronic acid ($P=0.007$) and collagen + 5% chondroitin sulphate ($P=0.022$) compared with growth on polystyrene alone. There was also a significant increase in cell number on the collagen + 5% hyaluronic acid compared to collagen alone ($P=0.031$) (Fig. 3). The growth of these cells on Matrigel was significantly reduced when compared with polystyrene and collagen (both $P=0.00$) (Table 1).

There was no significant difference in growth of the WI38 VA13 cells on any of the collagen substrates or the Matrigel when compared with the conrol polystyrene.

Discussion

The fluorimetric assay described was useful for quantifying cell growth on collagen substrates. It does not depend on any form of cell detachment, and the fluorescence values are directly proportional to the number of viable cells. In contrast, when cell growth was determined using a Coulter counter, cell numbers were lower than anticipated

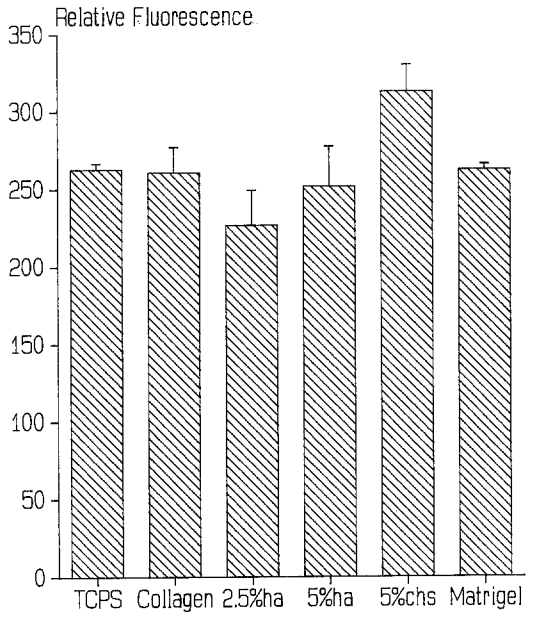

Fig. 4. Histogram of the relative fluorescence of WI38 VA13 cells on each substrate

and there was considerable variance within sample replicates. A substantial number of cells remained attached to the collagen substrates even after 20 min exposure to trypsin/EDTA. Cells of both types also formed aggregates when suspended in Isoton.

We regard the fact that the fluorimetric assay can yield reproducible quantitative results as an important prerequisite to further studies of cell growth on collagen membranes.

There was no significant difference in the growth of WI38 VA13 cells on any of the collagen substrates compared with polystyrene. In contrast, there was enhanced growth of the HT1080 cells on the collagen + 5% hyaluronic acid and on the collagen + 5% chondroitin sulphate substrates, but more significantly there was reduced growth on Matrigel. This reduction in growth and the morphological changes noted may reflect penetration of the cells into Matrigel layer [7]. Growth on the various substrates may be cell-dependent, and optimal conditions may have to be determined separately for each cell type.

Our results show that both cell types grow well on native type-I collagen without additional glycosaminoglycans. The WI38 VA13 (non-metastatic) cells also grew adequately on a substrate containing type-IV collagen (Matrigel) but, as noted, the growth of the HT1080 cells (metastatic) on this substrate was reduced.

Isolation and culture of human urothelium and transitional cell carcinoma have proved difficult [2, 9, 12] but this has been achieved in our department [8]. The results in the present study suggest that type-I collagen and Matrigel are adequate substrates for cell growth, and we now intend to use them for culturing human urothelium and transitional cell carcinoma.

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