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A NOVEL ARTIFICIAL SUBSTRATE FOR CELL CULTURE: EFFECTS OF SUBSTRATE FLEXIBILITY/MALLEABILITY ON CELL GROWTH AND MORPHOLOGY

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Summary

Gels of glyoxyl agarose (GA) are evaluated as a novel flexible substrate for cell culture with physical properties comparable to extracellular matrix (ECM) gels. We show here that cells adhere well to pure GA gels; in addition, specific interactions involving matrix receptors can be studied when individual matrix molecules are bound to the gel covalently. When cells are grown on such substrates, morphology is comparable to that observed on "natural" matrix gels (reconstituted gels of collagen type I or of Matrigel): rather than being flattened as in monolayer cultures on tissue culture plastic the cells assume a rounded morphology and tend to form tissue-like aggregates. The effects of the artificial matrix gels are discussed in the context of previous publications on cell interactions with the extracellular matrix, suggesting that in addition to specific recognition of matrix molecules the physical properties of ECM by themselves can be decisive for cell differentiation. We conclude that gels of glyoxyl agarose a) provide a useful model to mimic the physical properties of matrix gels without the presence of specific adhesion factors; b) may be useful as a general, non-specific ECM allowing cells to be cultured in vitro under conditions favorable for differentiation; and c) allow to design a variety of "synthetic" ECM models composed of a chemically defined gel matrix, which can be supplemented with covalently bound molecules to be recognized by cell surface receptors.

Key words: cell culture; extracellular matrix; artificial matrix gels; differentiation.

Introduction

Cell phenotypes are determined not only by internal genetic programs but also by important external signals exerted by the environment in organs and tissues. These are, as a rule, only incompletely mimicked by the majority of cell and tissue culture methods. In particular, rigid plastic materials as routinely used for growing anchorage dependent cells are problematical: Numerous recent publications on cell interactions with the extracellular matrix (ECM) have given ample evidence that ECM can be decisive for differentiation of cultured cells. In most publications, these effects have been attributed to chemical signalling by the component molecules of the ECM. However, there is evidence that physical properties of the ECM may be at least as important in this respect: Three-dimensional flexible substrates are much more favorable for cell specific differentiation than rigid coatings of the same chemical composition (e.g., Barcellos-Hoff and Bissell, 1989; Bissell and Barcellos-Hoff, 1987; Daniels and Solursh, 1991; Doillon et al., 1988; Hadley et al., 1990; Hohn et al., 1992; Lawley and Kubota, 1989; McDonald, 1989; Nakanishi and Ishii, 1989; Ormerod and Rudland, 1988; Shannon et al., 1990; Streuli et al., 1991; Tinois et al., 1987).

The advantage of matrix gels over tissue culture plastic simply coated with matrix molecules is their flexibility and malleability, which allows the cells to rearrange their architecture in a three-dimensional orientation and to establish an equilibrium of internal and external tensile forces. The balanced interaction of the cytoskeleton with components in matrix gels is thought to result in sort of a relaxed cytoarchitecture, which favors differentiation rather than in cells being forced into flat distorted structures as on rigid plastic that resists the tension exerted by the cytoskeleton through matrix receptors. The relevance of architectural regulation of differentiation with cells being regarded as tensegrity structures, the importance of mechano-chemical signalling, and the involvement of the extracellular matrix, of matrix receptors, and of the cytoskeleton have been argued for particularly by Ingber and coworkers (Ingber, 1991a,b; Ingber and Jamieson, 1985) and also by other authors (Ben-Ze'ev, 1992; Damsky and Werb, 1992; Juliano and Haskill, 1993).

Arguments derived from these considerations and their consequences for cell culture are suffering from methodological drawbacks: The influence of different physical properties of cell culture substrates can be studied, so far, only by comparing cell behavior on matrix gels with behavior on tissue culture plastic coated with the equivalent molecules. In particular, in these studies cells were grown on gels of collagen type I or of the basement membrane-like matrix extracted from the EHS-tumor (Matrigel, Kleinman et al., 1986) and compared with cells grown on rigid plastic coated with individual matrix molecules like fibronectin or collagen type I or with Matrigel (references as cited above). From experiments with

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such gels, it has not been possible to conclude whether changes in cell behavior are due to differences in molecular composition of the gels, to general differences in matrix structure, or to altered arrangement of certain matrix components, and what role purely physical properties of the substrate may play. Model substrates that would allow us to perform systematic series of experiments along these lines in order to discriminate between these various possibilities have not been available so far. Therefore, we intended to create artificial flexible substrate gels that a) have physical properties comparable to those of matrix gels, b) allow cells to interact with, and c) that cells can grow on. Variants of these gels are then created by crosslinking to them molecules for which cell surface receptors are known to exist. This study demonstrates that gels of glyoxyl agarose are a flexible substrate that can be modified by crosslinking molecules to the gel matrix for which receptors are expressed on the cell surface. It is shown that cells do attach to and grow on plain gels of glyoxyl agarose and of agarose derivatized with matrix molecules or with other adhesion factors. Cell morphology on these substrates was comparable to that observed on "natural" matrix gels.

Materials and Methods

Cells and Reagents

BeWo cells, a choriocarcinoma cell line established by Pattillo and Gey (1968), were obtained from the American Type Culture Collection (Rockville, MD). The cells produce different placental hormones and were free of mycoplasms in routine checks in our laboratory.

NuFix glyoxyl agarose was a generous gift from FMC BioProducts (Rockland, ME; distributed in Germany by Biozym, Hameln). Human fibronectin was obtained from the New York Blood Center (New York, NY), bovine collagen type I (Vitrogen) from Collagen Corporation (Palo Alto, CA), human collagen type IV from Southern Biotechnology Associates (Birmingham, AL), mouse laminin from Collaborative Research (Bedford, MA), and poly-L-lysine, M_r 35 000–70 000 D, from Sigma (St. Louis, MO). The basement membranelike matrix extracted from the EHS tumor (Matrigel, Kleinman et al., 1986), consisting of laminin, collagen type IV, heparan sulfate proteoglycan, and entactin, was purchased from Collaborative Research. All other chemicals were research grade reagents provided by Sigma, Merck (Gibbstown, NJ), or Serva (Heidelberg, Germany).

Cell Culture

BeWo cells were cultured in Ham's F12 (GIBCO, Grand Island, NY) containing 15% fetal bovine serum (FBS; Flow Laboratories, McLean, VA). The medium was replaced every second day. For experiments, the cells from subconfluent monolayers were dispersed using Trypsin-EDTA (Flow Laboratories), seeded at a density of 15 000 cells/cm^2 into 96-well flat bottom tissue culture plates (Falcon, Becton Dickinson, Oxnard, CA), containing different types of substrates as described below, and incubated with 100 μl medium per well at 37° C in a moist atmosphere of air with 5% CO_2. Cell growth was determined by estimation of total cell protein as described below (Landegren, 1984). Photographs of cultures were taken on Polaroid film 667 (Polaroid, Cambridge, MA) with a Nikon Diaphot TMD microscope equipped with a Nikon Microflex PFX photomicrographic attachment (Nikon, Tokyo, Japan).

Cell Culture Substrates

Tissue culture plastic coated with proteins. Proteins were dissolved in serum-free and antibiotic-free Ham's F12 medium and were allowed to adsorb to the plastic at 4° C overnight (100 μl/well). For attachment assays, the remaining free binding capacities of the plastic were saturated with 1% BSA (bovine serum albumin, Fraction V, Sigma; denatured for 10 min at 80° C) for 1 h at room temperature. After three rinses with medium, the plates were used immediately for attachment assays. Plates for growth experiments were not treated with denatured BSA but were sterilized with UV light for 1 h. Cell monolayer cultures for histological examination were grown on Thermanox tissue culture plastic cover slips (Nunc, Naperville, IL) with or without protein coatings.

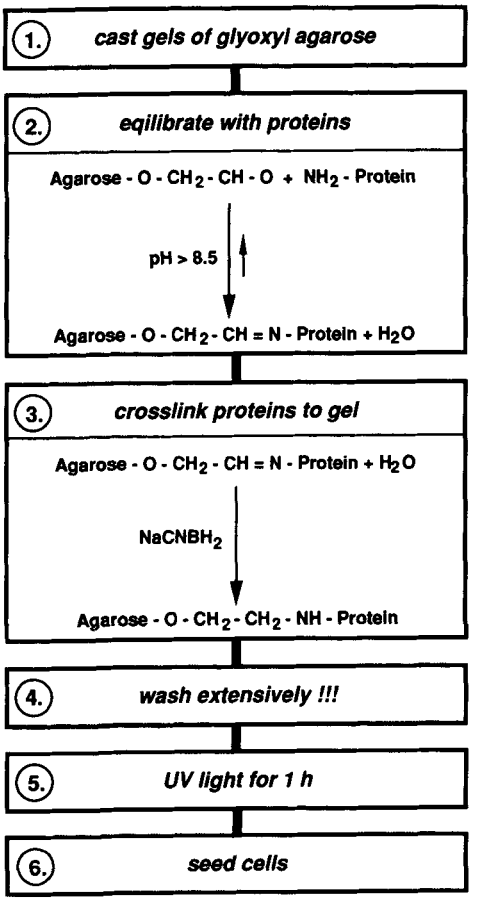

Fig. 1. Flow chart for the experimental design, modified after FMC product information bulletin No. 14 (Lane, 1975; Hutchins and Natale, 1979; Shainoff and Dardik, 1984).

Normal matrix gels. Normal matrix gels were reconstituted under aseptic conditions by incubating 35 μl/well of a solution of 1.5 mg/ml collagen I in PBS (phosphate buffered saline) or of undiluted Matrigel at neutral pH at 37° C in humidified air for 1 h.

Artificial flexible substrates. Artificial flexible substrates for cell culture were prepared from NuFix glyoxyl agarose (originally developed for electrophoresis and immobilization of molecules containing primary amines in the gel after electrophoresis). Aldehyde moieties of the agarose form Schiff bases with organic nucleophilic amines (FMC product information no. P-14; Shainoff and Dardik, 1984). Above pH 8.5, the equilibrium lies far on the side of the reaction product. An irreversible bond can be generated by reducing the Schiff base intermediate to its corresponding amine with dilute sodium cyanoborohydride (*see* Fig. 1; c.f. FMC product information bulletin no. P-14; Lane, 1975; Hutchings and Natale, 1979). Gels were prepared as recommended by the manufacturer (*see* flow chart in Fig. 1). Different amounts of glyoxyl agarose (0.2%, 0.35%, 0.5%, and 1% final concentrations) were dissolved by boiling in distilled water. After the solution had cooled down to 45° C, 50 μl aliquots were pipetted into wells of 96-well tissue culture plates that had been precoated with poly-L-lysine (100 μl of 50 μg/ml overnight at 4° C) in order to stabilize the agarose gels in the wells after chemical crosslinking. Polymerized gels were equilibrated at 4° C overnight with proteins dissolved in 50 μl PBS (pH 8.5) or with PBS

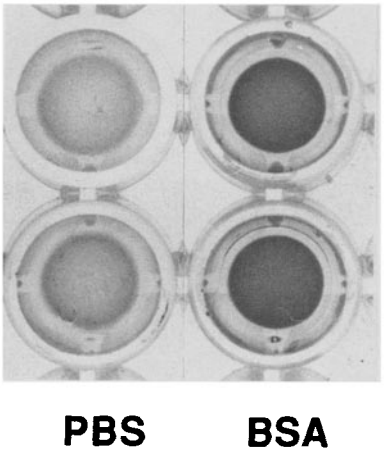

FIG. 2. Covalent linkage of BSA was demonstrated by staining bound protein with Coomassie Brilliant Blue. Gels were equilibrated with 1 mg/ml BSA in PBS, which was then crosslinked to the matrix. In contrast to gels incubated with PBS alone, such gels could not be destained.

alone. Concentrations used were: 50 μg/ml for fibronectin and for poly-L-lysine; 1 mg/ml for bovine serum albumin (BSA, fraction V, Sigma); and 0.2 mg/ml for Matrigel. After the supernatant had been removed carefully, the gels were treated with cyanoborohydride (0.1 *M* in distilled water, 100 μl/well) for 2 h at room temperature in order to crosslink the proteins to the agarose. Then the gels were washed very extensively (at least 10 washes for 10 min each) before they were rinsed with serum-free media, followed by equilibration with culture medium. Because cyanoborohydride is highly toxic, the crosslinking reaction and the first washes were performed under a fume hood. Before cells were seeded, these substrates were exposed to UV light for 1 h for sterilization. The successful crosslinking of proteins to gels was demonstrated by staining protein-derivatized agarose gels with Coomassie Brilliant Blue.

Adhesion Assays

For attachment assays, about 15 000 cells suspended in 0.1 ml serum-free medium were transferred into each well (50 000 cells/cm^2). The plates were then incubated at 37° C in a humid atmosphere of air with 5% CO_2. After 1 h, the wells were rinsed with medium three times and the number of attached cells was estimated indirectly by measuring the activity of their internal hexosaminidase as described below (*Estimation of Cell Mass*, Landegren, 1984). Plastic coated with denatured BSA, which allowed no cell attachment, served as a control.

Estimation of Cell Mass

Direct determination of total cell protein was not feasible in this study because cells were grown on substrates with varying protein contents. DNA measurements are not a reasonable alternative for choriocarcinoma cells that fuse and form syncytia to a varying degree depending on experimental conditions. In the indirect method chosen here, the activity of the ubiquitous lysosomal enzyme hexosaminidase was determined according to Landegren (1984) and used as an equivalent to total cell protein. Cells attached to the substrate were washed with PBS and incubated at 37° C in a humidified atmosphere for 1 h with 40 μl/well of 10 m*M* p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma) in 0.1 *M* citric acid trisodium salt (pH 5.0) containing 1% Triton X-100 to permeabilize the cells. After the reaction had been stopped with 100 μl/well 50 m*M* glycine (pH 10.4) with 5 m*M* EDTA (ethylenediaminetetraacetic acid), the absorbance was determined at a wavelength of 405 nm. The activity of the cellular hexosaminidase was linear to total cell protein content determined by the method of Herbert et al. (1971) using BSA as a standard.

Histology

After culture, cells were fixed in 2.5% (vol/vol) glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.4) for 4 h, postfixed in 1% (wt/vol) osmiumtetroxide, dehydrated in a graded series of ethanol and embedded in Epon/Araldite. Semithin sections (0.75 μm) were stained with 1% toluidine blue in 1% (wt/vol) sodium borate. The sections were examined and photographed in a Zeiss Axiophot (Zeiss, Oberkochem, Germany).

RESULTS

Preparation and handling of gels. Gels of glyoxyl agarose cast into wells of 96-well tissue culture plates were of varying solidity depending on agarose concentrations, with gels of 0.2% being very soft, gels of 1% being rather rigid, and gels of 0.35 to 0.5% intermediate. Because gels containing 0.2% glyoxyl agarose appeared similar in consistence to gels of collagen type I and because glyoxyl agarose gels of 0.35% were comparable to gels of Matrigel, it was planned to use lower concentrations of glyoxyl agarose in order to create artificial cell culture substrates. However, the softer the gels were the lower was their stability during further treatment for protein crosslinking and the higher was the risk of loosing these softer gels, particularly during the extensive washing procedures that were considered to be necessary in order to remove the highly toxic cyanoborohydride. Gels of collagen or of Matrigel were never lost, inferring that such gels are stabilized in the wells by proteins adsorbing to the plastic. Correspondingly, the contact of softer gels of glyoxyl agarose to the wells could be improved by coating the wells with poly-L-lysine before the gels were cast. That proteins were stably retained in the gels after chemical crosslinking was demonstrated after coupling BSA (dissolved in PBS) to the gel matrix and staining with Coomassie Brilliant Blue (Fig. 2).

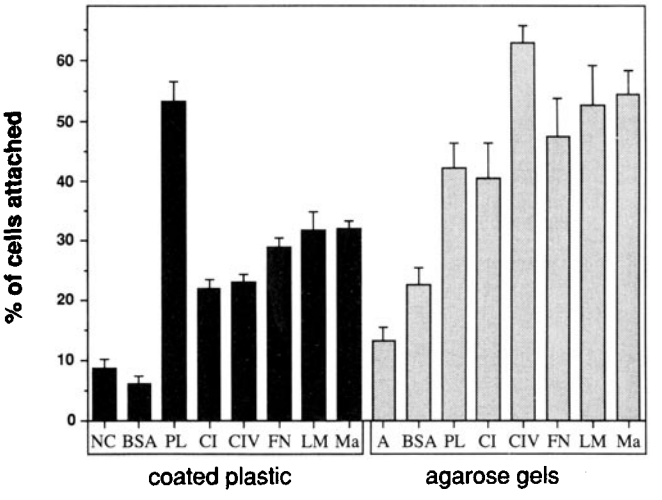

FIG. 3. Adhesion of BeWo cells to derivatized agarose gels as compared to plastic substrates coated with matrix molecules. 0.1 ml of cell suspension containing 150 000 cells/ml were incubated for 1 h at 37° C in humidified air with 5% CO_2 in microtiter wells coated with protein (25 μg/ml of protein except for BSA, 1 mg/ml, and Ma, 0.2 mg/ml) or containing glyoxyl agarose gels (0.35%) that were modified with covalently bound proteins (50 μg/ml). Proteins were BSA, poly-L-lysine (PL), collagens type I (CI) and type IV (CIV), fibronectin (FN), laminin (LM), and Matrigel (Ma). Controls were noncoated plastic (NC) and nontreated glyoxyl agarose (A). Averages are from 5 parallel wells ± SD.

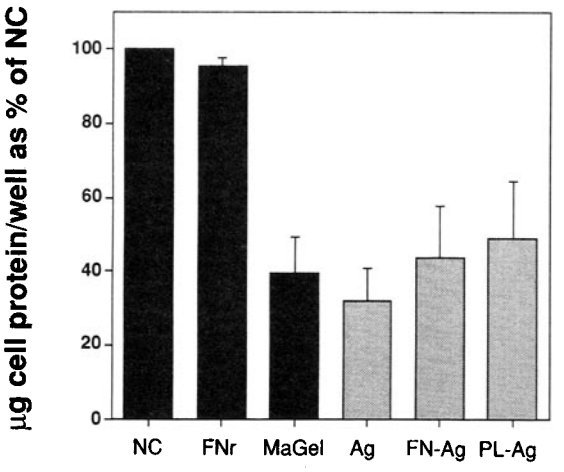

FIG. 4. Growth rates were compared between BeWo cells grown either on glyoxyl agarose gels or on plastic substrates coated or not with different matrix proteins or on Matrigel gels. 15 000 cells/cm^2 were seeded into 96-well flat bottom tissue culture plates containing proteins adsorbed to the plastic surface or covalently bound to 0.35% gels of glyoxyl agarose. Noncoated tissue culture plastic (NC) served as control. After 4 d the amount of total cell protein was estimated. Because growth on plastic coated with laminin, fibronectin, and on collagens gave the same results, only the growth rate on such rigid substrate with fibronectin is shown (FNr, 25 µg/ml). Other substrates were gels of Matrigel (MaGel), gels of either untreated glyoxyl agarose (Ag), fibronectin-agarose (FN-Ag), or of poly-L-lysine-agarose (PL-AG). FN-Ag and PL-Ag both contained 50 µg/ml of these molecules. Data are from three different experiments with five parallel wells in each given as averages ± SD.

Cell adhesion. Attachment was supported by artificial 0.35% glyoxyl agarose gels equally well to or even better than by protein-coated plastic substrates when adhesion factors were crosslinked to the agarose matrix (Fig. 3). Plain glyoxyl agarose and agarose modified with BSA also exhibited some adhesiveness.

Growth. Cell growth was retarded on agarose gels as compared to growth rates on rigid plastic substrates to a degree similar to that found previously on physiological matrix gels, particularly similar to that on gels of Matrigel (Hohn et al., 1992). Major differences between agarose gels derivatized with different molecules and also between different agarose concentrations were not observed. Estimation of total cell protein (Fig. 4) or ^{3}H-thymidine incorporation (data not shown) gave comparable results.

Morphology. Cells cultured on soft gels of glyoxyl agarose (0.2%) formed colonies that appeared similar to colonies obtained on normal matrix gels (Fig. 5). Except for poly-L-lysine, these colonies were rounded aggregates, almost appearing like spheroids, in phase contrast microscopy. On gels containing poly-L-lysine the colonies and aggregates were less compact and the cells grew more as sheets of cells that were much more rounded than cells in regular monolayers. While attachment of colonies was very stable on agarose gels derivatized with matrix molecules, and even more on agarose with poly-L-lysine, adhesion of the much more rounded aggregates on BSA-agarose appeared quite weak. However, on all agarose substrates, colonies appeared to be anchored to the support by cells that were in close contact with the gel showing lamellipodia- or spike-like protrusions extending from the edge of the colonies (Fig. 5). When the concentration of agarose in the gels was increased to 0.5%, colony morphology was shifted to monolayer-like aggregates (patches) of cells. Such patches (vs. spheroid-like colonies) were found only occasionally on 0.35% agarose but frequently on 0.5% agarose. In addition to such patches, on the other hand, on 0.5% agarose and even more on 1% agarose gels derivatized with matrix proteins, there were often larger monolayer-like areas similar to those on tissue culture plastic. Chord-like arrangements of cells (Fig. 5 *i*) were only sometimes observed on gels with lower agarose concentrations but more frequently on higher agarose concentrations.

In cross sections (Fig. 6), the spheroid-like arrangement of cells on the more flexible gels with low concentrations of agarose was obvious. In all colonies, the cells established a few to multiple layers of cells very similar to BeWo cells growing on gels of Matrigel or of collagen type I. However, the plane of contact between cells and agarose gels was always very smooth and even while collagen gels appeared to be degraded by the cells (representing their invasive potential) resulting in an irregular contact zone. Gels of Matrigel were also altered by the cells with tongues of cells penetrating into the gel. On agarose gels, in general, the cells were more rounded than cells cultured on protein-coated or noncoated tissue culture plastic. Cell morphology was comparable to that of BeWo cells grown as cell spheroids in suspension culture with cells usually exhibiting characteristics of cytotrophoblast-like cells (Grümmer et al., 1990). Large symplasms indicating cell fusion were not detected by light microscopy. Superficial cells exhibited numerous microvilli as an evidence for polarization, a morphology reminiscent of outgrowth although actual growing out of cells was not observed. Occasionally, flat extensions of individual cells in contact with the surface of the gel protruded from aggregates.

DISCUSSION

This communication describes a novel artificial substrate for cell culture, which allows to mimic certain physical properties of the extracellular matrix without needing to include any natural matrix molecules (although those may be added in covalent bondings if desired). Gels are composed of glyoxyl agarose to which molecules may be bound covalently. Attachment, cell growth, as well as cell morphology are comparable to cells interacting with physiological matrix gels in vitro.

The important role of the extracellular environment for cell behavior has been substantiated by numerous studies investigating cell interactions with the extracellular matrix (Hay, 1991). Furthermore, several publications suggest that not only the biochemical composition of any matrix substrate but also its physical properties contribute significant information for cell differentiation (partly reviewed in Hohn et al., 1992, 1993). This information, however, was derived only from experiments in vitro using substrate gels composed of matrix molecules that form flexible gels physiologically (i.e., gels of collagen type I or of the basement membrane-like matrix from the EHS tumor termed Matrigel). Artificial substrates for cultured cells have been mainly developed for reconstructive surgery (i.e., for prosthetic implants or for skin or vascular grafts) (Hanker and Giammara, 1988; Thomas et al., 1988; Ziats et al., 1988; Friedman, 1992; Kohn, 1992; Louise and Borghetti, 1992; Pongor et al., 1992). Such biomaterials, however, have been designed to be biocompatible and to meet certain mechanical requirements for implants in order to resist tensile forces and, therefore, are quite rigid. Although these materials are biocompatible, al-

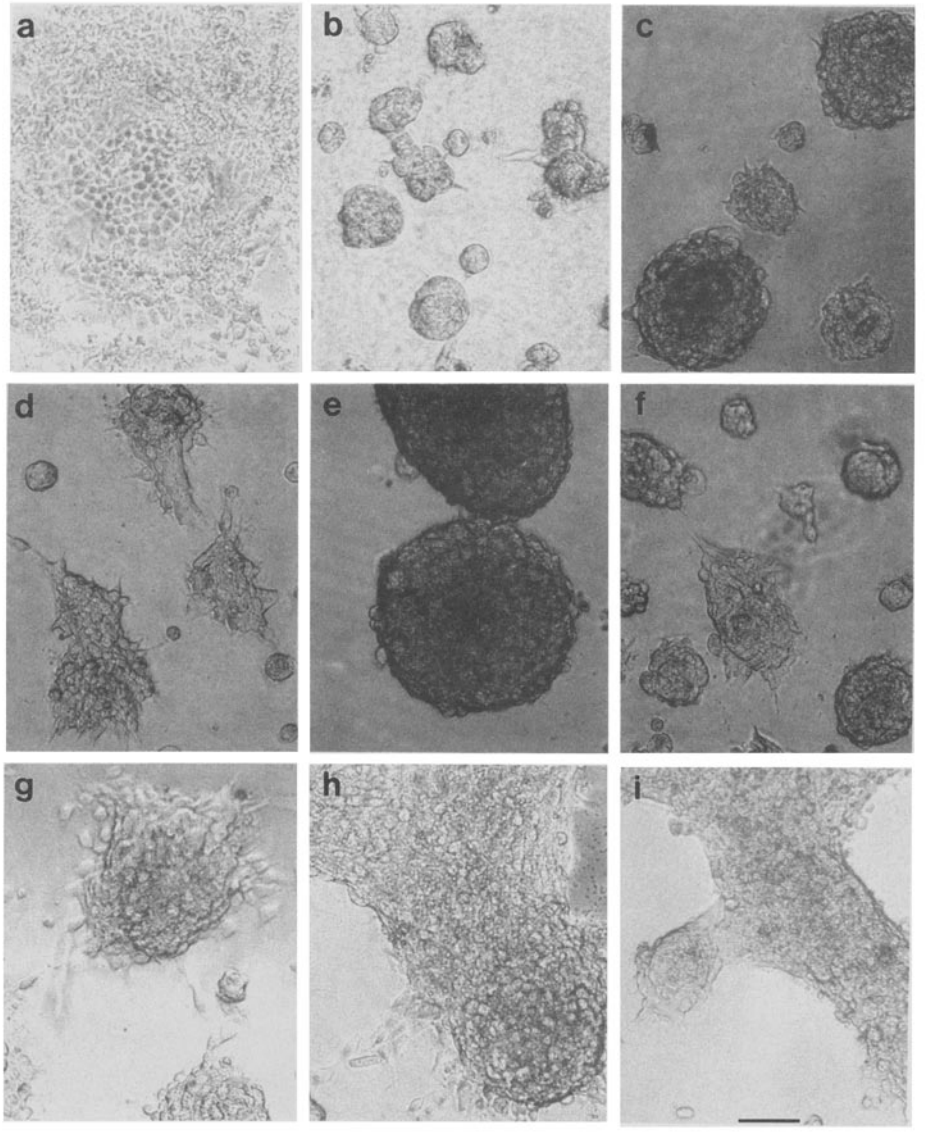

FIG. 5. Phase contrast images taken on Day 4 of culture showing rounded BeWo colonies formed on gels of Matrigel (*b*) or of glyoxyl agarose (*c–i*), as compared with cell monolayers grown on plastic with or without proteins adsorbed (*a*). On plain 0.2% glyoxyl agarose (*c*) and on 0.2% agarose gels containing matrix molecules (*f*, exemplified by fibronectin-agarose), colonies were similar to those grown on gels of Matrigel (*b*), although on plain glyoxyl agarose adhesion was weaker. Larger spheroids formed on 0.2% agarose with BSA (*e*) and adhesion was even weaker than on plain glyoxyl agarose. Stronger adhesiveness of 0.2% agarose containing poly-L-lysine (*d*) resulted in relatively flat colonies of more or less rounded cells. Raising the concentration of agarose in the gels decreased gel flexibility and caused progressive changes in morphology to more flattened clusters of rounded cells on 0.35% and 0.5% gels (*g*, 0.35% agarose with collagen type IV as an example), and generation of monolayer-like morphology with formation of cell chords on 1% gels (*h,i*, fibronectin-agarose as an example). Flat cellular protrusions reaching out from colonies are seen frequently. *Bar* in (*i*) = 100 μm.

though it has been shown that cells are able to attach to and to grow on such substrates, and although such implants are very helpful in reconstructive surgery, it cannot be denied that the natural extracellular environment for most cells is rather flexible and malleable. In the physiological situation, these physical essentials of the extracellular matrix support a balanced tensegrity system of external and cell-internal mechanical forces (Ingber and Jamieson, 1985; Ingber and Folkman, 1989; Ingber, 1991a,b). When the relevance of these mechanical forces for cell behavior is investigated using collagen gels or gels of Matrigel specific responses of the cells to components of the substrates or to specific arrangements of the components in the gels cannot be excluded (Hohn et al., 1992). To exclude effects of chemical recognition of matrix components in an in vitro model, we adapted gels of glyoxyl agarose as cell culture substrate. This derivative of regular agarose had originally been developed for covalent immobilization of biologicals with primary amines, particularly of peptides and proteins, within agarose gels after electrophoresis (Shainoff and Dardik, 1984). Using glyoxyl agarose, proteins are immobilized directly to the gel through stable covalent bonds under gentle conditions and yet remain in their native state.

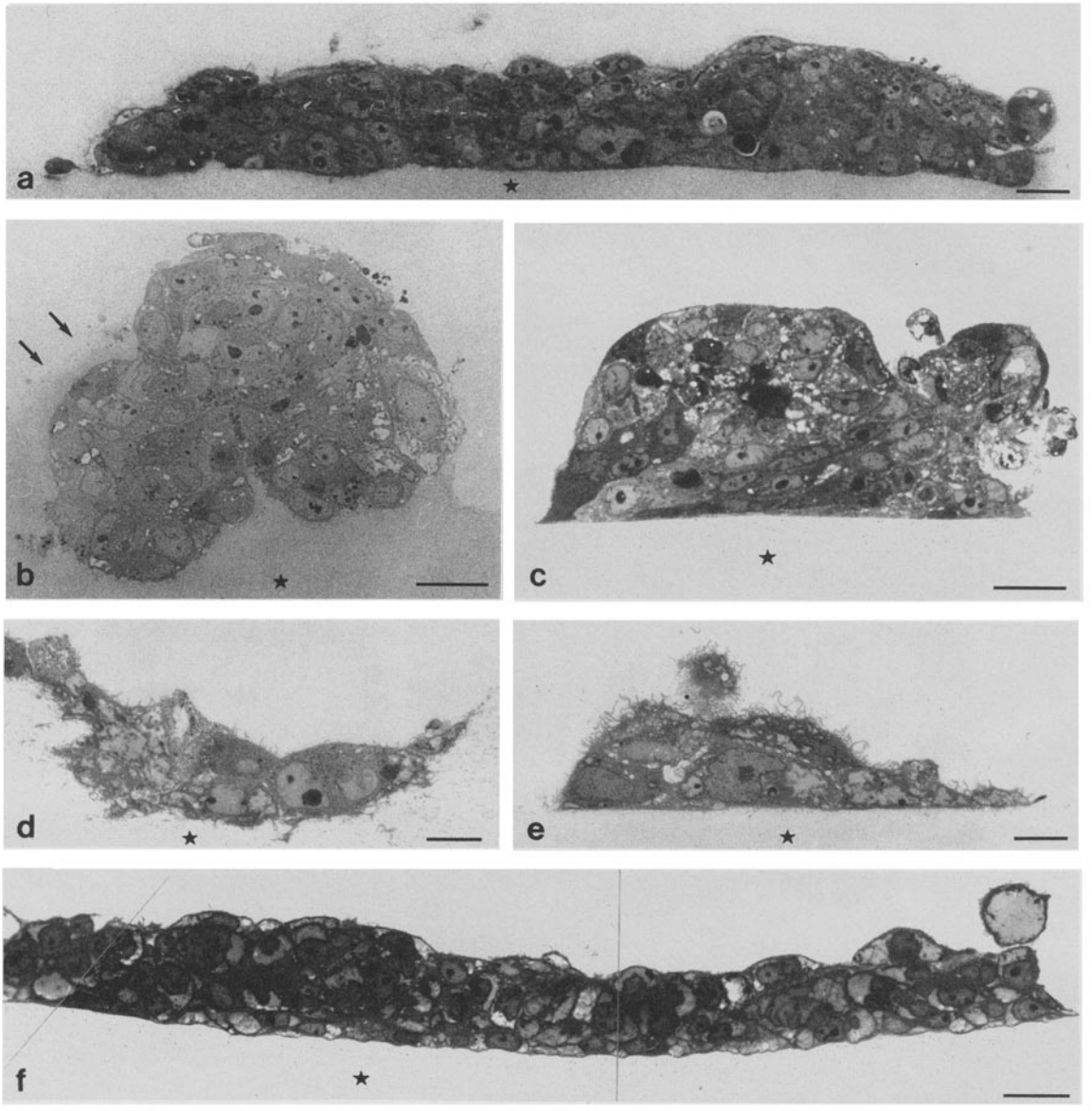

FIG. 6. Light micrographs of semithin sections taken from BeWo colonies grown on gels (location marked by *asterisks*) of Matrigel (*a,b*), of collagen type I (*d*), or of glyoxyl agarose (*c,e,f*). Colonies could take different shapes, i.e., more rounded (*b,c*) or more flattened (*a,d–f*), on Matrigel as well as on glyoxyl agarose. Colonies on 0.35% plain agarose gels (*c*) were comparable to those formed on matrix-derivatized agarose or on poly-L-lysine agarose (*e*), while colonies on higher agarose concentrations (*f* = 1% agarose with fibronectin) were more flattened. On glyoxyl agarose gels, the plane of contact between cells and gel was rather smooth whereas on Matrigel it appeared to be distorted by the cells that tended to partly surround themselves with gel (*b, arrows*). *Bars* = 25 μm in (*a,b,c,f*) and 10 μm in (*d,e*).

This procedure can also be used to create matrices for affinity separations.

A comparable but still different approach to develop a cell culture substrate to which molecules can be bound covalently was described by Raja et al. (1986) who constructed a new acrylamide derivative containing terminal 1,2-dihydroxy groups. Dihydroxyacrylamide could then be copolymerized with acrylamide and bisarylamide, and was then oxidized with $NaIO_4$ to generate reactive aldehyde groups to which proteins could be crosslinked in a similar way as described for glyoxyl agarose. At that time, however, this substrate was only used with respect to covalent linkage of matrix molecules to an inert matrix, thus providing a culture substrate allowing specific cell adhesion only, but not with respect to its physical properties. Dihydroxyacrylamide after activation with $NaIO_4$ and glyoxyl agarose (as it comes from the manufacturer) both contain the same reactive groups (i.e., aldehyde groups) to which molecules can be bonded. Therefore, as for dihydroxy-acrylamide, non-specific interaction of cells with the agarose gel may be prevented by reducing unreacted aldehyde groups to hydroxyl groups with $NaBH_4$. In contrast to glyoxyl agarose, dihydroxyacrylamide is not available commercially.

The possibility to bind molecules covalently to artificial cell cul-

ture substrates is of considerable interest to the investigator because molecules simply adsorbed to tissue culture substrates still can dissociate and diffuse away and may, therefore, be replaced by other molecules secreted by the cells or present in the culture media thus making interpretation difficult (Grinnell, 1978). This disadvantage is overcome by covalently immobilizing adhesive proteins or peptides to glass surfaces (Hubbell et al., 1992). However, these glass substrates are too rigid to meet cytomechanical requirements (Ingber, 1991a,b; Wang et al., 1993). A substrate like glyoxyl agarose mimics the normal cytomechanical situation much better; it is flexible and malleable, and adhesion factors can be linked to it covalently. While on rigid glass surfaces or on comparable polymeric biomaterials with covalently immobilized molecules the cells spread and form regular cell monolayers (Massia and Hubbell, 1991, 1992; Massia et al., 1993), cells grown on glyoxyl agarose are more rounded and form more or less rounded clusters as they do on gels of collagen or of Matrigel (c.f., Hohn et al., 1992). A similar behavior was indicated by Raja et al. (1986, *see* above), who stated that the cells on polyacrylamide gels containing dihydroxyacrylamide derivatized with different molecules were less flat and more rounded than on plastic substrates. However, in their studies they used rather high concentrations of acrylamide (20%), which usually results in gels that are relatively inflexible.

Major advantages of glyoxyl agarose over other biomaterials, in addition to the most important flexibility and malleability, are that a) glyoxyl agarose is readily available and does not require laborious preparation, b) the handling is rather simple, c) preactivated gels can also be cast before any protein of interest can be covalently coupled to the gel in order to provide specific attachment factors in a nonreversible bond, d) the concentration of molecules bound to the gels only depends on the high total binding capacity of glyoxyl agarose, and e) the matrix can be used as a cell culture substrate even without derivatization with proteins as shown in this study. These advantages are partly shared by dihydroxyacrylamide as described by Raja et al. (1986, *see* above). The model described here has the potential to study cell-substrate interactions involving adhesion molecules and adhesion receptors in a new physical arrangement in vitro.

Acknowledgments

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Part of these data have been presented previously in abstract form (J. Cancer Res. Clin. Oncol. 117 (Suppl), S 49; Abstr. Z2, 1991; Eur. J. Cell Biol. 54 (S 32) 48, 1991; Verh. Anat. Ges. 87 (Anat Anz. Suppl. 174), 62, 1992).

References

Barcellos-Hoff, M. H.; Bissell, M. J. Mammary epithelial cells as a model for studies of the regulation of gene expression. In: Satir, B. H., ed. Modern cell biology. Functional epithelial cells in culture. Vol. 8. New York: Alan R. Liss; 1989:399–433.

Ben-Ze'ev, A. Cytoarchitecture and signal transduction. Crit. Rev. Eukaryotic Gene Expression 2:265–281; 1992.

Bissell, M. J.; Barcellos-Hoff, M. H. The influence of extracellular matrix on gene expression: is structure the message? J. Cell Sci. Suppl. 8:327–344; 1987.

Damsky, C. H.; Werb, Z. Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. Curr. Opin. Cell Biol. 4:772–781; 1992.

Daniels, K.; Solursh, M. Modulation of chondrogenesis by the cytoskeleton and extracellular matrix. J. Cell Sci. 100:249–254; 1991.

Doillon, C. J.; Wasserman, A. J.; Berg, R. A., et al. Behaviour of fibroblasts and epidermal cells cultivated on analogues of extracellular matrix. Biomaterials 9:91–96; 1988.

Friedman, R. J. Advances in biomaterials and factors affecting implant fixation. Instr. Course Lect. 41:127–136; 1992.

Grinnell, F. Cellular adhesiveness and extracellular substrata. Int. Rev. Cytol. 53:65–144; 1978.

Grümmer, R.; Hohn, H.-P.; Denker, H.-W. Choriocarcinoma cell spheroids: an in vitro model for the human trophoblast. Trophoblast Res. 4:97–111; 1990.

Hadley, M. A.; Weeks, B. S.; Kleinman, H. K., et al. Laminin promotes formation of cord-like structures by sertoli cells in vitro. Dev. Biol. 140:318–327; 1990.

Hanker, J. S.; Giammara, B. L. Biomaterials and biomedical devices. Science 242:885–892; 1988.

Hay, E. D. Cell biology of the extracellular matrix. 2nd ed. New York: Plenum Press; 1991.

Herbert, D.; Phipps, P. J.; Strange, R. P. Chemical analysis of microbial cells. In: Norris, J. R.; Ribbons, D. W., eds. Methods in microbiology. Vol. 58. London: Academic Press; 1971:210–310.

Hohn, H.-P.; Boots, L. R.; Denker, H.-W., et al. Differentiation of human trophoblast cells in vitro is stimulated by extracellular matrix. Trophoblast Res. 7:181–200; 1993.

Hohn, H.-P.; Parker, C. R.; Boots, L. R., et al. Modulation of differentiation markers in human choriocarcinoma cells by extracellular matrix: on the role of a three-dimensional matrix structure. Differentiation 51:61–70; 1992.

Hubbell, J. A.; Massia, S. P.; Drumheller, P. D. Surface-grafted cell binding peptides in tissue engineering of the vascular graft. Ann. NY Acad. Sci. 665:253–258; 1992.

Hutchins, R. O.; Natale, N. R. Cyanoborohydride. Utility and applications in organic synthesis. A review. Org. Prep. Proced. 11:201–246; 1979.

Ingber, D. Extracellular matrix and cell shape: potential control points for inhibition of angiogenesis. J. Cell. Biochem. 47:236–241; 1991a.

Ingber, D. Integrins as mechanochemical transducers. Curr. Opin. Cell Biol. 3:841–848; 1991b.

Ingber, D. E.; Folkman, J. Mechanochemical switching between growth and differentiation during fibroblast growth factor-stimulated angiogenesis in vitro: role of extracellular matrix. J. Cell Biol. 109:317–330; 1989.

Ingber, D. E.; Jamieson, J. D. Cells as tensegrity structures: architectural regulation of histodifferentiation by physical forces transduced over basement membrane. In: Anderson, L. C.; Gahmberg, C. G.; Ekblom, P., eds. Gene expression during normal and malignant differentiation. London: Academic Press; 1985:13–32.

Juliano, R. L.; Haskill, S. Signal transduction from the extracellular matrix. J. Cell Biol. 120:577–585; 1993.

Kleinman, H. K.; McGarvey, M. L.; Hassel, J. R., et al. Basement membrane complexes with biological activity. Biochemistry 25:312–318; 1986.

Kohn, D. H. Overview of factors important in implant design. J. Oral Implantol. 18:204–219; 1992.

Landegren, U. Measurement of cell numbers by means of the endogenous enzyme hexosaminidase. Applications to detection of lymphokines and cell surface antigens. J. Immunol. Methods 67:379–388; 1984.

Lane, C. F. Sodium cyanoborohydride—a highly selective reducing agent for organic functional groups. Synthesis 3:135–146; 1975.

Lawley, T. J.; Kubota, Y. Induction of morphologic differentiation of endothelial cells in culture. J. Invest. Dermatol. 93:59S–61S; 1989.

Louise, F. R.; Borghetti, A. F. New developments in synthetic bone replacement materials. Curr. Opin. Dent. 2:97–103; 1992.

Massia, S. P.; Hubbell, J. A. Human endothelial cell interactions with surface-coupled adhesion peptides on a nonadhesive glass substrate and two polymeric biomaterials. J. Biomed. Mater. Res. 25:223–242; 1991.

Massia, S. P.; Hubbell, J. A. Vascular endothelial cell adhesion and spreading promoted by the peptide REDV of the IIICS region of plasma fibronectin is mediated by integrin alpha4/beta1. J. Biol. Chem. 267:14019–14026; 1992.

Massia, S. P.; Rao, S.; Hubbell, J. A. Covalently immobilized laminin peptide Tyr-Ile-Gly-Ser-Arg (YIGSR) supports cell spreading and colocalization of the 67-kilodalton laminin receptor with alpha-actinin and vinculin. J. Biol. Chem. 268:8053–8059; 1993.

McDonald, J. A. Matrix regulation of cell shape and gene expression. Curr. Opin. Cell Biol. 1:995–999; 1989.

Nakanishi, Y.; Ishii, T. Epithelial shape change in mouse embryonic submandibular gland: modulation by extracellular matrix components. Bioessays 11:163–167; 1989.

Ormerod, E. J.; Rudland, P. S. Mammary gland morphogenesis in vitro: extracellular requirements for the formation of tubules in collagen gels by a cloned rat mammary epithelial cell line. In Vitro Cell. Dev. Biol. 24:9–16; 1988.

Pattillo, R. A.; Gey, G. O. The establishment of a cell line of human hormone-synthesizing trophoblastic cells in vitro. Cancer Res. 28:1231–1236; 1968.

Pongor, P.; Betts, J.; Muckle, D. S., et al. Woven carbon surface replacement in the knee: independent clinical review. Biomaterials 13:1070–1076; 1992.

Raja, R. H.; Herzig, M.; Grissom, M., et al. Preparation and use of synthetic cell culture surfaces. A new reagent for the covalent immobilization of proteins and glycoproteins on a nonionic inert matrix. J. Biol. Chem. 261:8505–8513; 1986.

Shainoff, J. R.; Dardik, B. N. Electrophoretic procedures for zonal immobilization of protiens on glyoxyl agarose. In: Lewised, L. A., ed. CRC handbook of electrophoresis. Vol. III. Boca Raton, FL: CRC Press; 1984:21–28.

Shannon, J. M.; Emrie, P. A.; Fisher, J. H., et al. Effect of a reconstituted basement membrane on expression of surfactant apoproteins in cultured adult rat alveolar type II cells. Am. J. Respir. Cell Mol. Biol. 2:183–192; 1990.

Streuli, C. H.; Bailey, N.; Bissell, M. J. Control of mammary epithelial differentiation: basement membrane induces tissue-specific gene expression in the absence of cell-cell interaction and morphological polarity. J. Cell Biol. 115:1383–1395; 1991.

Thomas, J. H.; Pierce, G. E.; Iliopoulos, J. I., et al. Vascular graft selection. Surg. Clin. North Am. 68:865–874; 1988.

Tinois, E.; Paure, M.; Chatelain, P., et al. Growth and differentiation of human keratinocytes on extracellular matrix. Arch. Dermatol. Res. 279:241–246; 1987.

Wang, N.; Butler, J. P.; Ingber, D. E. Mechanotransduction across the cell surface and through the cytoskeleton. Science 260:1124–1127; 1993.

Ziats, N. P.; Miller, K. M.; Anderson, J. M. In vitro and in vivo interactions of cells with biomaterials. Biomaterials 9:5–13; 1988.