Use of collagen–hydroxyethylmethacrylate hydrogels for cell growth

(connective tissue/cell surface/cell biology/fibroblasts)

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ABSTRACT Collagen–hydroxyethylmethacrylate hydrogels were prepared by polymerizing monomeric hydroxyethylmethacrylate in the presence of various concentrations of soluble native collagen. The resulting transparent hydrogels were evaluated as substrata for growth of IMR-90 human embryonic lung fibroblasts. Without collagen no significant growth occurred, whereas a dose-response curve expressing maximal cell growth against collagen concentration could be constructed quite readily by the use of appropriate hydrogels. The method allows for quantification of the collagen contribution to cell growth and, in a more general sense, provides the foundation for a relatively easy procedure to probe mechanisms of cell adhesion and cell differentiation.

Among the many synthetic polymers presently used as biomaterials are those known as hydrogels. The term "hydrogel" refers to a broad class of polymeric materials that are swollen extensively in water but that do not dissolve in water (1). They have been used in a wide variety of biomedical applications and may be synthesized from monomers or monomers mixed with polymers. Hydrogels are attractive as biomaterials; they are highly permeable to water, ions, and small molecules (1). Hydrogels such as poly(2-hydroxyethylmethacrylate) (HEMA) and other synthetic polymers are relatively nontoxic and well tolerated when implanted in vivo (2, 3) or when added to an actively metabolizing tissue-culture system (4–6).

The implantation of a biological material such as collagen results in good tissue tolerance both in vivo (7–15) and in vitro (16–21). Collagen has been used in many biomedical applications such as in the dialysis membrane of an artificial kidney (7, 22–24), an artificial corneal membrane (8, 9), as vitreous body (10–12), in skin and blood vessels (13, 14), and as a surgical hemostatic agent (15). In 1969, Chvipil et al. (25) described good tissue tolerance in vivo of a collagen-hydrophilic polymer that they had constructed from a calf-hide collagen sponge immersed in HEMA monomer, allowing polymerization to occur on the sponge surface. They attributed the biocompatibility of this material to the porosity of the sponge, which permitted the ingrowth of blood vessels. In 1977, Shinnick et al. (26) reported high tissue compatibility of laminar copolymers of bovine collagen and various synthetic polymers that they had constructed by applying plasma discharge and γ-irradiation to effect crosslinkage between a layer of collagen coated onto a layer of synthetic polymer. More recent studies involving collagen–cell surface interactions have focused on the role of fibronectin (27, 28).

In a study by Folkman and Moscona (29), poly(HEMA) coating was used as a means of preventing fibroblast growth on standard tissue-culture flasks (Falconware). Similarly, a morphologic study of mouse fibroblasts grown on synthetic-coated coverslips found poly(methylmethacrylate) and other polymers to be unfavorable surfaces for cell adhesion and growth (30). In the present communication, we have attempted to construct a collagen hydrogel copolymer, in which collagen is incorporated into the hydrogel structure by addition of the aqueous protein solution to aqueous monomer solution prior to free-radical-initiated polymerization of the mixture. This approach circumvents the problems inherent in bonding a layer of protein to a synthetic polymer. The growth patterns of human embryonic lung fibroblasts (IMR-90) in cell culture by use of poly(HEMA) polymerized in the presence of various concentrations of protein as the substrata for cell adherence and growth are described. The nature and content of the sub-stratum, which is responsible for initial cell adhesion and possibly cell growth, can be controlled. The effect of prior collagenase treatment of the protein hydrogel surface is examined as well.

In general, we believe that this system may well serve as a reasonable assay for evaluating the efficiency of several biologic polymers, such as collagen, to support cell adhesion, growth, and even motility.

MATERIALS AND METHODS

Materials. Commercial HEMA was purchased from Aldrich (lot no. 060377); ammonium persulfate was purchased from Bio-Rad; bovine serum albumin and porcine pepsin were obtained from Sigma. Bacterial collagenase (Clostridium histolyticum) type I, which was purchased from Sigma, was further purified by Sephadex G-200 gel filtration as described by Peterskofsky (31) and assayed as described by Hu et al. (32).

Pepsin-soluble collagen was prepared by stirring the ground, shaved skin from a 1-week-old calf in 0.5 M acetic acid at 4°C. The residue after centrifugation was resuspended in 0.5 M acetic acid containing porcine pepsin at a final enzyme-to-tissue ratio of 1:50 (wt/wt) and allowed to stir overnight. The solubilized collagen was then precipitated by addition of solid NaCl to a concentration of 5% (wt/vol). The resulting precipitate was resolubilized in 0.5 M acetic acid, then dialyzed exhaustively against 20 mM Na₂HPO₄ (pH 7.44) at 4°C. After dialysis, the precipitate was subjected to differential NaCl precipitation at pH 7.44 as described (33), and the precipitates were lyophilized. The 10% NaCl-precipitated collagen was used in all of the following experiments.

Protein Solutions. The lyophilized NaCl-precipitated collagen described above was suspended in 0.5 M acetic acid at a concentration of 1.2–1.4 mg/ml, as determined by hydroxy-
proline content, and allowed to stir overnight at 4°C. The resulting solution was dialyzed against 0.15 M NaCl/50 mM Tris-HCl, pH 7.4, overnight at 4°C. This represents the stock collagen solution which is added to the hydrogel monomer mixture as described below. Bovine serum albumin was dissolved in the same NaCl/Tris buffer at a concentration of 10 mg/ml for addition to HEMA hydrogel mixtures.

Preparation of Hydrogel–Protein Membranes. Polymers of HEMA were essentially prepared as described (34, 35). One milliliter of HEMA, 1.0 ml of ethylene glycol, 1.0 ml of H2O or buffer, 0.1 ml of 6% (wt/vol) ammonium persulfate, and 0.1 ml of 12% (wt/vol) sodium metabisulfite were added in sequence. After mixing, the resulting clear, viscous monomer solution was polymerized by heating for 2 hr at 38°C between two glass slides separated by two coverslips. The approximate thickness of the gel was 0.5 mm. The resulting clear flexible hydrogel membrane was then dialyzed exhaustively against the Tris/NaCl buffer (pH 7.4) to remove residual monomer and ethylene glycol. During this procedure the membranes become opaque, but transparency returns once the ethylene glycol has been exchanged for water.

If in place of the 1.0 ml of H2O, the buffered solutions of collagen or albumin described above are substituted, then the resulting hydrogels that are formed contain protein. Hydrogels were prepared by using 1.0-ml aliquots of various dilutions of the stock collagen solution and the final collagen content of the resulting individual hydrogel buttons prepared from these mixtures was calculated from the appropriate dilution.

Preparation of Hydrogels for Growth Studies. The hydrogel–buffer or hydrogel–protein membranes were sterilized in Puck’s Ca2+- and Mg2+-free saline containing 1000 units of penicillin, 50 μg of Aureomycin, and 0.25 μg of Fungizone per ml of medium by placing them under ultraviolet lights for 2 hr. The hydrogels were then transferred to Puck’s saline containing penicillin and streptomycin and stored at 4°C prior to use. Before sterilization, the hydrogels were cut into 1.4-cm-diameter buttons with a size 10 cork borer.

Cell Cultures. Human embryonic lung fibroblasts (IMR-90) were used in 8th passage (23rd population doubling) for the hydrogel growth curves. Into each chamber of a Costar cluster dish (24 wells, 1.6 cm diameter) was placed an individual hydrogel button. To each chamber was then added a suspension of cells that contained 5 × 10⁴ cells in 0.5 ml of medium. Dulbecco’s modified Eagle’s medium supplemented by the addition of 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) was used in these studies. After the cells were allowed to settle directly onto the hydrogel button for 4 hr, the medium in the individual chambers was removed and the buttons were transferred to new chambers containing 2.0 ml of medium. The number of cells adhering to the buttons in the initial 4-hr period was small relative to the total number of cells suspended. The numbers varied from approximately 4000 cells on those gels with no collagen to 7000–5000 cells on those gels containing collagen. The buttons were then incubated at 37°C in a humidified atmosphere of 5% CO2/95% air. Cells were fed three times weekly with 2.0 ml of medium. Cells growing on hydrogels were photographed with a phase-contrast microscope at various times (Olympus Inverted Phase Microscope). Cell number was determined by transfer of separate hydrogels that had been washed with Isoton to a Coulter Counter vial containing 2 ml of 0.05% trypsin in 0.02% EDTA (GIBCO). After 5 min the cell suspension was vigorously disrupted with a Pasteur pipette in order to prevent cell clumping. The suspension was then diluted to 10 ml with Isoton and counted in the Coulter Counter. Microscopic examination of the hydrogel buttons after such treatment revealed that no cells remained attached to the HEMA gels with or without collagen.

Enzymatic Treatment of Protein Hydrogels for Cell Culture. Prior to antibiotic treatment for cell culture, certain of the hydrogel buttons containing collagen or albumin were incubated individually with 20 μg of purified bacterial collagenase in 0.5 ml of 0.15 M NaCl/50 mM Tris-HCl/0.36 mM CaCl2, pH 7.5, for 20 hr at room temperature with shaking. The buttons were then treated with 2.0 ml of 50 mM EDTA for 15 min, washed with Tris/NaCl buffer, and prepared for cell culture as described above. Enzymatic incubation does not appear to alter the gross physical integrity of the hydrogel membranes.

Amino Acid Content of Protein Hydrogels. Membranes prepared with collagen as described above were finely minced to increase surface area and hydrolyzed, under reduced pressure, in 6 M HCl at 110°C for 20 hr. The HEMA membrane itself does not dissolve under these conditions and, therefore, analyses were easily performed on the supernatant of these membranes with a Beckman model 119-CL amino acid analyzer. Collagen content could be estimated on the basis of hydroxyproline content of the hydrolysate.

In another experiment, hydrogels containing collagen were minced and treated with bacterial collagenase as described above and then hydrolyzed in 6 M HCl. Control hydrogels were incubated with buffer as above but with no enzyme and then hydrolyzed. The control and collagenase supernatants were also hydrolyzed, and amino acid analyses were performed on all samples.

RESULTS

Growth of IMR-90 Fibroblasts on HEMA–Collagen Hydrogels. Growth of IMR-90 fibroblasts cultured on HEMA hydrogels containing various amounts of collagen was evaluated. Fig. 1 expresses the number of cells present as a function of time on several of the hydrogels. Each cell number determination is the average of eight individual hydrogels. The standard deviation in the number of cells within a single time point and at a single collagen concentration was approximately 20%. Although all collagen concentrations listed in Table 1 were studied, only six of these are represented in the figure. Photomicrographs of cells growing for 4, 8, and 10 days on a typical collagen hydrogel prior to trypsinization are shown in Fig. 2B.

Figs. 1 and 2A show that HEMA–buffer hydrogels are unfa-
favorable surfaces for support of fibroblast growth. Figs. 3 and 4 display the effect of varying concentrations of collagen in the gels as a function of maximal obtainable cell number. For convenience, we used a maximal cell number obtained regardless of the day it was maximal. This was necessitated by the observation that maximal cell number did not always occur on the same day in culture, as shown in Fig. 1. The hydrogels that marginally support cell growth display cell "sluffing" earlier than those gels displaying maximal cell growth. The amount of collagen in the hydrogel preparation capable of supporting approximately 80% of maximal cell number observed was 16 μg. The minimal collagen content of a hydrogel button necessary to detect any significant cell growth was 0.2 μg.

Treatment of collagen hydrogels with purified bacterial collagenase eliminated cell growth. The cell number on these hydrogels was the same as the number of cells on hydrogels that contained no collagen. Morphologically, cells grown on the HEMA-collagen hydrogels that were incubated with collagenase appeared round and sparsely distributed, similar to cells grown on hydrogels without protein (Fig. 2 A and C). For comparative purposes, hydrogels that contained bovine serum albumin were used. Certain of these albumin-containing hy-

![Diagram](image)

**Fig. 2.** Photomicrographs of IMR-90 cells on various HEMA hydrogels. (A) Control hydrogel, 1.0 ml of buffer alone. (B) HEMA-collagen hydrogel contains 1.0 ml of stock collagen solution (31 μg). (C) Same hydrogel as in B but treated with purified bacterial collagenase prior to cell culture. (D) HEMA-albumin hydrogel contains 1.0 ml of albumin solution (10 mg/ml).

**Fig. 3.** Maximal cell growth obtained on HEMA hydrogels containing various amounts of collagen.

**Fig. 4.** Semilogarithmic plot of maximal cell growth obtained in HEMA hydrogels against collagen content.
dorgels were treated with collagenase prior to incubation with the cells. Although these albumin hydrogels (Fig. 2D) clearly did not support cell growth as well as the collagen hydrogels, the maximal cell number on the albumin hydrogels was the same with or without collagenase treatment. This would indicate that the manipulations during the collagenase treatment itself did not affect cell growth.

**Hydroxyproline Analyses of Protein Hydrogels.** Hydrolysates of collagen-containing hydrogels yielded amino acid analyses consistent with that of collagen. However, all of the collagen could not be recovered from the hydrogel by hydrolysis in 6 M HCl, suggesting that the collagen incorporated into the hydrogel is tightly bound or not readily accessible to hydrolysis. Approximately 32% of the hydroxyproline added to the hydrogel (31.0 µg total collagen) was recovered after hydrolysis. In a separate series of experiments, bacterial collagenase was used to solubilize the collagen in the hydrogels. However, after collagenase treatment no hydroxyproline was detected in the supernatant. Such data suggest that the collagenous material originally incorporated into the hydrogel might be covalently bound or entrapped within the hydrogel structure and, as such, might not be susceptible to the action of the bacterial collagenase. In the same vein, the interior collagen is most likely not accessible to the cells adhered to the surface of hydrogels because the HEMA gel is not easily penetrated by the cells.

**DISCUSSION**

Collagen has been used as a substratum for promotion of cell adhesion and growth in several laboratories. Although tissue-culture surfaces are coated routinely with collagens from various sources, the molecular interactions responsible for this adhesion of cells to such surfaces are poorly understood. The discovery of fibronectin, a protein that apparently serves to mediate cell-surface interactions with collagen, has been an exciting recent development (27, 28). It has renewed interest in many laboratories to reinvestigate the relationships between cell and collagen interactions. A major shortcoming in such studies is the fact that one cannot readily quantify the collagen contribution to the system. This communication describes a simple procedure to produce a transparent, smooth plastic surface that supports cell growth and can be used to assess the collagen contribution to cell growth. By polymerizing monomeric HEMA in the presence of native collagen solutions of various concentrations, one can control the amount of protein added to the surface. An added feature of the system is that, as shown previously, poly(HEMA) does not support cell growth alone (29). When cell growth is supported on such surfaces, the responsible agent for such growth must be the added collagen. From such studies a dose–response curve can be constructed expressing maximal growth of cells against collagen concentration. It becomes obvious from such studies (Fig. 1) that a certain minimal content of collagen is needed for support of cell growth.

It is worth considering the nature of the substratum that we have described. Hydrogels by their very nature imbibe water. The protein present during the initial polymerization gets trapped in the aqueous pockets that are created by the polymer and is usually not accessible to the external milieu. This is not true, of course, for that portion of the protein that is bound on the surface of the hydrogel. Because only the surface-bound proteins are accessible to the cells that are being cultured and the cells grown on these surfaces do not appear to penetrate the hydrogel, one can, with appropriate assumptions, make estimations of the number of cells supported by a specific number of collagen molecules on the surface. To calculate the number of molecules of collagen present on the surface of the hydrogel, one needs to calculate the volume of the hydrogel buttons used. Knowing the volume of a collagen molecule, one can estimate the maximal number of collagen molecules that the hydrogel could theoretically accommodate. By calculating the area of the surface of the button and assuming that all of the collagen molecules present on the hydrogel surface lie with their long axis parallel to this surface, one can estimate the number of collagen molecules present by assuming that the collagen molecule is 3000 Å long and 15 Å wide. This number will be equivalent to the minimal number of collagen molecules on the hydrogel surface. On the other hand, the maximal number of molecules on the surface can be calculated by assuming that all collagen molecules are stacked with their long axis perpendicular to the surface of the hydrogel so that the area each occupies is calculated from the cross-sectional area of a collagen molecule. Using the minimal and maximal number of molecules on the hydrogel surface and the total number of molecules that can maximally be incorporated within the hydrogel volume, one can then calculate the minimal and maximal percentage of collagen molecules present at the surface. Applying these values to the final cell number grown on the hydrogels, one can calculate the possible minimal and maximal number of collagen molecules present per cell. These data, given in Table 1, suggest that the number of collagen molecules on the surface (regardless of what that number is) determines the final

<table>
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<th>Collagen content of hydrogel buttons, µg</th>
<th>Maximum cell no. attained × 10^5</th>
<th>No. of collagen molecules at hydrogel surface^1</th>
<th>Molecules of collagen per cell</th>
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<tbody>
<tr>
<td></td>
<td>Minimum × 10^5</td>
<td>Maximum × 10^5</td>
<td>Minimum</td>
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<tr>
<td>31.0</td>
<td>3.09</td>
<td>780</td>
<td>192</td>
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<td>24.0</td>
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<td>1.89</td>
<td>98</td>
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<tr>
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<td>1.56</td>
<td>49</td>
<td>12.4</td>
</tr>
<tr>
<td>0.9</td>
<td>0.52</td>
<td>22</td>
<td>6.2</td>
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<td>0.3</td>
<td>0.19</td>
<td>7.4</td>
<td>1.86</td>
</tr>
<tr>
<td>0.2</td>
<td>0.13</td>
<td>4.9</td>
<td>1.24</td>
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^ Average of eight separate determinations.

^1 See Discussion.
cell density of the culture that is propagated on the surface. The actual number of collagen molecules per cell can be as low as 40–50 to approximately 10,000, depending on the orientation of the molecules at the cell surface.

One legitimately might question the contribution that the cells themselves might make to the collagen content of the surface by producing their own collagen. In the studies described, the IMR-90 cells were grown without added ascorbate. Faris et al. (36) have shown that without added ascorbate the IMR-90 cells do not produce insoluble collagen. It may well be that when such studies are repeated in the presence of appropriate amounts of ascorbate the amount of collagen contained in the gels necessary for maximal growth will be less than the amount needed in the absence of ascorbate.

Although this present study describes the use of HEMA-collagen hydrogels to study fundamental aspects of cell growth and to a lesser extent cell adhesion, one could use this same system to examine more intricate aspects of adhesion, growth, and motility. As noted in the Results, the number of cells that initially adhered to the collagen-containing HEMA was slightly higher than the number of cells that adhered to the HEMA without collagen. Although these data are not sufficiently different to explain the effects of the collagen on the maximal cell growth obtained, it obviously plays an important role and is being considered at present. An additional advantage of these gels are their transparency; they allow one to evaluate the growth and behavior of cells through the microscope. Manipulation of the cells adhered to the surface is also feasible; transfer to other medium or to large surfaces would not require trypsinization, for example. Surfaces could well be constructed with other purified macromolecules and directed toward understanding cell differentiation and transformation. Fig. 2D shows phase-contrast micrographs of cells grown on bovine-albumin-containing gels. Although these studies are not completed, the cells do not appear as healthy as those grown on collagen, and growth was qualitatively much slower. In fact, the cells on the albumin-containing buttons never become confluent; yet, growth was better than on HEMA gels with no added protein.

By slight modification of the polymers (for example, by addition of other crosslinking agents or the use of copolymers), one could create surfaces that could be stretched so that mechanical stress on cells could be evaluated. By the procedure described in this paper we can incorporate different proteins, both soluble and insoluble, into the hydrogels, which then can be used to evaluate a variety of biological phenomena.

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