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IMPROVED ATTACHMENT AND SPREADING IN PRIMARY CELL CULTURES OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*

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SUMMARY

At present, establishment of a cell line from bivalve molluscs has been unsuccessful, and in vitro work is limited to primary cell cultures. We sought to improve attachment and spreading of cells of the eastern oyster, *Crassostrea virginica*, to aid primary cultures and to assist development of a bivalve cell line. Our objectives were to examine the effects of substrate on ventricle cell viability, attachment, and spreading by testing of collagen I, collagen IV, fibronectin, laminin, poly-D-lysine, and two types of uncoated tissue culture plates (Falcon® and Corning®). Experiments were conducted by incubating cells with the varions snbstrates for 24 h and 5 d. An assay with a tetrazolium compound (MTS) was used to estimate cell nnmbers based on metabolic activity. Although differences in MTS assay values for substrate effect on cell viability were detected at 24 h and at 5 d ($P > 0.0001$), these were attributed to variations in metabolic activity due to different levels of attachment and spreading among treatments. Differences among treatments were detected in attachment and spreading at 24 h and 5 d (for all, $P > 0.0001$). At 24 h, poly-D-lysine induced the highest levels of attachment and spreading; no other factor performed better than the uncoated Falcon® substrate, and collagen I performed most poorly. At 5 d, poly-D-lysine and the uncoated Corning® substrate induced significantly higher levels of attachment and spreading than did the uncoated Falcon® substrate, and collagen I performed most poorly. From these results, poly-D-lysine best promoted cell attachment and spreading. Fibronectin (at 24 h) and laminin (at 5 d) warrant further stndy. Along with improvements in medium composition, future work should involve screening of other attachment factors and combinations of factors, including those of bivalve origin.

Key words: Crassostrea virginica; attachment factors; MTS; bivalve cell culture.

INTRODUCTION

Although techniques for primary cell culture of bivalve tissues have been developed (Perkins and Menzel, 1964; Odinstava and Khomenko, 1991), attempts to produce a bivalve cell line have been unsuccessful (Hetrick et al., 1981; Ellis and Bishop. 1989). Within the mollusca, the only established cell line is from the freshwater snail, *Biomphalaria glabrata* (Hansen, 1976). The importance of cell lines has long been recognized in in vitro studies (Freshney, 1994). The development of a bivalve cell line would provide an invaluable tool for research in areas such as pathobiology, cytogenetics, and gene transfer.

Improving cell attachment and spreading in primary cell culture would be of benefit in the eventual development of a bivalve cell line. Cell detachment and lysis have been noted as problems in the long-term primary culture of bivalve cells (Hetrick et al., 1981; Miahle et al., 1988; Chen et al., 1989). Attachment and spreading are known to play important roles in cellular functions such as differentiation, migration, proliferation, and gene expression, as well as in maintaining cell viability (Ben-Ze'ev et al., 1980; Guan and Chen, 1996). Several attachment factors have been isolated from bivalve cells (Suzuki et al., 1991; Panara et al., 1996), and some studies have addressed improved attachment in bivalve primary cell cultures (Table 1). However, these studies did not quantitatively assay improvement in cell attachment and spreading. In this study, we used an objective method to quantify attachment and spreading.

The goal of this work was to improve attachment and spreading in oyster primary cell culture. To this end, oyster ventricle cells were tested with the attachment factors, collagen I, collagen IV, fibronectin, laminin, poly-D-lysine, and two types of uncoated tissue culture plates to investigate effects on cell viability, improvement of cell attachment, and improvement of cell spreading. To our knowledge, this is the first quantitative report on improvement of attachment and spreading in bivalve primary cell culture.

MATERIALS AND METHODS

Cells and cell culture. Eastern oysters were collected from coastal Louisiana and were held at least 5 d in filtered artificial sea water (ASW) (Fritz Super Salt. Fritz Industries Inc., Dallas, TX) before use. The osmolality of all solutions, including ASW and medium, was measured with a vapor pressure

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TABLE 1

REPORTS ON UTILITY OF ATTACHMENT FACTORS IN BIVALVE CELL CULTURE

Scientific name	Common name	Cell source	Beneficial substrates	Non-beneficial substrates	Assay for utility of factors[a]	Reference
Mytilus edulis	Blue mussel	Digestive gland, mantle, male gonad, larvae	Polylysines (137, 190 kDa), collagen III, fibronectin, fetal calf serum, adhesive protein from *M. edulis*	Polylysines (24, 100 kDa) histone H1 and protamine-like protein from *M. edulis*	Cell viability with fluorescein diacetate and ethidium bromide, [H^3]uridine assay for RNA production	Odintsova et al., 1994
Mizuhopecten yessoensis	Japanese scallop	Larvae	Collagen (type not reported)	None reported	Subjective criteria	Odintsova and Khomenko, 1991
Patinopecten yessoensis	Scallop	Digestive gland, mantle, male gonad, larvae	Polylysines (137, 190 kDa), collagen III, adhesive protein from *M. edulis*	Polylysines (24, 100 kDa) fibronectin, fetal calf serum, histone H1 and protamine-like protein from *M. edulis*	Cell viability with fluorescein diacetate and ethidium bromide, [H^3]uridine assay for RNA production	Odintsova et al., 1994
Crassostrea gigas	Pacific oyster	Larvae	None reported	Collagen (type not reported), fibronectin, gelatin, Primaria™ (Falcon®) plates	Subjective criteria	Takeuchi et al., 1994
Crassostrea gigas	Pacific oyster	Ventricle	Poly-D-lysine	None reported	Subjective criteria	Le Deuff et al., 1994
Crassostrea virginica	Eastern oyster	Larvae	Primaria™ (Falcon®) plates	None reported	Subjective criteria	Ellis and Bishop, 1989
Crassostrea virginica	Eastern oyster	Heart, mantle, gonad, muscle, labial palp, pericardial membrane	None reported	Poly-D-lysine	Subjective criteria	Hetrick et al., 1981
Crassostrea virginica	Eastern oyster	Embryo	Fibronectin	Collagen III	Subjective criteria	Hetrick et al., 1981
Ostrea edulis	Edible oyster	Whole heart	Poly-D-lysine	None reported	Subjective criteria	Renault et al., 1995
Mercenaria mercenaria	Northen quahog	Larvae	Primaria™ (Falcon®) plates	None reported	Subjective criteria	Ellis and Bishop, 1989
Mya arenaria	Softshell clam	Hemocytes	None reported	Poly-D-lysine	Subjective criteria	Hetrick et al., 1981
Mya arenaria	Softshell clam	Ventricle	Primaria™ (Falcon®) plates	None reported	Subjective criteria	Kleinschuster et al., 1996

[a]Subjective criteria include general observations (such as observations on cell health, culture longevity, noticeable differences in cell spreading) on utility of attachment factors with no attempts at quantification.

osmometer (Model 5500, Wescor Inc., Logan, UT) and maintained at 425 mOsmol/kg. Ventricles were removed and decontaminated with two 30-min incubations in an antibiotic solution consisting of (per l) 100 mg penicillin G, 100 mg streptomycin, 100 mg gentamicin, 200 mg kanamycin, 100 mg neomycin, 100 mg polymyxin B, 200 mg erythromycin, and 2.8 mg amphotericin B dissolved in sterile ASW. Primary cell cultures were obtained by dissociation of ventricles in a saline solution of (per l) 0.635 g $CaCl_2 \cdot 2H_2O$, 1.46 g $MgSO_4$, 2.18 g $MgCl_2 \cdot 6H_2O$, 0.310 g KCl, 11.61 g NaCl, and 0.35 mg $NaHCO_3$) containing 0.1 mg Pronase (CalBiochem, La Jolla, CA) per ml. Pronase has proven to be the most effective dissociation enzyme tested in our laboratory (unpublished data). Cells were washed three times in a rinsing solution of (per l) 15.00 g NaCl, 0.54 g KCl, 0.60 g $NaHCO_3$, 0.50 g glucose, 0.10 g galactose, 0.10 g trehalose, and resuspended in the culture medium JL-ODRP-4 (Appendix). This medium was chemically defined and thus allowed for examination of attachment and spreading without interaction from unknown medium components (i.e., fetal bovine serum). All cells were cultured in a humid chamber at 25° C.

Substrate evaluation. Seven types of substrates were tested in these experiments. Commercially available 24-well plates (Falcon Biocoat™, Becton Dickinson, Bedford, MA) were purchased precoated with the following attachment factors: collagen I, collagen IV, fibronectin, laminin, and poly-D-lysine. Also used were uncoated 24-well plates from Falcon® (Becton Dickinson, Bedford, MA) and Corning® (Corning Inc., Corning, NY). All plates were made of polystyrene and were tissue-culture treated (treated to reduce the negative charges associated with polystyrene).

Estimation of cell number. Cell number was estimated by the Cell Titer 96™ AQ_{ueous} Cell Proliferation Assay (Amersham Pharmacia Biotech, Piscataway, NJ), modified for use with 24-well plates. This kit contained the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), and an electron-coupling agent, phenazine methosulfate (PMS). In solution with metabolically active (viable) cells, MTS (in the presence of PMS) is reduced to a soluble formazan dye by mitochondrial dehydrogenases. The amount of reduction of MTS can be measured as an increase in absorbance at a wavelength of 490 nm. A relationship between cell number and total metabolic activity is assumed to estimate cell number. This technique will be referred to as the MTS assay throughout this paper. Other studies have used a similar technique to estimate cell number (Buttke et al., 1993; Berg et al., 1994). In this study, MTS assays were performed on cells in 500-µl volumes of medium in 24-well plates with MTS at 333 µg/ml final concentration and PMS at 25 µ*M* final concentration. After a 6-h incubation, a 300-µl sample was transferred from each well to a 96-well assay plate (Corning Inc., Corning, NY). Absorbance of each well was measured with a microtiter plate reader (Dynatech, Chantilly, VA) at 490 nm.

The validity and sensitivity of this technique was demonstrated by our preparing serial dilutions of ventricle cells in 500-µl volumes in 24-well plates and assaying these cells with the MTS assay. Also, interaction of the assay with the substrates used in this study was tested in 500-µl of medium (without cells) in wells coated with substrates used in this study.

Cell viability assay. Eight wells of each substrate were seeded with 1×10^6 cells in 500 µl of medium, and the effect on cell viability was assayed at 24 h and 5 d. An MTS assay was performed to examine any change in total cell number after incubation with each substrate. Wells without cells were prepared with medium, MTS, and PMS to serve as blanks. After 5 d, the remaining four wells were assayed identically to the initial sample. This experiment was repeated three times.

Cell attachment assay. Eight wells of each substrate were seeded with 1×10^6 cells in 500 µl of medium, and effects on cell attachment assayed at 24 h and 5 d. After 24 h, four wells of each plate were washed and assayed for cell attachment. Each well was washed three times with 500 µl of medium, and 500 µl of fresh medium was added to the attached cells remaining in the well. These cells were examined for cell spreading (see below). The cells and medium washed from the wells were centrifuged at 200 ×g for 10 min, and the supernatant was removed. The cells were resuspended in 500 µl of fresh

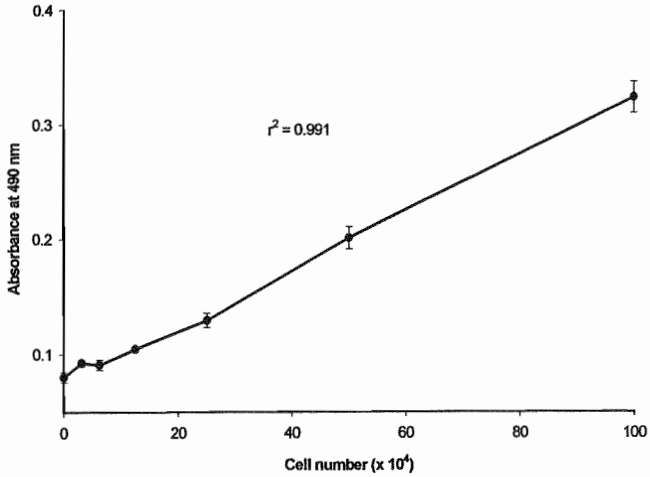

FIG. 1. Relationship of absorbance at 490 nm to number of *Crassostrea virginica* ventricle cells after MTS assay.

medium and transferred to a new 24-well plate. An MTS assay was performed to estimate the number of cells remaining attached in a well and the number of cells washed from a well. Wells without cells were prepared with medium, MTS, and PMS to serve as blanks. After 5 d, the remaining four wells were assayed identically to the 24 h sample. This experiment was repeated three times.

Cell spreading assay. Assessment of the percentage of spreading cells in each well was performed after washing and before the cell attachment assay. Two hundred cells from each well were examined at 200× with phase-contrast microscopy. Cells were classified as "spread" or "not-spread." Spread cells exhibited flattening and extension of the cytoplasm. Cells that were not spread remained spheroid. Representative portions of each well were photographed with color slide film (ASA 200). The slides were examined for assessment of morphological features of attached cells. All wells examined for cell attachment were also examined for cell spreading. This experiment was also repeated three times.

Statistical analysis. Data were analyzed with the General Linear Models Procedure of SAS version 6.12 (SAS Inc., Cary, NC). We removed variability associated with repetition of each experiment by incorporating each repetition as a block. Separately, the effect of plate type on cell viability, number of cells remaining attached in a well, number of cells washed from a well, and percentage of cells spreading in a well were each tested by one-way analysis of variance (ANOVA). A Tukey's studentized range test was used to separate means post hoc. A significance level of $P < 0.05$ was used in all statistical analyses.

RESULTS

Estimation of cell number. The MTS assay provided a linear estimate of cell number and had a resolution of $\sim 1 \times 10^5$ cells (Fig. 1). The assay proved to be objective, reproducible, and consistent. Testing of all attachment factors with the MTS and PMS reagents revealed no interactions (data not shown).

Cell viability assay. Significant effects on cell viability ($P < 0.0001$) were detected among substrates at 24 h. Cell number in wells coated with poly-D-lysine (mean absorbance at 490 nm: $A_{490} = 0.343$) was significantly greater than in all other treatments. Cell number in wells coated with collagen I ($A_{490} = 0.288$) was significantly lower than in other treatments. All other treatments, including the uncoated Falcon® substrate ($A_{490} = 0.329$), were not significantly different from each other and formed a group between these two values (Table 2).

TABLE 2

THE EFFECT OF SUBSTRATE ON VIABILITY OF *CRASSOSTREA VIRGINICA* VENTRICLE CELLS AFTER 24 H AND 5 D[a]

Substrate	24 h	5 d
Collagen I	0.288 ± 0.025 x	0.358 ± 0.030 w
Collagen IV	0.329 ± 0.019 y	0.375 ± 0.015 y
Fibronectin	0.330 ± 0.036 y	0.367 ± 0.031 x
Laminin	0.328 ± 0.029 y	0.366 ± 0.010 x
Poly-D-lysine	0.344 ± 0.034 z	0.349 ± 0.040 v
Uncoated Corning®	0.323 ± 0.025 y	0.384 ± 0.015 z
Uncoated Falcon®	0.329 ± 0.020 y	0.367 ± 0.036 x

[a]Absorbance values (mean ± standard deviation) at 490 nm after MTS assay for cell metabolic activity. Cells were incubated in 24-well plates on the substrates listed. Higher values indicate greater cell numbers or increased cell metabolic activity. Values were averaged from three repeated experiments with four replicate wells of each treatment within each experiment. Values sharing letters within columns were not significantly different ($P > 0.05$).

Significant effects on cell viability ($P < 0.0001$) were detected among substrates at 5 d as well. Cell number on the uncoated Corning® substrate ($A_{490} = 0.384$) was significantly greater than in all other treatments. Cell number in wells coated with poly-D-lysine ($A_{490} = 0.349$) was significantly lower than in all other treatments. The other treatments formed three statistical groupings between these two values (Table 2).

Cell attachment assay. Cell attachment was influenced by attachment factors at 24 h. Significantly more cells ($P < 0.0001$) remained attached in the presence of poly-D-lysine ($A_{490} = 0.329$) than for all other treatments. Significantly fewer cells remained attached in the presence of collagen I ($A_{490} = 0.082$). Cell attachment on the uncoated Falcon® substrate ($A_{490} = 0.304$) was not significantly different from attachment on fibronectin ($A_{490} = 0.288$), and lower only than attachment on poly-D-lysine (Fig. 2).

The number of cells washed from a well at 24 h was influenced by substrate ($P < 0.0001$), and was inversely related to the results for cell attachment at 24 h (Fig. 2). Significantly more cells were washed from wells coated with collagen I ($A_{490} = 0.234$). Significantly fewer cells were washed from wells coated with poly-D-lysine ($A_{490} = 0.041$). The number of cells washed from the uncoated Falcon® substrate ($A_{490} = 0.086$) was not significantly different from the number of cells washed from fibronectin-coated wells, and fewer cells were washed only from poly-D-lysine-coated wells (Fig. 2).

Cell attachment was influenced by attachment factors at 5 d ($P < 0.0001$); however, cell attachment among treatments was different from that of the 24-h sample. Significantly more cells remained attached to the uncoated Corning substrate ($A_{490} = 0.224$) than all other treatments. Significantly fewer cells remained attached in the presence of collagen I ($A_{490} = 0.131$). Cell attachment on the uncoated Falcon® substrate ($A_{490} = 0.195$) was not significantly different from attachment on poly-D-lysine or laminin, and lower only than attachment on the uncoated Corning® substrate (Fig. 3).

The number of cells washed from a well at 5 d was influenced by substrate ($P < 0.0001$), and was inversely related to the results for cell attachment at 5 d (Fig. 3). Significantly more cells were washed from wells coated with collagen I ($A_{490} = 0.248$) or collagen IV ($A_{490} = 0.246$). Significantly fewer cells were washed from wells with the uncoated Corning® substrate ($A_{490} = 0.199$) or poly-D-lysine (A_{490}

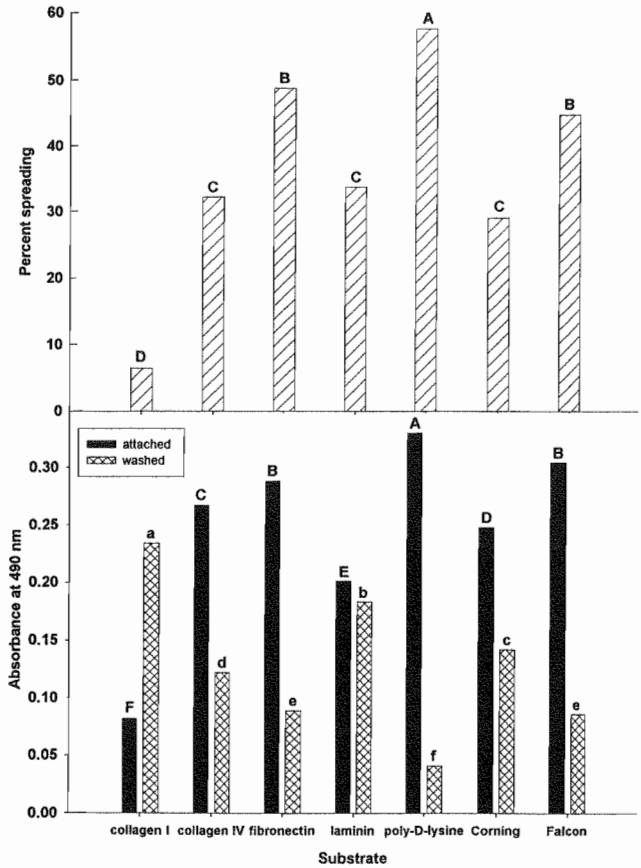

FIG. 2. *Top panel:* Effect of substrate on percent spreading of *Crassostrea virginica* ventricle cells in 24-well plates at 24 h. Columns sharing letters were not significantly different ($P > 0.05$). *Bottom panel:* Effect of substrate on attachment of *C. virginica* ventricle cells in 24-well plates at 24 h. Absorbance at 490 nm is correlated with cell number. Black columns indicate number of cells remaining attached to substrate after washing, and hatched columns indicate number of cells washed from a substrate. Within a column type (black or hatched), columns sharing letters were not significantly different ($P > 0.05$). The substrates "Corning®" and "Falcon®" refer to uncoated plates. All other substrates refer to coatings on Falcon® plates.

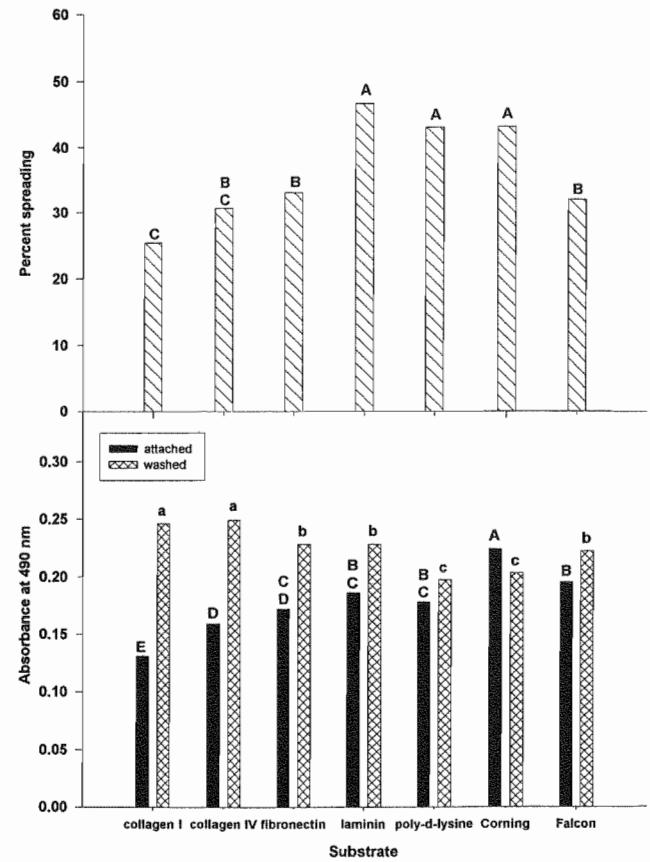

FIG. 3. *Top panel:* Effect of substrate on percent spreading of *Crassostrea virginica* ventricle cells at 5 d. Columns sharing letters were not significantly different ($P > 0.05$). *Bottom panel:* Effect of substrate on attachment of *C. virginica* ventricle cells in 24-well plates at 5 d. Absorbance at 490 nm is correlated with cell number. Black columns indicate number of cells remaining attached to substrate after washing, and hatched columns indicate number of cells washed from a substrate. Within a column type (black or hatched), columns sharing letters were not significantly different ($P > 0.05$). The substrates "Corning®" and "Falcon®" refer to uncoated plates. All other substrates refer to coatings on Falcon® plates.

= 0.196). All other treatments including the uncoated Falcon® substrate (A_{490} = 0.221) were not significantly different from each other and formed a group between these two groups (Fig. 3).

Cell spreading assay. Significant differences ($P < 0.0001$) in the percentage of spreading cells were detected among attachment factors at 24 h. A significantly higher percentage of cells (58.6%) spread in the presence of poly-D-lysine than for all other attachment factors. Collagen I (6.4%) was less effective than the other treatments at enhancing cell spreading. Cell spreading on the uncoated Falcon substrate (44.7%) was not significantly different from that of fibronectin (48.7%). Cell spreading on these substrates was lower only than the spreading on poly-D-lysine (Fig. 2). Generally, higher spreading was associated with higher attachment.

Significant differences ($P < 0.0001$) in the percentage of spreading cells were detected among attachment factors at 5 d as well. A significantly higher percentage of cells were spread in the presence of laminin (46.4%), the uncoated Corning® substrate (42.9%), or poly-D-lysine (42.8%). Collagen I (25.3%) was significantly less effective than all other treatments at enhancing cell spreading. The percentage of cells spreading on all other substrates, including the uncoated Falcon® substrate (31.8%), formed one statistical grouping between these values (Fig. 3). Generally, higher spreading was associated with higher attachment.

DISCUSSION

Attachment and spreading of cells in vitro can increase cell function, metabolism, and gene expression (Folkman and Moscona, 1978; Ben-Ze'ev et al., 1980), all of which are important to effectively utilize cell culture. To our knowledge, this is the first report to quantify effects of substrate on bivalve cell attachment and spreading. In this study, poly-D-lysine promoted cell attachment and spreading better than any other attachment factor. Poly-D-lysine is a highly positively charged molecule. Coating wells with this synthetic polymer has been used to improve the adhesive properties of many cell types by allowing the negatively charged cells to attach to and interact with the

well bottom (Freshney, 1994). This may allow the cells to produce an extracellular matrix and facilitate attachment and spreading. Polylysines of less than 100 kDa have been found to be toxic to bivalve cells, but larger polylysines promoted attachment (Odinstava et al., 1994). The molecular weight of the poly-D-lysine used in this study was 100 to 150 kDa. Poly-D-lysine has also been reported to enhance attachment and spreading in primary cultures from the oysters *Ostrea edulis* (Renault et al., 1995), and *Crassostrea gigas* (Le Deuff et al., 1994). Interestingly, poly-D-lysine was reported to be of no benefit to *C. virginica* primary cultures, and even toxic to the clam *Mya arenaria* cultures (Hetrick et al., 1981). However, the molecular weight of the poly-D-lysine used in these studies was not reported.

Cells on poly-D-lysine appeared to have a higher viability than other cells at 24 h. As no evidence of rapid cell proliferation on poly-D-lysine or cell death on other substrates was observed, this is most likely an artifact of the MTS assay. This assay estimates cell number based on metabolic rate. It is known that attachment and spreading can increase the levels of metabolism in cell culture. For bivalve cells, it has been demonstrated that substrate can cause increased rates of mRNA synthesis (Odinstava et al., 1994). Because wells with poly-D-lysine had more attached and spread cells than did other substrates, the higher metabolic activity of these cells could have caused an increased MTS assay value.

It should be noted that this MTS artifact did not affect the cell attachment assay. For this assay, MTS data were collected for cells remaining attached in a well and for cells washed from a well. Any effect of substrate on metabolic activity would be detected as incongruous results between the two data sets. Data collected on attached and washed cells revealed the expected inverse relationship, implying that attached cells had similar metabolic rates regardless of substrate, and likewise for suspended cells. These data were also supported by the cell spreading assay. This supports observations on cell attachment based on MTS data.

The results for attachment, spreading, and viability differed between 24 h and 5 d for poly-D-lysine and other treatments. Generally, after 5 d, treatments with initially high MTS values (at 24 h) became lower and treatments with low MTS values became higher. This can be explained by considering the MTS assay and medium composition. Because wells with high initial attachment also had cells with higher metabolic activity, nutrients in the defined medium may have been depleted more rapidly, thus lowering attachment and metabolic activity within the well over time. Wells with higher initial levels of attachment and spreading would be affected the most (e.g., poly-D-lysine), and wells with low initial attachment and spreading would be affected the least (e.g., collagen I).

Collagen I was the only substrate to have more cells washed from a well than remained attached at 24 h. Unlike poly-D-lysine, collagen I depends on interaction of the factor with cell membrane receptors to facilitate attachment and spreading. Coating the plate with collagen I reduced the positive charge of the surface, and *C. virginica* cells apparently did not recognize this molecule effectively. This is surprising in that a collagen I-like molecule has been isolated and characterized from the extracellular matrix secreted by hemocytes of the oyster *Pinctada fucata* (Suzuki and Funakoshi, 1992). There may be differences between the bivalve and mammalian forms of this molecule.

Viability on collagen I appeared to be lower than in other treatments. Similar to the case for poly-D-lysine, this result was potentially an artifact of the MTS assay. Because cells on collagen I were inhibited from attaching and spreading more than with other treatments, the metabolic activity of these cells was probably lower than on other substrates. It should be noted that on occasion, patches of attached and spread cells could be found in the collagen I wells. This may be attributed to uneven coating of the well with collagen I, allowing cells access to the uncoated Falcon® surface.

There were few discernable trends among the other substrates tested. No attachment factor tested, other than poly-D-lysine, induced attachment and spreading better than uncoated Falcon® wells (the control treatment), although fibronectin (at 24 h) and laminin (at 5 d) performed comparably. It is interesting to note that there were differences in attachment and spreading between the uncoated Falcon® and Corning® plates. As both plate types were tissue-culture-treated by the manufacturer to impart a slight positive charge, it may be possible that different treatments by the manufacturer affected the ability to induce cell attachment and spreading. The factors fibronectin, collagen IV, and laminin are similar to collagen I in depending on interaction of cell membrane receptors with the attachment factors. The fact that uncoated tissue-culture-treated plates performed as well or better than these attachment factors indicates that charge may play an important role in the attachment and spreading of oyster cells, especially if these cells are not able to recognize traditional attachment factors well. Except for poly-D-lysine, all of the factors used in this study were isolated from mammalian sources but have been effective for a wide variety of cell types (Freshney, 1994). Because poly-D-lysine is a synthetic polymer primarily used to impart a positive charge, it does not specifically react with cell membrane receptors. However, given the variability in the response of other bivalve cells to attachment factors (Table 1), there may be different optimal factors for each cell type and organism. Poly-D-lysine appears to be useful as a general factor to enhance attachment. Optimization will probably involve the isolation and use of attachment factors derived from bivalves.

Recently, several attachment factors have been isolated from bivalves, including a fibronectin-like molecule from the oyster *P. fucata* (Suzuki and Funakoshi, 1992), a collagen I-like molecule from *P. fucata* (Suzuki et al., 1991), other types of collagen-like molecules and proteoglycans from *P. fucata* (Suzuki et al., 1991), a fibronectin-like molecule from the mussel *Mytilus galloprovincialis* (Panara et al., 1996), and an adhesive plaque protein from *M. galloprovincialis* (Inoue et al., 1995). Future work in improving bivalve cell attachment should involve further screening of different factors, including some of bivalve origin. For example, hemocytes could be used to produce an extracellular matrix upon which primary cell cultures could be initiated.

This study indicates that substrate can influence attachment and spreading of oyster cells. We found an effect of duration of incubation, which we attributed to medium depletion rather than substrate toxicity. We found poly-D-lysine promoted cell attachment and cell spreading best. Combinations of commercially available attachment factors may prove to be more effective than the use of single factors and should be tested. Different cell types from different tissues must also be examined. Finally, improvements in dissociation techniques and medium composition must occur along with improvements in cell attachment and spreading for the establishment of bivalve cell lines to be realized.

Appendix

Composition of Medium JL-ODRP-4

Medium JL-ODRP-4 is a chemically defined medium developed for the cultivation of oyster cells in vitro. It was developed from a medium designed for the cultivation of *Perkinsus marinus* (La Peyre and Faisal, 1997), a parasite of *C. virginica*. Though proven to maintain oyster cells, this medium is still under development, and further improvements are needed for the long-term propagation of oyster cells. All chemicals used in medium were of tissue-culture grade and from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. A balanced salt solution was prepared by dissolving (per l) 0.48 g $CaCl_2$-$2H_2O$, 1.09 g $MgSO_4$, 1.64 g $MgCl_2$-$6H_2O$, 0.23 g KCl, 8.71 g NaCl, 0.35 g $NaHCO_3$, 0.02 g Na_2HPO_4 in tissue-culture grade water to a final volume of 909 ml. This solution was filter sterilized (0.22 μm). The following components were added under sterile conditions to produce 1 L of medium:

1) 1 ml of a solution containing (per ml) 0.834 mg ferrous sulfate and 0.143 mg zinc sulfate

2) 1 ml of a solution containing 0.249 mg cupric sulfate per ml

3) 10 ml of minimal essential medium (MEM) amino acids solution without glutamine (GIBCO BRL, Gaithersburg, MD)

4) 10 ml of MEM nonessential amino acids solution (GIBCO BRL)

5) 10 ml of a solution containing (per ml) 10 mg alanine, 5 mg glycine, 5 mg serine, 15 mg taurine, and 5 mg glutamine

6) 6 ml of RPMI 1640 vitamin solution

7) 10 ml of a solution containing (per ml) 50 mg glucose, 10 mg trehalose, and 10 mg galactose

8) 10 ml of chemically defined lipid concentrate (GIBCO BRL)

9) 2 ml of a solution containing (per l) 0.5 mg each of adenosine 5′-monophosphate, cytidine 5′-monophosphate, uridine 5′-triphosphate, and coenzyme A

10) 10 ml of a solution containing 0.5 mg chloramphenicol per ml

11) 10 ml of 100× selenium, pyruvate, insulin, transferrin (SPIT) solution

12) 1 ml of a solution containing 16 mg soybean trypsin inhibitor per ml

13) 10 ml *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer.

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