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LG4-5 domains of laminin-2 binds α-dystroglycan to allow myotube attachment and prevent anoikis

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Abstract

Poly(2-hydroxyethyl methacrylate) (PolyHEMA) prevents cell attachment was used here to study anoikis, the process where cells die when unattached or attached to an inappropriate matrix, in mouse C_2C_{12} myotubes. A method was developed to efficiently embed proteins into PolyHEMA and the effect on cultured myotubes was determined. Myotubes grown on PolyHEMA-coated plates fail to attach to the surface and remain as rounded, suspended cells, undergo dramatic increases in apoptosis and necrosis, and the number of viable cells decreases,. Incorporation of merosin (laminin-211) or the short laminin globular (LG4-5) modules of the laminin α2 chain C-terminus (called 2E3) that binds α-dystroglycan diminishes both apoptosis and necrosis and increases viability while bovine serum albumin had a much lesser effect, showing the specificity of this effect for these matrix proteins. One sarcolemma receptor for laminin-binding is α-dystroglycan. An antibody which binds α-dystroglycan but which does not block laminin-binding (VIA4) had little effect on apoptosis or viability on merosin or 2E3 embedded plates while another antibody (IIH6) which specifically blocks binding dramatically decreased viability and increased apoptosis. When merosin or 2E3 are added to culture media rather than embedded on plates these can also increase viability and decrease apoptosis even though the cells remain in suspension, though the effect is not as great as found for the embedded proteins where the cells attach. Thus, we conclude that the binding of a small LG4-5 modules of laminin-211 to α-dystroglycan is important in preventing anoikis and that attachment plus binding is necessary for maximal cell survival.

Introduction

The extracellular matrix includes both loose connective tissue and basement membrane. The basement membrane is a sheet-like structure that is fashioned from collagen and laminin bilayered networks that are positioned under epithelial and endothelial cells (Ghohestani et al., 2001; Timpl and Brown, 1996; Tisi et al., 2000; Tzu and Marinkovich, 2008). The role of the basement membrane is to affix the epithelium to the loose connective tissue via cell - matrix adhesions, and is present surrounding the sarcomere. Several aspects of cell phenotype including gene expression, differentiation and proliferation are regulated by binding to the extracellular matrix (Adams and Watt, 1993; Blau and Baltimore, 1991; Ingber, 1993). Therefore, binding of adherent cells to the extracellular matrix is critical for cellular development and the stabilization of tissue structures (Frisch and Francis, 1994). The laminins, a component of the ECM, are a family of large (≥ 800 kDa) heterotrimeric (α, β and γ) multidomain glycoproteins with each domain containing different structures and functions. Currently, five α, three β and three γ chains have been identified that assemble into 12 different laminin isoforms (Aumailley et al., 2005; Aumailley and Smyth, 1998; Iivanainen et al., 1999; Koch et al., 1999; Meinen et al., 2007; Miner and Yurchenco, 2004; Tisi et al., 2000).

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Laminin-211 (merosin) has been found in the ECM of muscle and provides a critical link where it binds integrins and α-dystroglycan, which in turn link to the cytoskeleton (Helbling-Leclerc et al., 1995; Henry and Campbell, 1999; Meinen et al., 2007; Shibuya et al., 2003; Tisi et al., 2000; Tzu and Marinkovich, 2008). The rod-like and globular domains of laminin-211 are arranged in a cruciform structure with all three chains (α, β and γ) contributing to the α-helical coiled-coil structure that give rise to the long arm of the cruciform (Beck et al., 1990; Colognato and Yurchenco, 2000; Tisi et al., 2000; Yurchenco and Cheng, 1993). The three short arms of the cruciform are formed from globular domains located at the N-terminus of each chain. These N-terminal arms of the cruciform have been implicated in the Ca^{2+}-dependent polygonal polymerization of laminin at the cellular membrane (Miner and Yurchenco, 2004; Yurchenco and Cheng, 1993). At the C-terminus of the α chain there are five tandem laminin globular (LG) domains, labeled as LG1-LG5, that can bind integrin and α-dystroglycan (Tzu and Marinkovich, 2008). The LG1 – LG3 domains of laminin-211 have been shown to bind to integrins α7β1 and α6β1 (Smirnov et al., 2002; Zhou et al., 2006). The LG4-LG5 pair of laminin-111 or -211 can be proteolytically cleaved from the rest of laminin to yield a fragment termed E3. The E3 region of laminin-211 will be referred to as 2E3. Recombinant expression of these fragments in mammalian cells aided the structure-function studies of 2E3 and allowed for precise mapping of the binding sites of these fragments to heparin, sulfolipids and α-dystroglycan on cell membranes (Andac et al., 1999; Mayer et al., 1993; Michele and Campbell, 2003; Talts et al., 1999; Talts et al., 1998; Timpl et al., 2000). Furthermore, studies have shown that the LG4 – LG5 domains bind with α-dystroglycan at acidic polysaccharide chains on α-dystroglycan (Sasaki et al., 2004; Tisi et al., 2000; Zhou et al., 2006). Thus, 2E3 has the binding sites that allow it to bind with membrane constituents, such as α-dystroglycan, but does not contain the domains for the Ca^{2+}-dependent polygonal polymerization.

An important aspect of multicellularity is that non-transformed, adherence-dependent cells will proliferate and differentiate when attached in the correct ECM environment. However, if located in an inappropriate environment they will remove themselves from the population through apoptosis. Apoptosis of this type has been coined anoikis (Greek: *homelessness*) by Frisch *et. al.* (Frisch and Francis, 1994) and is a response induced by either a loss of cell anchorage, anchorage to inappropriate ECM proteins or disrupted cell adhesion to the extracellular matrix. In order for cells to sense if they are in an appropriate attachment environment they use cell – ECM and cell – cell interactions for their determination (Ingber, 2003a; Ingber, 2003b; Katsumi et al., 2004). The ECM not only provides for discrete cellular anchorage points but also mediates important cell survival signals. Interaction between the ECM and cell surface receptors leads to the formation of an adhesion-dependent signaling scaffold consisting of a number of adaptor proteins and kinases that activate pathways involved in cell proliferation, differentiation or survival (Giancotti, 2000; Gilmore, 2005; Hynes, 2002). Consequently, decreasing the interaction between the ECM and their cell surface receptors by changing the physicochemical surface characteristics of the culture substrate would therefore alter cellular adherence, morphology and survival.

Studies related to anoikis have been conducted *in vitro* by culturing cells on vessels coated with poly (2-hydroxyethyl methacrylate) (PolyHEMA) (Bretland et al., 2001). PolyHEMA is a hydrogel that has biomedical potential due to its ability to form a three-dimensional, hydrophilic polymeric network, with a soft and rubbery consistency, low interfacial tension, which closely resembles the ECM environment (Kudela, 1976; Lombello et al., 2002; Seidel and Malmonge, 2000). However, the high degree of hydophilicity and non-ionic nature of PolyHEMA are factors that contribute to a poor substrate for binding secreted ECM proteins that are positively charged; thereby, decreasing the ability of cells to adhere to PolyHEMA coated surfaces. Thus, anoikis studies have utilized PolyHEMA coated culture vessels since tissue culture plastic adhesivity can be greatly reduced by the application of PolyHEMA to the surface (Folkman and Moscona, 1978). Furthermore, adhesivity can be partially restored by

the permanent inclusion of ECM proteins within the hydrogel matrix (Carbonetto et al., 1982). As a result of being able to incorporate ECM proteins in the PolyHEMA matrix the effects of these proteins on cellular adherence and subsequent survival can be better defined.

Two methods for generating PolyHEMA hydrogels are investigated for their ability to stably incorporate proteins within the hydrogel. The method described by Carbonetto et al. polymerizes 2-hydroxyethyl methacrylate (HEMA) monomers through free radical-directed polymerization (Carbonetto et al., 1982). This method has the advantage of being able to stably incorporate protein within the hydrogel. Also, cultured cells were able to adhere to the incorporated proteins indicating that these ECM proteins were displayed on the surface of the hydrogel in a sufficiently native form. The use of free radical generators in this method is a disadvantage because of the possibility of free radical damage to the incorporated protein that would decrease their ability to bind adhesion receptors on the cell. Another disadvantage of this method is incomplete polymerization of monomeric HEMA since monomeric HEMA is highly cytotoxic. An alternative method was later described by Frisch and Francis in which poly(2-hydroxyethyl methacrylate) (PolyHEMA) was solubilized in ethanol (Frisch and Francis, 1994). The ethanolic solution of PolyHEMA produced a polymeric film that remained tightly associated with the plastic culture surface following the evaporation of the alcohol. This method has the advantage of starting with polymeric HEMA and would have relatively no cytotoxic monomer present. A disadvantage of this method is that it has not been used to incorporate proteins and the possible denaturation of the protein in the ethanol if this were attempted.

This article reports that anoikis can be induced in anchorage-dependent C_2C_{12} cells through the disruption of cell-matrix interactions by the application of an ethanolic solution of the hydrogel PolyHEMA that forms a non-adhesive film on the cell culture plastic following the evaporation of the alcohol. Furthermore, the role of laminin-211 in the regulation of anoikis in C_2C_{12} cells was investigated by selectively incorporating merosin (laminin- 211/221) or 2E3, the LG4-LG5 domains of laminin-211, within the hydrogel. By selectively introducing this ECM protein into the PolyHEMA hydrogel, or its respective fragment, their role in cellular adherence and survival can be better understood.

Materials and Methods

Poly(2-hydroxyethyl methacrylate) (PolyHEMA), 2-hydroxyethyl methacrylate (monomeric HEMA) and Trypsin-EDTA solution (1x) were purchased from Sigma Scientific. Micro BCA protein assay kit was purchased from Pierce. Dulbecco's Modification of Eagle's Medium (DMEM) was purchased from Cellgro. The BD Annexin V-FITC apoptosis detection kit was purchased from BD Bioscience (Bedford, MA). Phosphate buffered saline (PBS) was purchased from Hyclone. The semi-pure rat merosin was isolated by the method described by Cheng et al. (Cheng et al., 1997). The expression and subsequent purification of recombinant 2E3 was conducted using the method described by Zhou et al. (Zhou et al., 2006) from hLNA2-E3 293 (HEK293) cells transfected with the LG4-5 domains of laminin α2. The hLNA2-E3 293 (HEK293) cells were a kind gift from Professor Jan F. Talts of Lund University (Lund, Sweden). The α dystroglycan specific antibodies IIH6 and VIA4-1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Preparation of PolyHEMA and HEMAp solutions and incorporating protein within the hydrogels

To prepare the poly (2-hydroxethyl methacrylate) (PolyHEMA) solution, 2.4 g of PolyHEMA was dissolved in 20 ml of 95% (v/v) ethanol in a 50 ml sterile conical tube with constant mixing at 65 °C for 8 hours. Dilutions of the PolyHEMA solution were made using 95% (v/v) ethanol. Protein incorporation throughout the investigation was conducted at a 1:10 ratio of protein

solution in 0.22-μm cellulose acetate filter-sterilized PBS to PolyHEMA solution due to increased hydro-bead formation and decreased transparency of the hydrogel at higher ratios.

The 2-Hydroxyethyl methacrylate (HEMA) monomer was polymerized (HEMAp) as previously described by Civerchia-Perez, et al. (1980) inside a biological safety cabinet. Briefly, the polymerization consisted of sequential addition of 1 ml of HEMA monomer, 1 ml of ethylene glycol, 1 ml of protein solution in sterile PBS, 0.1 ml of a 6% (w/v) solution of ammonium persulfate and 0.1 ml of a 12% (w/v) solution of sodium metabisulfite in a sterile 15-ml conical tube.

The PolyHEMA and HEMAp solutions were added at 0.1 ml aliquots for 96-well plates, 0.5 ml aliquots for 24 well plates and 2 ml aliquots for 6-well plates within a biological safety cabinet and left to dry at 22° C into a transparent film on the polystyrene. The hydrogels were UV-sterilized for 6 hours within the biological safety cabinet and then rinsed three times with sterile PBS in a 1 hour period in order to rehydrate the hydrogels prior to use. These sterilization techniques proved effective since no microbial contamination occurred in any experiment.

The PolyHEMA and HEMAp films were inspected via light microscopy at 400x magnification for any surface defects. Any hydrogel films that were observed to have excessive amounts of air bubbles within the hydrogel or incomplete coating of the polystyrene surface were excluded from the study.

In order to determine which hydrogel stably incorporated proteins with greater efficacy, BSA (2 μg/well) was mixed into both hydrogels. Hydrogels were lightly rinsed with sterile PBS once to rehydrate the hydrogel (−PBS) or vigorously rinsed three times with sterile PBS (+PBS) prior to determining protein concentration. Embedded protein concentrations for each hydrogel were determined comparing it to a standard curve of BSA on the same plate. The sodium metabisulfite was found to interfere with the microBCA Protein Assay kit (Pierce) therefore the Bradford method was utilized (Bradford, 1976).

Optimization of protein incorporation within PolyHEMA was conducted by altering the protein incubation method. These alterations included changing temperature, time and agitation of the solution. PolyHEMA (24 μg/ml) was added into six 15 ml conical tubes at 5 ml aliquots. The tubes were divided into three pairs and pre-incubated at 4° C, 22° C or 37° C for 1 hour. BSA was added at a concentration of 40 μg/ml per tube and mixed. The six PolyHEMA – BSA solutions were then incubated at these temperatures with or without agitation. From these conical tubes, 0.1 ml aliquots were taken at times specified and added to the wells of a 96 well plate. The alcohol in the PolyHEMA solution was allowed to evaporate in a biological hood, UV-sterilized and the hydrogels were rinsed three times with sterile PBS. Protein concentrations were determined using the Micro BCA Protein Assay kit per manufacturer's protocol.

When merosin, 2E3 or BSA were incorporated within PolyHEMA in subsequent anoikis studies, the ethanolic polyHEMA solution used to coat the wells contained 2 μM of that protein assuming merosin (800 kDa.), 2E3 (50 kDa.) and BSA (67 kDa.). In Supplemental Data is presented a Coomassie Blue stained SDS polyacrylamide gel of the merosin and 2E3 used. The gel shows that the proteins are of high purity. The merosin appears as a large aggregate, which occurs with laminin when Ca^{2+} is not thoroughly removed and when SDS denaturation is not prolonged.

C_2C_{12} mouse myogenic cell culture and differentiation

Initially, C_2C_{12} mouse myoblasts were cultured in 100 mm polystyrene cell culture plates with Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% (v/v) heat-

inactivated fetal bovine serum (FBS) in a NuAire IR Autoflow CO_2 Water-Jacketed incubator set at 37° C with 5% CO_2 and 95% atmospheric air. Differentiation of C_2C_{12} myoblasts into myotubes was initiated by rinsing the myoblasts twice with sterile PBS and then culturing the cells in DMEM supplemented with 1% (v/v) heat-inactivated FBS for five days and then harvesting the cells for the experiments. Dissociation of myotubes from the polystyrene plates was by the addition of 3 ml trypsin-EDTA solution (0.5 g porcine trypsin and 0.2 g EDTA in 100 ml Hank's buffered saline solution) at 37° C for 5 minutes. The trypsin reaction was inhibited by adding 3 ml of DMEM supplemented with 10% FBS. The cells were then centrifuged at 500 × g and the pellet rinsed with sterile PBS. Cell densities were determined by the method of Freshney (Freshney, 1987) and cells passaged onto PolyHEMA coated (± embedded protein) or uncoated wells. C_2C_{12} myoblasts were seeded at densities of 2×10^3 cells / well in 96-well plates, 5×10^4 cells/well for 24 well plates and 2.5×10^5 cells / well for 6 well plates.

Determination of cell mitochondrial activity using the MTS cytotoxicity assay

Mitochondrial activity was determined using the CellTiter AQueous One (MTS) solution as per manufacturer's protocol. Briefly, a 1:100 dilution of MTS solution in the cell culture was generated and allowed to incubate for 2 hours at 37 °C in a humidified incubator as suggested by the manufacturer. Dehydrogenase activity in the mitochondria of metabolically active cells converts the tetrazolium salt to formazan inducing a colorimetric change that was detected at 490 nm using a TECAN Infinite M200 multi-well plate reader.

Flow cytometry of C_2C_{12} myotubes

The cells were assessed for viability, necrosis and apoptosis using the BD Annexin V-FITC apoptosis detection kit as per manufacturer's protocol. Briefly, the cell pellets were resuspended in 50 μl of 1x Annexin Binding Buffer and 5 μl of Annexin VFITC solution, 5 μl of propidium iodide (250 μg/ml) solution were mixed and incubated for 15 minutes in the dark at 4° C. The solution was then diluted by an additional 440 μl of 1x Annexin Binding Buffer to achieve a final volume of 500 μl to minimize cell aggregation. Cells were passed through a 70 μm nylon Cell Strainer (BD Biosciences) using a 1000 μl pipette tip to further minimize cell aggregates. Aliquots of 50 μl aliquots were analyzed on a Beckman Coulter CellLab Quanta SC flow cytometer. Cells that were not consistent in electronic volume or side scatter with a standard myotube culture or apoptotic cultures treated with 4 μM camptothecin were excluded from analysis to avoid analyzing cell aggregates and cell fragments. Exclusion of lysed cells was confirmed by the absence of cell counts following incubation of a myotube culture with RIPA buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml Sigma Protease Inhibitor Cocktail, 1 mM sodium orthovanadate and 1 mM sodium fluoride) for 15 minutes at 4° C and then staining with Annexin V-FITC and propidium iodide solutions.

Cells negative for both Annexin V-FITC and propidium iodide were labeled as viable cells. Cells positive for propidium iodide only were labeled as necrotic. Cells positive for Annexin V-FITC only were labeled as apoptotic. However, late apoptotic and late necrotic cells would stain positive for both Annexin V and propidium iodide. Because cells had been gated according to electronic volume and side scatter, late necrotic cells that would stain positive for both dyes would have been excluded from analysis due to increased cell volume prior to cell lysis and increased side scatter following cell lysis. Therefore, cells that stained positive for both Annexin V-FITC and propidium iodide were considered to be undergoing secondary necrosis (late apoptosis) and were included in the apoptotic population.

α-Dystroglycan antibodies

C_2C_{12} myotubes (5×10^4) were trypsinized and pre-incubated with α-dystroglycan specific mouse monoclonal antibodies, IIH6 and VIA4-1, at a dilution of 1:500 each in DMEM

supplemented with 1% FBS and allowed to incubate for 15 minutes prior to seeding onto 24-well plates coated with 24 μg/ml PolyHEMA incorporated with merosin or 2E3. Myotubes were cultured for 24 hours and the suspension and adherent cells for each treatment were pooled and analyzed. Differences in cell number and percent viable and percent apoptotic cells were determined by flow cytometry using the BD Annexin V-FITC apoptosis detection kit.

Anoikis studies using soluble proteins

C_2C_{12} myotubes were trypsinized and seeded at 2.5×10^5 cells/well into 6 well plates coated with 24 μg/ml PolyHEMA and cultured for 48 hours in DMEM culture media supplemented with 1% FBS and 2 μM of merosin, 2E3 or BSA in solution. Trypsinization of these cells was necessary to dissociate and exclude the laminin bound to the adhesion receptors prior to passaging them into the PolyHEMA coated wells (Zhou et al., 2006). PolyHEMA coated wells were used because merosin, in solution, is unable to bind to the 24 μg/ml PolyHEMA surface even after a 72 hour incubation period (data not shown). Cultures were grown for 48 hours and differences in cell number and percentage of viable and apoptotic cells were determined by flow cytometry and compared to myotubes cultured on 24 μg/ml PolyHEMA alone.

Statistical analyses

All descriptive statistics were conducted using Microsoft Excel (Microsoft Corporation, WA) and graphed using GraphPad Prism v4.0 (GraphPad Software, Inc., CA). All data are represented as mean ± standard deviation. Comparisons between two groups were conducted by two-tailed unpaired t-tests. One-Way ANOVA followed by Tukey's post-hoc analysis was conducted for comparisons between multiple data sets using GraphPad Prism v4.0 (GraphPad Software, Inc., CA). Statistical significance was set at $p < 0.05$ for all assays.

Results

In order to conduct our studies of anoikis several parameters first had to be established. These parameters were: 1) determine which of the two previously published methods for incorporating protein in the PolyHEMA hydrogel would achieve greater results, 2) determine what concentration of the hydrogel would affect cell viability and 3) determine what concentration of PolyHEMA would most effectively incorporate protein.

Embedding proteins in PolyHEMA and Optimization of PolyHEMA concentration

First, the method that would stably incorporate the highest amount of BSA within the hydrogel was investigated. Fig. 1A represents the amount of BSA that was retained by either method following the addition of PBS to allow the hydrogels to hydrate. These data show that HEMAp retained 0.49 ± 0.04 μg of BSA (24.5% of initial amount of BSA), which is significantly ($p < 0.05$) greater than the 0.34 ± 0.05 μg of BSA (17.2% of initial) retained with PolyHEMA. Vigorously rinsing the hydrogels with PBS removed loosely bound protein from the hydrogels. This revealed that PolyHEMA had stably incorporated 0.21 ± 0.02 μg of BSA (10.5% of initial), which was significantly ($p < 0.05$) greater than the 0.05 ± 0.04 μg of BSA (2.5% of initial) stably incorporated in HEMAp. PolyHEMA stably incorporated BSA at a higher percentage than HEMAp and was used in all subsequent studies.

Next, the minimal concentration of PolyHEMA needed to significantly decrease cell survival in C_2C_{12} mouse myotubes was investigated. This was conducted by increasing the concentration of PolyHEMA (12 – 120 μg/ml), passaging C_2C_{12} myotubes (2×10^3 cells/well) and culturing the cells for 48 hours. Fig. 1B shows that 24 μg/ml of PolyHEMA reduced mitochondrial activity in C_2C_{12} cells with no further significant decreases following this concentration of PolyHEMA. Also, this concentration of PolyHEMA incorporated the highest amount of protein within the hydrogel as shown in Figure 1 Panel C. Because 24 μg/ml of

PolyHEMA could effectively incorporate protein and reduce mitochondrial activity in these cells this concentration of PolyHEMA was utilized for all subsequent investigations.

The optimization of protein incorporation within the PolyHEMA hydrogel was investigated by altering time, temperature and mixing conditions prior to coating plates. The results are shown in Fig. 1D. The best incorporation occurred upon 1 h, 37°C incubation with agitation. Since this condition resulted in the highest amount of stably incorporated BSA within the hydrogel it was used throughout the rest of the study in order to incorporate proteins within the hydrogel.

Myotube anoikis on PolyHEMA

In order to determine if C_2C_{12} cells could be made to undergo apoptosis the cells were cultured on polystyrene, PolyHEMA or 1% (w/v) agarose. Culturing adherent cells on agarose-coated plates is a well-utilized method that blocks cellular adherence and increases apoptosis. Fig. 2 reveals that 24 μg/ml PolyHEMA significantly reduces cell mitochondrial activity compared to cells cultured on polystyrene. The reduction in mitochondrial activity was associated with culturing cells on PolyHEMA and is similar to that observed for cells cultured on agarose. The results show that culturing cells on ethanol treated wells does not significantly decrease the mitochondrial activity of cells compared to cells cultured on polystyrene alone. Cells were then cultured on wells coated with 0.12 μg/ml monomeric HEMA or 120 μg/ml PolyHEMA in order to determine if the reduction in mitochondrial activity was due to monomer HEMA-induced cytotoxicity. Culturing cells on 0.12 μg/ml monomeric HEMA treated wells significantly decreased cell mitochondrial activity compared to any of the other substrates indicating high cytotoxicity of the monomeric HEMA solution. Next, the 5-fold increase in the concentration of PolyHEMA did not significantly decrease mitochondrial activity compared to cells cultured on 24 μg/ml PolyHEMA. These data indicate that the reduction in mitochondrial activity observed for PolyHEMA cultured cells was not due to either ethanol or monomer HEMA cytotoxicity when employing the ethanolic solution of PolyHEMA. The decreased mitochondrial activity was similar to that of cells cultured on agarose. This suggests that loss of cell adherence and subsequent cell death are likely reasons for the observed decrease in mitochondrial activity. PolyHEMA is a well-defined polymer that allows for incorporation of protein within the hydrogel and is therefore deemed preferable to agarose as a model system to test for anoikis in this study.

PolyHEMA reduces cell adherence and viability while embedded proteins ameliorate

Figure 3 shows that culturing myotubes on 24 μg/ml PolyHEMA significantly reduced both the number of cells and percent viable cells compared to cells cultured on polystyrene. The reduction in percent viable cells coincided with a significant increase in percent apoptotic and necrotic cells. Fig. 3 also shows the morphology of the myotubes cultured on polystyrene or PolyHEMA. The cells cultured on polystyrene display a normal, adherent morphology while cells cultured on PolyHEMA are spherical in morphology, consistent with the morphology of suspension cells. The cells cultured on PolyHEMA were loosely associated with the surface of the PolyHEMA, which was revealed after lightly tapping the plate and observing cells dissociate from the substrate through an inverted microscope. These results show that 24 μg/ml PolyHEMA reduces cell adherence and cell viability and increases apoptosis.

Merosin is an integral component of the ECM surrounding muscle tissue and is important for the adherence of muscle tissue to the basal lamina. Figure 4 shows that incorporating merosin within PolyHEMA significantly increases cell number, and decreases apoptosis and necrosis compared to PolyHEMA cultured cells. Incorporating 2E3 within PolyHEMA also significantly decreases apoptosis and increases cell number compared to PolyHEMA cultured cells but the effects of 2E3 were significantly less compared to cells cultured on PolyHEMA

with merosin. The incorporation of BSA, a non-ECM protein, within PolyHEMA was also investigated. The incorporation of BSA within PolyHEMA resulted in a significant difference in cell number, percent viable and percent apoptotic cells compared to cells cultured on PolyHEMA incorporated with merosin or 2E3. These data indicate that the differences in cell number and cell survival observed with PolyHEMA incorporated with merosin or 2E3 are mediated by cell adhesion receptor binding. Digital images of cells cultured on each substrate are shown in Fig. 5. Cells cultured on PolyHEMA (Fig. 5A) developed a spherical morphology consistent with cells that have lost adherence to the substrate due to the poor adhesive property of PolyHEMA. Upon incorporating either merosin (Fig. 5B) or 2E3 (Fig. 5C) in the PolyHEMA the cells re-establish an adherent-like morphology but was not completely identical to the morphology of cells cultured on polystyrene (Figure 3). The morphology of cells cultured on PolyHEMA incorporated with BSA (Figure 5D) is similar to that of PolyHEMA alone. These data reveal that PolyHEMA disrupts cell adherence, incorporation of a non-ECM protein (BSA) within the hydrogel is insufficient to disrupt the non-adhesive property of PolyHEMA, and incorporation of merosin and 2E3 within PolyHEMA allows cells to attach to the substrate and restores cell survival.

The role of α-dystroglycan in anoikis

We next examined if the merosin and 2E3 embedded in PolyHEMA was binding to α-dystroglycan, a component of the dystrophin glycoprotein complex (DGC) and a known cell surface adhesion receptor of laminin-211. Two α-dystroglycan specific antibodies, IIH6 and VIA4-1, were used to dissect the role of α-dystroglycan in binding to merosin or 2E3. Figure 6 shows that pre-incubating cells with IIH6 prior to culturing on PolyHEMA with merosin significantly reduced cell viability and number and increased apoptosis compared to cells cultured on PolyHEMA with merosin alone or when pre-incubated with VIA4-1. Cells pre-incubated with IIH6 and then cultured on PolyHEMA embedded with 2E3 also exhibited a significant reduction in cell viability and increased apoptosis compared to cells cultured on PolyHEMA with 2E3 alone or with VIA4-1. These results indicate that α-dystroglycan plays a role in merosin and 2E3 associated cellular adherence and the subsequent reduction in apoptosis. However, VIA4-1 was not without effect. While VIA4-1 gave the same viability and apoptosis as the no antibody control, it did significantly decrease cell number. On merosin, it decreased cell number though not as much as IIH6. On 2E3, it decreased cell number by the same amount as IIH6. This would be accounted for if the VIA4-1 antibody is loosening attachment enough so that cell recovery for counting is affected but allowing sufficient attachment so that apoptosis is avoided. While this effect is reproducible and significant, we currently have no explanation for it. Thus, since two different α-dystroglycan antibodies affect cell counts and the one known to block laminin-binding increases apoptosis and decreases cell viability, we conclude that α-dystroglycan serves a role in cell attachment and that attachment involves the LG4-5 modules of laminin-α2.

Attachment is required for the maximal effect

Culturing C_2C_{12} cells on PolyHEMA with protein in suspension is a model system that allows merosin or 2E3 to bind to the adhesion receptors on the myotubes but does not allow for cell adhesion on the PolyHEMA substrate. The results of this study are shown in Figure 7. The addition of merosin or 2E3 to the culture media significantly decreased the percentage of apoptotic cells by almost 45%; however, a 17% increase in necrotic cells attenuated the increase in cell viability to only 28% when compared to cells cultured on PolyHEMA alone or 2 μM BSA. The addition of 2 μM BSA to the myotubes resulted in a marginal increase (7%) in percent viable cells with no significant differences in percent apoptotic cells compared to polyHEMA cultured myotubes. There were no significant differences in cell numbers between any of the treatment groups. These results indicate that adhesion receptor binding to merosin or 2E3 is able to increase cell survival and ameliorate PolyHEMA associated apoptosis.

However, the level of amelioration is small compared to cells grown on PolyHEMA embedded with these proteins, which allows for cell adhesion. This indicates that while binding alone plays a role in cell survival, adhesion plays a greater role in regulating cell survival in myotubes.

Discussion

The results, as a whole, indicate that 24 μg/ml PolyHEMA decreases adherence of C_2C_{12} myotubes to the substrate and increases apoptosis. The PolyHEMA associated apoptosis is surmountable upon incorporating either merosin (laminin- 211/221) or the LG4-LG5 fragment of laminin-211, 2E3, within the PolyHEMA, which re-establishes cell anchorage. The cell surface adhesion receptor, α dystroglycan, is clearly involved in attachment and preventing apoptosis. Binding alone to merosin or 2E3 plays a minor but significant role in ameliorating the PolyHEMA-induced apoptosis while binding followed by adherence plays a greater role in myotube survival.

The MTS assay and flow cytometry revealed differences in the effect of PolyHEMA on cell survival. These differences can be explained by MTS cytotoxicity assay limitations. In the MTS assay a tetrazolium compound is reduced by mitochondrial activity to produce a colored formazan product that is soluble in cell culture medium and has an absorption maxima at 490 nm. This assay tests for differences in mitochondrial activity between mitochondrially active and mitochondrially inactive (necrotic) cells but it cannot distinguish between mitotically active, senescent and apoptotic cells within the culture. PolyHEMA increases apoptosis but not necrosis in these cells and the apoptotic cells would add to the production of formazan product within the assay. Therefore, the MTS assay would underestimate the negative effects of PolyHEMA on cell survival. Because of this limitation by the MTS assay all subsequent investigations regarding cell survival on PolyHEMA were done via flow cytometry.

PolyHEMA is used extensively in anoikis studies because it is able to reduce tissue culture plastic adhesivity and disrupts cell-matrix interactions. However, cell adhesion can be restored, to some extent, by the incorporation of ECM proteins within the hydrogel matrix (Carbonetto et al., 1982) Therefore, the initial goal was to determine the method that would stably incorporate protein within the hydrogel. The ethanolic solution of PolyHEMA was an easier, more consistent, method in forming a polymeric film on polystyrene that stably incorporated protein within the hydrogel. The PolyHEMA film coated on polystyrene was able to disrupt cell-matrix interactions and induce apoptosis in adherent-dependent C_2C_{12} mouse myogenic cells as indicated by changes in cellular morphology, cell number and increased apoptosis. This was consistent with the observations made by both Carbonetto and Frisch when they were conducting their anoikis studies using PolyHEMA (Carbonetto et al., 1982; Frisch and Francis, 1994). Cell number on PolyHEMA coated plates were not only reduced by apoptosis but possibly due to decreased proliferation of lingering myoblasts within the myotube culture. Frisch had suggested that the spherical morphology of non-adherent cells affected cell proliferation and cell number. Also, non-adherent cells formed spheroids that decreased cell proliferation through density dependent inhibition of growth as cells packed tightly together, thereby simulating confluency (Frisch and Francis, 1994). Folkman and Moscona observed that plating cells onto substrates with varying degrees of adhesiveness would affect the degree of DNA synthesis and this was directly correlated with cell flatness (Folkman and Moscona, 1978). Later, it was revealed that cell proliferation of adherent-dependent cells was directly related to cell shape. Cells were shown to proliferate when they were adherent and possessed a flat conformation but proliferation would gradually decline as cells lost adherence and possessed a more spherical morphology (Tucker et al., 1981). Moreover, protein synthesis, RNA synthesis and actin gene expression have all been shown to have strict cell shape – responsive metabolic controls (Aggeler et al., 1984; Ben-Ze'ev, 1984; Ben-Ze'ev et al., 1980; Benecke et al., 1978; Farmer et al., 1983; Wittelsberger et al., 1981). Therefore, changes in

morphology and apoptosis were associated with the loss of adherence and incorporating merosin or 2E3 within the hydrogel mitigated these changes.

PolyHEMA is a defined substrate that disrupts the deposition of extracellular matrix proteins on the surface of the hydrogel and can selectively incorporate merosin (laminin- 211/221) or the recombinantly expressed 2E3, the LG4-LG5 domains of the laminin α2 subunit. This allowed for investigation into the role of merosin on cellular adherence and apoptosis. Incorporating merosin within the hydrogel increased cellular adherence and cell number compared to PolyHEMA cultured cells. However, cell morphology was not identical to cells cultured on polystyrene. A plausible explanation for this difference is that a number of positively charged ECM proteins are synthesized by the cells and deposited on the negatively charged polystyrene surface. However, cells cultured on PolyHEMA incorporated with merosin or 2E3 would be limited to binding with only the properly oriented peptides in the PolyHEMA film. Also, laminin can form polygonal polymers that induce the aggregation of several transmembrane adhesion receptors, including α-dystroglycan (Barroso et al., 2008; Colognato et al., 1999; Colognato and Yurchenco, 2000; Yurchenco et al., 1992). These transmembrane adhesion receptors are tethered indirectly to actin and would aid in cytoskeletal reorganization, which can alter cell morphology. Incorporation of merosin within the hydrogel may hide much of this polymeric network within the hydrogel thereby affecting aggregation of the receptors and consequently cell morphology. Incorporation of 2E3 within PolyHEMA also increased cellular adherence and cell number compared to cells cultured on PolyHEMA alone, but was different compared to cells cultured on polystyrene. Cell number was also significantly reduced compared to those grown on PolyHEMA incorporated with merosin. Laminin polymerization and receptor clustering can also explain differences in cell morphology between polystyrene and PolyHEMA with incorporated merosin. 2E3 can bind to α-dystroglycan but cannot form polygonal polymers and has been shown to inhibit *in vitro* laminin polymerization and receptor clustering (Colognato et al., 1999). Therefore, incorporation of 2E3 within PolyHEMA would allow for increased cellular adherence by α dystroglycan binding but would not be able to form the polygonal polymers needed for adhesion receptor aggregation, which would consequently affect morphology.

The proteolytic fragment, 2E3, contains only the LG4 -5 domains of laminin-211 and binds α-dystroglycan but not integrins. Therefore, any positive cell survival effects observed from incorporating laminin-211 or 2E3 within PolyHEMA should be through binding to α dystroglycan, a component of the dystrophin glycoprotein complex. This hypothesis was investigated by pre-incubating C_2C_{12} myotubes with IIH6, an α-dystroglycan specific antibody that blocks laminin binding, or VIA4-1, an α dystroglycan specific antibody that does not directly block laminin binding. Given that merosin or 2E3 can be selectively incorporated within the PolyHEMA hydrogel and the properties of PolyHEMA prohibit deposition of ECM proteins on the surface of the hydrogel it can be concluded that α dystroglycan is binding to incorporated merosin and 2E3. Our data does not, however, rule out the possibility that other receptors, the integrins, are involved in preventing apoptosis.

From these studies it was shown that PolyHEMA could disrupt adherence in C_2C_{12} cells, which was associated with an increase in apoptosis. Since anoikis is defined as apoptosis following the loss of cell anchorage, these data indicate that myotubes undergo anoikis. Also, PolyHEMA serves as a model system for dissecting the role of ECM proteins in apoptosis of adherent-dependent cells since the hydrogel surface is non-adhesive and disrupts the deposition of extracellular matrix proteins from associating with the surface. The hydrogel lends itself to selective incorporation of proteins in order to investigate the roles of these ECM proteins in cellular adherence, morphology, differentiation and viability. This system was able to show that apoptosis in C_2C_{12} cells can be mitigated by laminin-211 through α dystroglycan binding *in vitro*. The differences in cell morphology and apoptosis between polystyrene and

PolyHEMA-laminin cultured cells begs the question of the importance of polygonal polymerization of laminin in the maintenance and cellular signaling within these cells and will be explored in subsequent investigations.

Smirnov, et al. concluded that cell attachment of C_2C_{12} myoblasts was via the LG1-3 modules of laminin-211 (Smirnov et al., 2002). Undifferentiated C_2C_{12} myoblasts do not have detectable levels of DGC proteins localized to the sarcolemma (Abdelmoity et al., 2000). These proteins have a perinuclear distribution during this stage of development (Trimarchi et al., 2006). Differentiation gradually alters the localization of the DGC proteins from the perinuclear region to the cytoplasm and finally to myotube tips, cytoskeleton and costameres (Abdelmoity et al., 2000; Belkin and Burridge, 1995; Blottner and Luck, 1998; Kostrominova and Tanzer, 1995; Noguchi et al., 2000). Our results were obtained with myotubes and used a different culturing protocol, and found that the isolated LG4-5 modules are also important for attachment. Clearly, myocyte attachment is likely a more complex phenomenon involving multiple receptors and laminin globular domains.

We conclude that one physiological role of the DGC, in muscle, is to prevent anoikis by binding the LG4-LG5 region of laminin and that this is also an important component of cell attachment.

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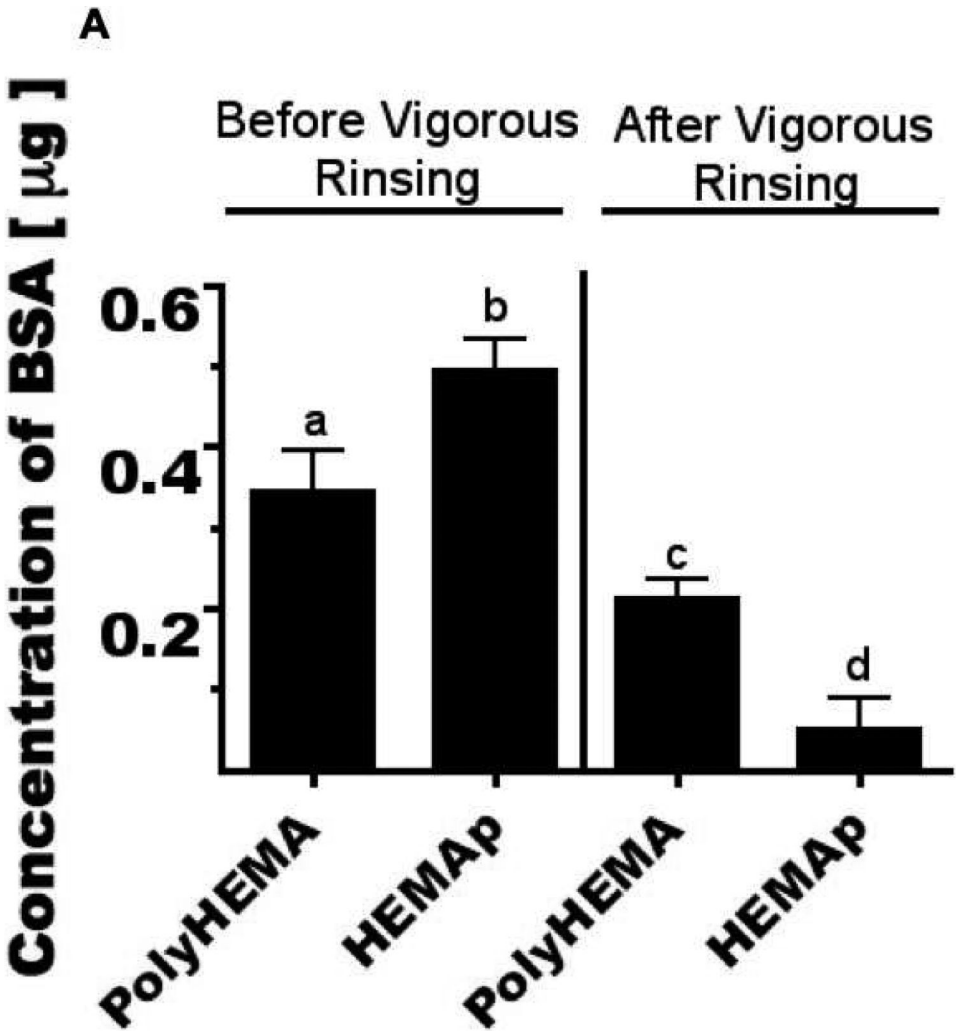

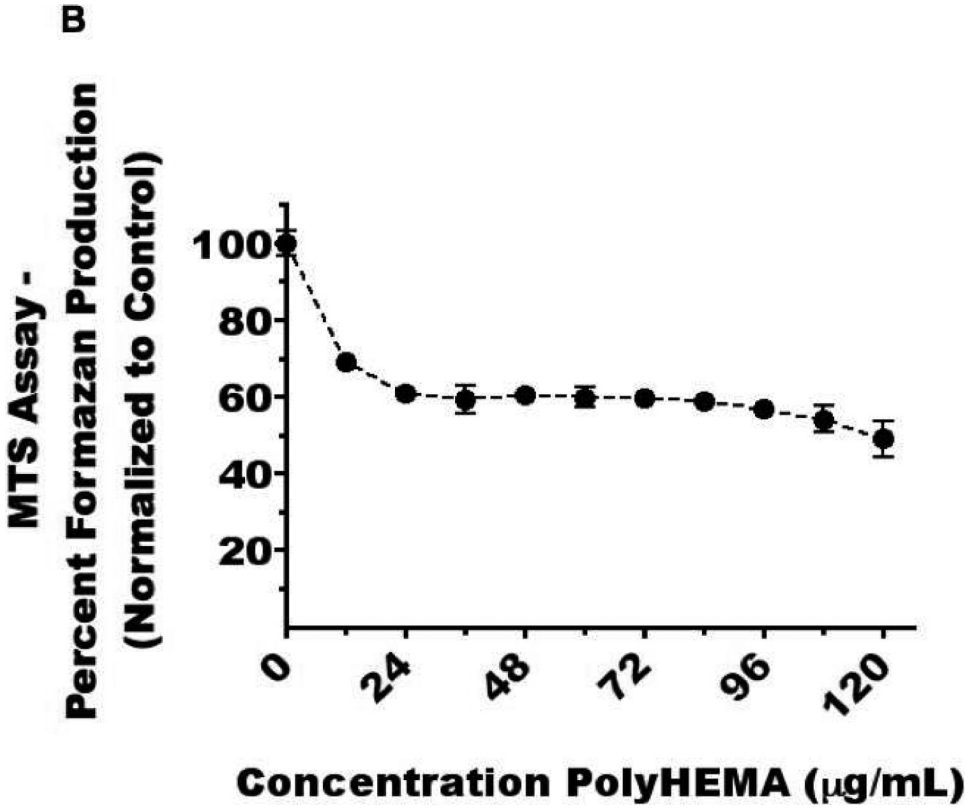

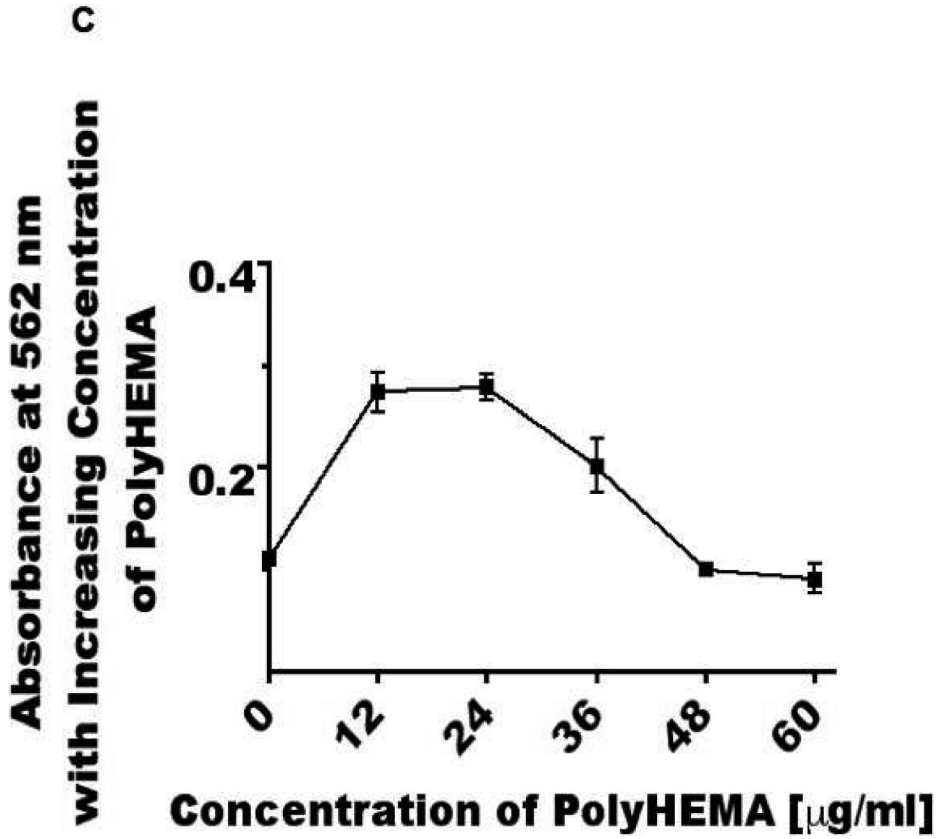

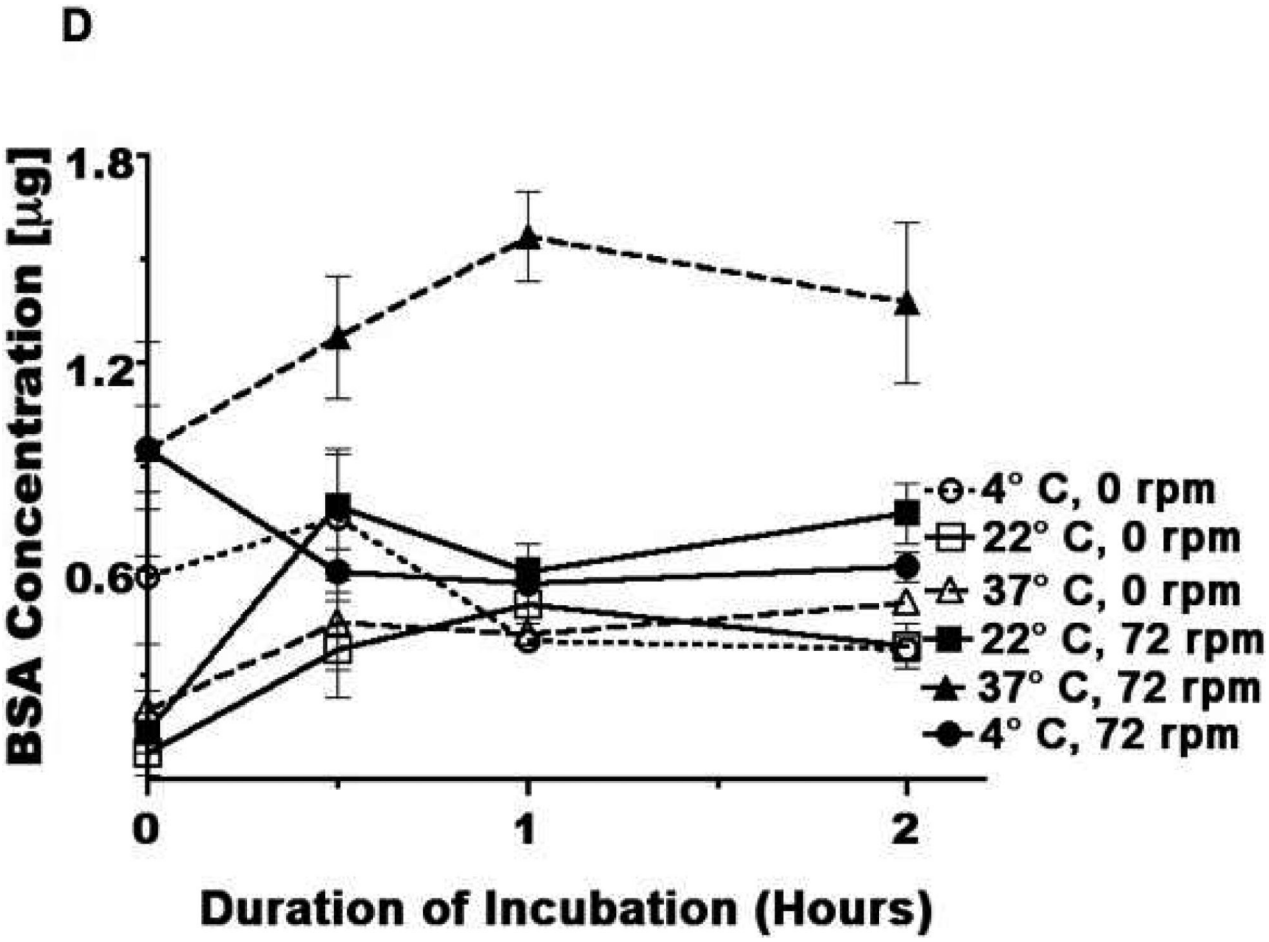

Figure 1.

Polymeric HEMA was investigated for its ability to stably incorporate proteins within the hydrogel and for its ability to prevent cell adhesion. **Panel A:** Polymeric HEMA (PolyHEMA; 24 μg/ml) was solubilized in 95% ethanol and the monomeric HEMA was chemically polymerized (HEMAp) as described in Methods. BSA (2 μg/well) was incorporated into both hydrogels and the solutions and a 96-well cell culture polystyrene plate coated. The final concentration of BSA incorporated before and after vigorous rinsing with PBS was determined. After rinsing, the PolyHEMA hydrogel stably incorporated 10.5% BSA while HEMAp only incorporated 2.5% of BSA. Bars with different letters (a-d) indicate a statistical difference ($p < 0.05$) between the means as revealed by One-Way ANOVA followed by Tukey's post-hoc analysis. **Panel B:** C_2C_{12} mouse myotubes (2×10^3) were cultured for 48 hours in a 96 well plate coated with increasing concentrations of PolyHEMA. Mitochondrial activity of metabolically active cells was determined using the MTS cytotoxicity assay and detecting the colorimetric change at 492 nm. Percent formazan production was determined by normalizing absorbances of each PolyHEMA concentration to the absorbance of the polystyrene culture. Mitochondrial activity declined the most with 24 μg/ml of PolyHEMA with no further significant decreases following this concentration. **Panel C:** Increasing concentrations of PolyHEMA (0 to 60 μg/ml) were mixed with 0.2 mg/ml BSA and added to a 96 well plate. The final concentration of BSA embedded in each PolyHEMA concentration was determined. The highest levels of BSA incorporation occurred at 12 μg/ml and 24 μg/ml concentrations of PolyHEMA. **Panel D:** BSA (4 μg/well) was mixed with 24 μg/ml PolyHEMA at the following conditions: 22° C with no agitation (□); 37° C with no agitation (△); 4° C with no agitation

(○); 22° C with 72 rpm agitation (■); 37° C with 72 rpm agitation (▲); 4°C with 72 rpm agitation (●). Mixing BSA with PolyHEMA at 37° C with 72 rpm agitation resulted in the highest (40%) incorporation of BSA within the hydrogel. Assays were conducted in triplicate and the data points and error bars represent mean ± standard deviation.

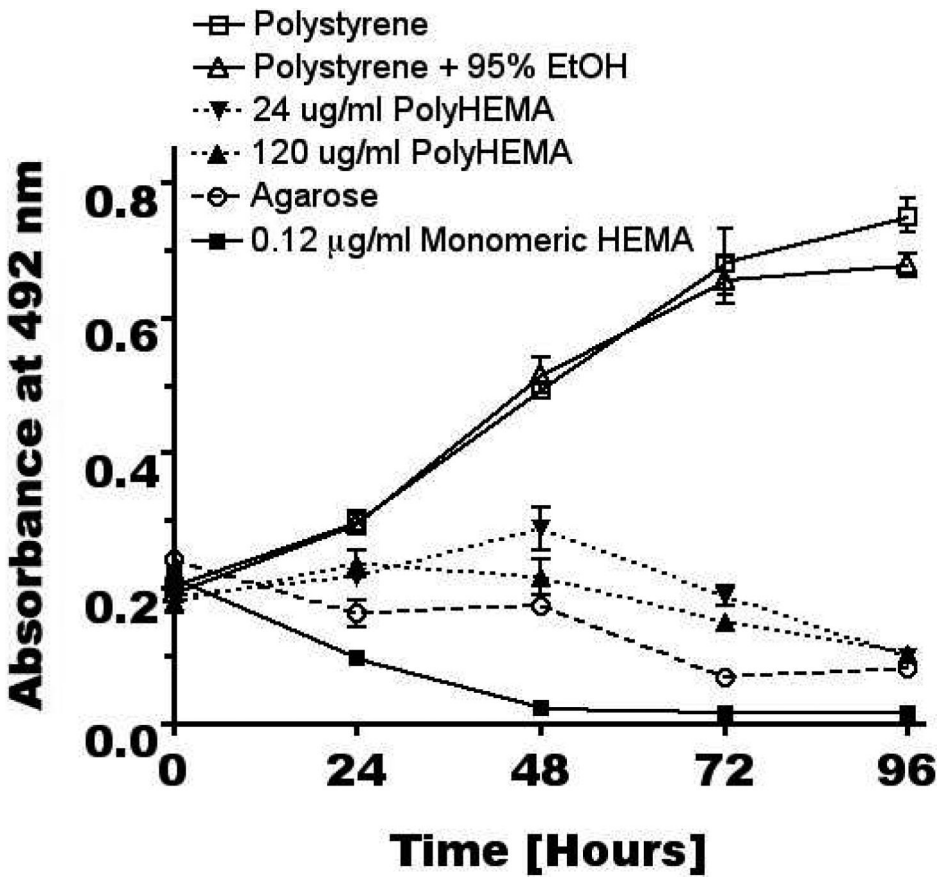

Figure 2.

C_2C_{12} mouse myotubes (2×10^3/well) were passaged into a 96 well plate. Cell culture substrates were: polystyrene (□); polystyrene treated with 95% (v/v) ethanol (□); 24 μg/ml PolyHEMA (▼); 120 μg/ml PolyHEMA (▲); 1% (w/v) agarose (○); or 0.12 μg/ml monomeric HEMA solution (■). Differences in mitochondrial activity, as determined by absorbance at 492 nm for each cell culture substrate, were assessed by the MTS cytotoxicity assay at different time intervals. The cultures were conducted in triplicate and the data points represent mean ± standard deviation of the absorbance at each incubation period.

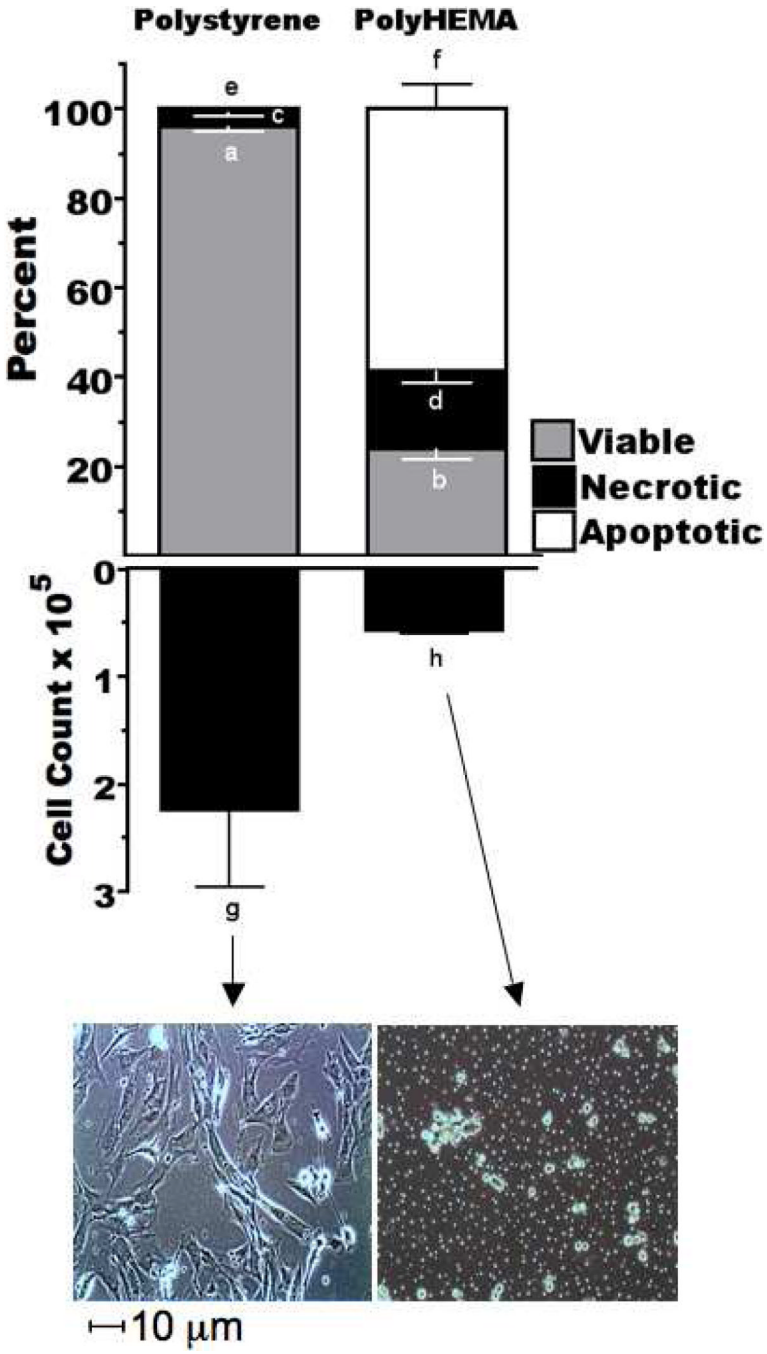

Figure 3.

C_2C_{12} myotubes (2.5×10^5 cells / well) were differentiated 5 days prior to use with DMEM supplemented with 1% (v/v) FBS for 48 hours at 37 °C with 5% CO_2 and 95% atmospheric air. The myotubes were passaged to PolyHEMA coated or uncoated wells of a 6-well plate and cultured for 48 hours. Adherent and suspension cells were collected, centrifuged, rinsed with PBS and resuspended in Annexin binding buffer prior to staining with propidium iodide and Annexin V-FITC. Stained cells were analyzed by flow cytometry to determine both cell number (inverted black bars) and the percentage of viable cells (grey bars), percentage necrotic cells (black bars) or percentage apoptotic cells (white bars) within each culture. The assays were conducted in triplicate and the bars represent the mean ± standard deviation. Bars with the

same color and orientation but with different letters (a-h) indicate a statistical difference ($p < 0.05$) between the means as revealed by a two-tailed Student's t-test. Digital images (100x) of C_2C_{12} myotubes cultured on polystyrene or 24 μg/ml PolyHEMA are shown. Polystyrene cultured cells exhibit normal myotube morphology while PolyHEMA cultured cells exhibit a spherical morphology with spheroid formation consistent with loss of substrate adhesion and greater emphasis on cell-cell adhesion. The bar on the micrographs depicts 10 μm.

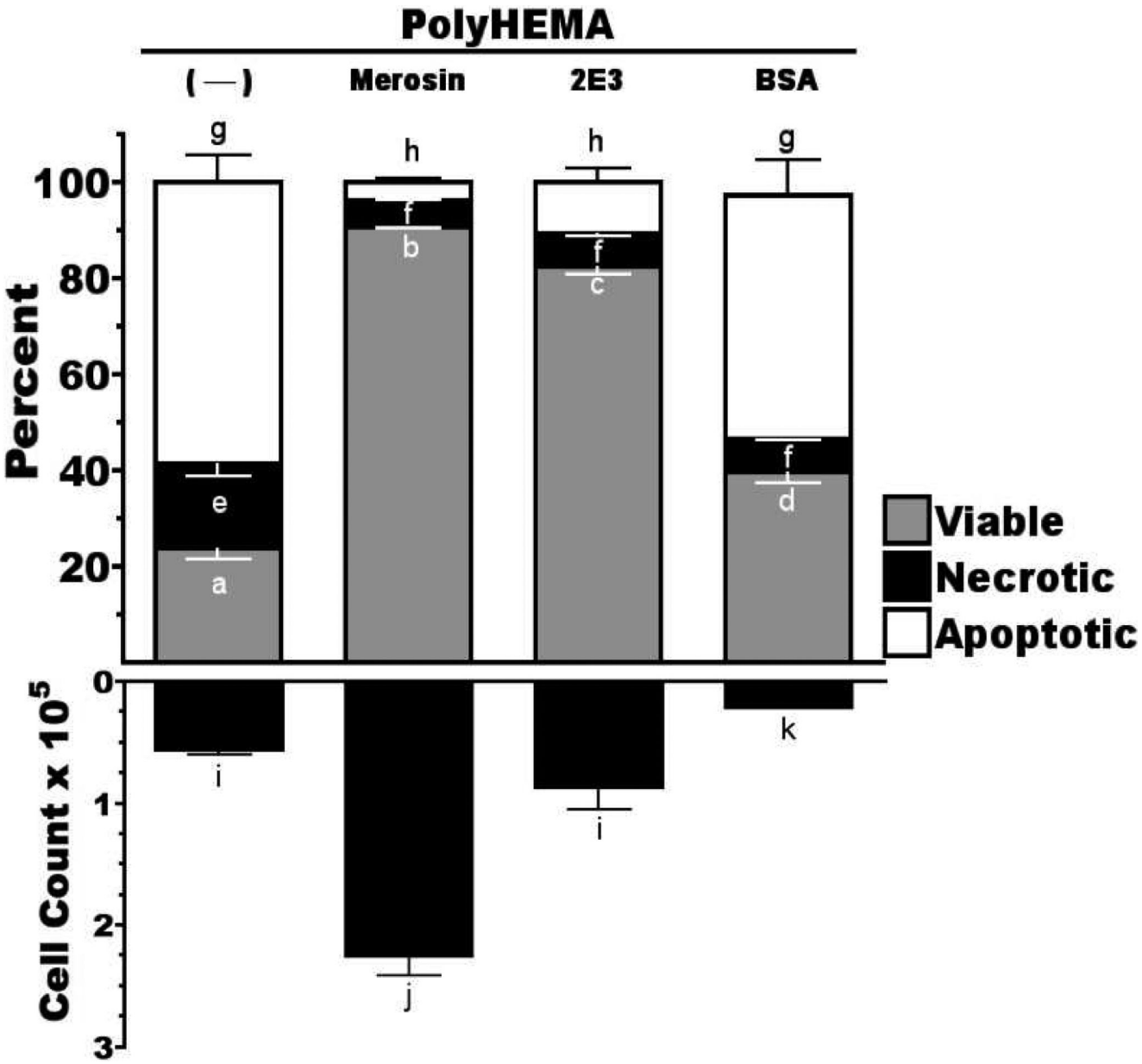

Figure 4.

Differentiated C_2C_{12} mouse myotubes (2.5×10^5 cells / well) were cultured on 24 μg/ml PolyHEMA coated wells (−) or on wells with 2 μM merosin, 2E3 or BSA incorporated in the PolyHEMA. Adherent and suspension cells were collected, centrifuged and rinsed with PBS. The cell pellets were resuspended in Annexin binding buffer prior to incubation with propidium iodide and Annexin V-FITC. Data analysis and presentation are as in Fig. 3 except in this case, statistical analysis between the means was by a One-Way ANOVA followed by Tukey's post-hoc analysis.

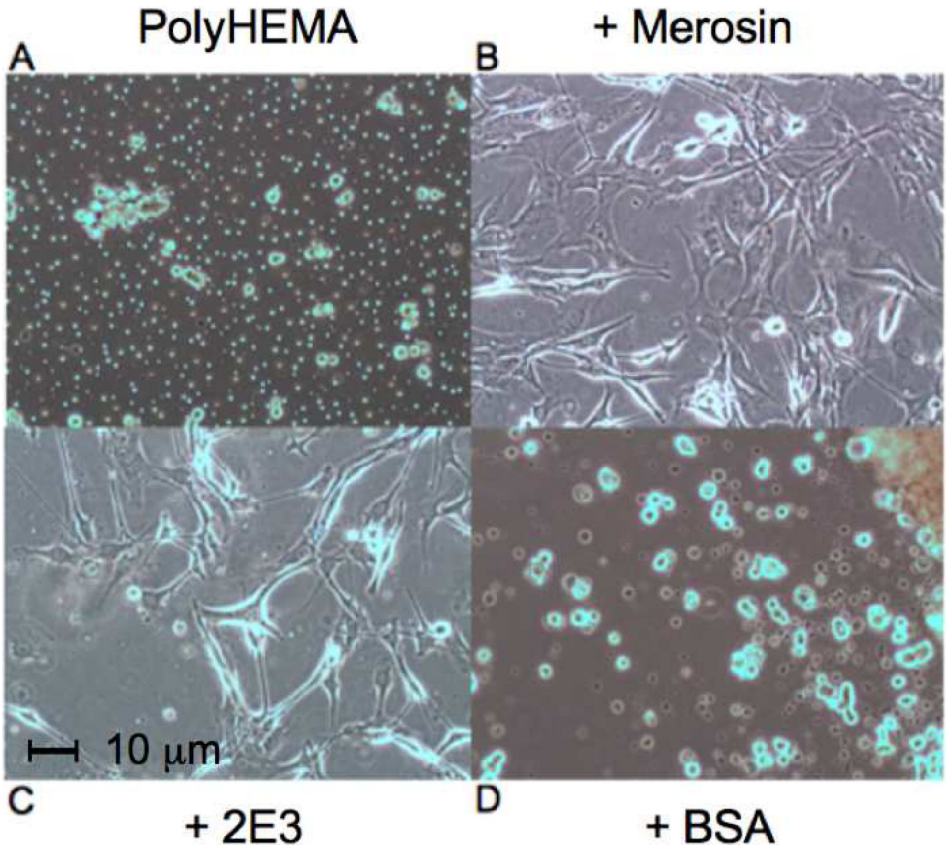

Figure 5.

The wells of a 6-well plate were coated with 2 ml of the following solutions: **A** 24 μg/ml PolyHEMA alone or the same PolyHEMA concentration containing 2 μM **B** Merosin; **C** 2E3; or **D** BSA. C_2C_{12} myotubes (2.5×10^5 cells / well) were cultured on the substrates for 48 hours. Digital images of cell morphology were captured at 100x magnification for each substrate. The bar on the micrograph depicts 10 μm.

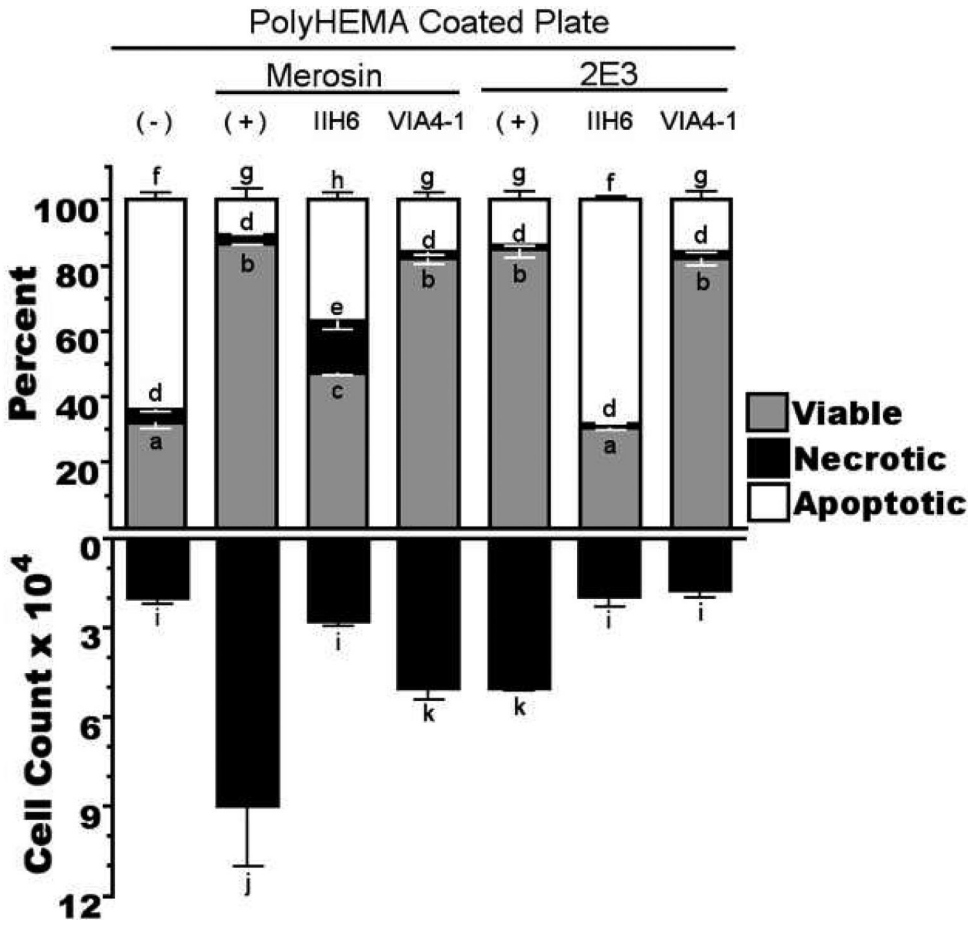

Figure 6.

The wells of a 24-well culture plate were coated with 2 ml of PolyHEMA without embedded protein (−) or PolyHEMA embedded with merosin (laminin-211/221) or 2E3 (+). C_2C_{12} mouse myotubes (5×10^4 cells/well) were trypsinized, rinsed with PBS and then pre-incubated with IIH6 (1:500), an α dystroglycan binding site blocking antibody, or VIA4-1 (1:500), an α dystroglycan antibody that does not directly block the laminin binding site. Cells were cultured for 24 hours and then dissociated from the substrates by trypsinization. The cells were rinsed with PBS and the cell pellets resuspended with Annexin binding buffer prior to staining with propidium iodide and Annexin V-FITC. Data analysis and presentation are as in Fig. 4.

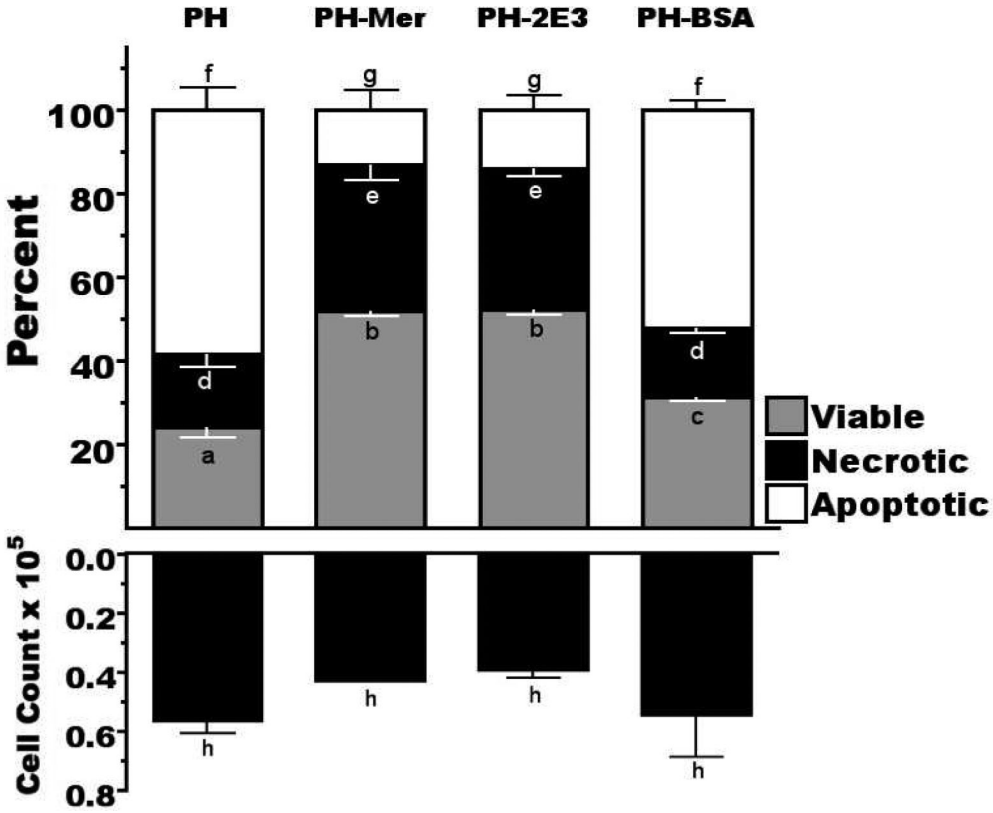

Figure 7.

The wells of a 6-well plate were coated with 2 ml of 24 μg/ml PolyHEMA. C_2C_{12} myotubes (2.5×10^5) were trypsinized and seeded into the 6-well culture in DMEM media supplemented with 1% FBS and containing 2 μM merosin, 2E3 or BSA. The cells were cultured for 48 hours prior to being stained by propidium iodide and Annexin V-FITC. Data analysis and presentation are as in Fig. 4.