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14. ABSTRACT The project objectives were to determine the composition and quantity of the extracellular matrix (ECM) that is deposited by the cells when placed in contact, either in vivo or in vitro, with electronic materials. In addition, a defined serum-free in vitro system developed in this laboratory was used to identify favorable and unfavorable materials, both biological and non-biological, that could be used to enhance interactions of cells and tissue with electronic components. Pure cultures of embryonic hippocampal neurons do not produce large areas of ECM spread over the coverslip. The cells deposit ECM proteins as a barrier to the inorganic DETA surface, possibly as a way to control their microenvironment, especially since the protein composition did not differ as a function of surface hydrophilicity or hydrophobicity. The neurons secrete laminin and collagen, but it is mostly concentrated around the soma and under the processes. In pure in vitro culture, the neurons do not secrete fibronectin, which seems to be the role of glial cells.					
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Project Objectives

- To determine the composition and quantity of the extracellular matrix (ECM) that is deposited by the cells when placed in contact, either *in vivo* or *in vitro*, with electronic materials.
- A defined serum-free *in vitro* system developed in this laboratory was used to identify favorable and unfavorable materials, both biological and non-biological, that could be used to enhance interactions of cells and tissue with electronic components.
- The primary goal of this project was to enable rapid integration of the research efforts to enable analysis of the ECM in real devices for use in operations-related systems.

Technical Approach

- Neurons were cultured on silicon dioxide coated with DETA & 13F.
- The neurons were characterized morphologically and immunocytochemically
- The surfaces were characterized both prior to and after cell culture, utilizing surface analytical methods including XPS and SIMS.
- Protein identification & quantification was accomplished using SDS-PAGE & Western blotting.
- Analysis was completed to correlate ECM amount and composition to cellular characteristics and surface composition in two model cultures:
 - Embryonic Hippocampal Cultures
 - Embryonic Dorsal Root Ganglion Cultures

Accomplishments/New Findings

- The cells cultured using the *in vitro* system reacts similarly to those in *in vivo* conditions; the cells secrete ECM proteins and interact.
- Pure cultures of embryonic hippocampal neurons do not produce large areas of ECM spread over the coverslip. The cells deposit ECM proteins as a barrier to the inorganic DETA surface, possibly as a way to control their microenvironment, especially since the protein composition did not differ as a function of surface hydrophilicity or hydrophobicity. The neurons secrete laminin and collagen, but it is mostly concentrated around the soma and under the processes. In pure *in vitro* culture, the neurons do not secrete fibronectin, which seems to be the role of glial cells.
- These results indicate the importance of a laminin matrix for hippocampal neuronal survival on a permissive surface such as DETA. An extensive network of ECM proteins does not cover the surface of the coverslip in hippocampal cultures, indicating that the primary function of the neuron is not the formation of an ECM network. In contrast, a much more extensive ECM network of laminin, collagen and fibronectin was present in the Dorsal Root Ganglion (DRG) co-culture at day 4 or 5 after culture.
- The quantity of protein produced in our *in vitro* system was as follows:
 - Laminin comprised 9.3 ($\pm$ 1.9) % of the protein deposited by the hippocampal cultures.
 - Fibronectin comprised 23.6 ($\pm$ 3.5) % of the protein deposited by the DRG cultures.

Technical Report is included as an Addendum that describes how to apply this research to wound healing.

Determining Extracellular Matrix Deposition Quantity and Composition from Cells in Response to Electronic Materials

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OUTLINE

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I. An Introduction to Extracellular Matrix (ECM) Dynamics in Neuronal/Electrical Neurointerfaces

There are many on-going efforts within the Department of Defense (DoD) to integrate non-biological systems with biological systems. These projects span the range from single cells to humans and many of these efforts are projected for operational integration within a 3 - 5 year time period. However, there are many fundamental issues that still need to be addressed such as the nature of the biological material at the interface of these two disparate systems. This research project addressed the question of the composition and quantity of the extracellular matrix (ECM) that is deposited by the cells when placed in contact, either *in vivo* or *in vitro*, with electronic materials. While much recent work has been devoted to fashioning and understanding communication between biological and non-biological systems, very little work has explicitly paid attention to the deposition of material by the cells in contact with these electronic materials.

This project applied surface analytical techniques in combination with biochemical and biological assays to both **quantify** and **identify** the materials deposited by neuronal cells, in response to various materials and conditions. We developed a defined, serum-free system in combination to identify favorable and unfavorable materials, both biological and non-biological, that could be used to enhance interactions of cells and tissue with electronic components. The primary goal of this project was to enable rapid integration of the research efforts listed above to enable analysis of the ECM in real devices for use in operations related systems.

III. The Importance of ECM Dynamics to DoD Neurointerface Objectives

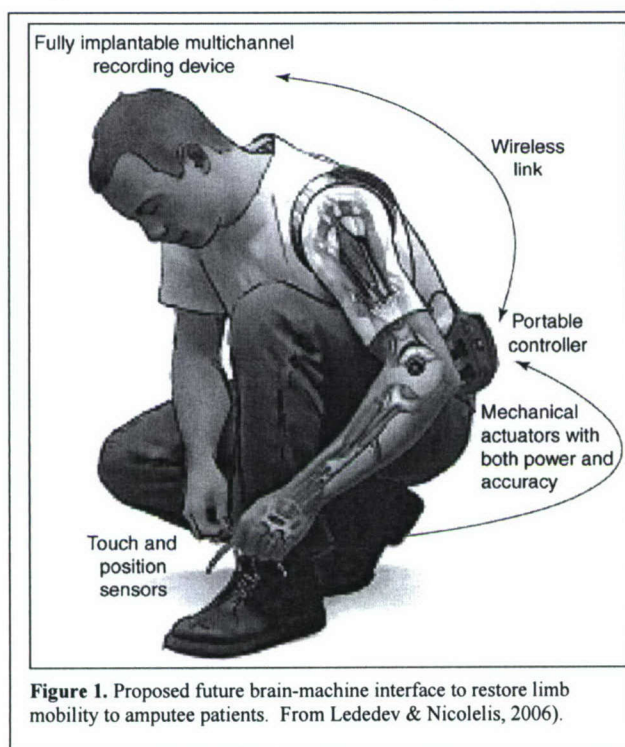
Understanding how cells react and adapt to their environment is important for tissue engineering, cell-based drug discovery, and the development of biomedical devices. Cells deposit an extracellular matrix, both *in vivo* and *in vitro* composed of a complex network of proteins and other biomolecules (Davis et al., 2000; Kalluri, 2003). Understanding the function and interaction of the ECM with cells is necessary for the development of successful techniques for tissue engineering and devices since the ECM will be present at the interface of the biological and the non-biological systems. A major component of the mammalian central nervous system (CNS) and peripheral nervous system (PNS) the ECM provides support, cell-to-cell recognition, cellular adhesion, and even neuronal connections through its composition of glycoproteins, proteoglycans, cell adhesion molecules (CAMs), and matrix metalloproteinases (MMPs). Therefore, ECM dynamics remains as a significant barrier to the implementation of effective neurointerfaces.

Specific ECM dynamics are important to the DoD because they limit ongoing neurointerface development efforts rapidly moving toward implementation. These *in vivo* applications could involve any type of implant, either temporary or permanent, that is placed in the body of a human, animal or invertebrate. These include various sensor systems as well as brain – machine interface probes that depend upon a tight coupling of the electronic materials to neurons or their processes.

External devices that monitor and guide the activity of the human nervous system have been a reality for more than two decades (Mussa-Ivaldi & Miller, 2003) with the cochlear implant being the best realized example thus far (Loeb, 1990). More sophisticated systems are under development that can translate information encoded in electrophysiologic signals from the brain into commands to control devices. These technologies rely upon electrical

interactions with large populations of neurons in which information passes between individual neurons and silicon-based electronics. While the development of these brain-machine devices has experienced impressive growth, this growth is restrained by a number of bottlenecks which must be overcome (Lebedev & Nicolelis, 2006; Wolpaw, 2004). As the transition from proof-of-concept demonstrations to reliable technologies occurs, it is important to consider potential future military applications of these devices because this information will shape the development process.

In the next decade it can be speculated that neurointerfaces will have two main applications of interest to the DoD: 1) *neuro-prosthetic devices* that compensate for damage, such as battle-related injuries in soldiers (see Figure 1); or 2) *neuro-*

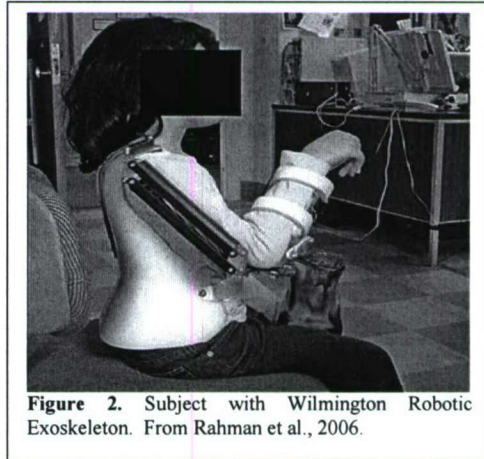


augmentation devices which are designed to increase the strength, accuracy, or peak physical/intellectual performance of healthy soldiers in the field. To explore this issue further we will focus on the current technological strategic goals of DoD's Defense Advanced Research Projects Agency (DARPA). The relevancy of this particular project will be

compared to current technology thrust areas within DARPA's Defense Sciences Office (DSO) Program, specifically to goals within DSO's Biological Sciences group.

Neuro-Prosthetic Devices

During the Vietnam War the mortality rate from brain injuries was 75% or greater due to the force of blast related injuries (Mayorga, 1997). However, because of advances in battlefield trauma treatment systems and the presence of Kevlar helmets and body armor, blast related mortality has improved substantially (Okie, 2005). Yet this increased survival rate has also been correlated with an increase in traumatic brain injury, limb amputations, and an increase in limb specific injuries which limit post-injury functioning (Fox et al., 2005; Okie, 2006). Therefore, the DoD has an active interest in recovering functional capabilities for soldiers to prepare them for a successful return to civilian employment. Therefore, human-assisted neural devices which



provide closed loop motor control of hands, arms, legs, feet, or external control of an external computer assistant are of interest to the DoD. For instance, DARPA has created a *Revolutionizing Prosthetics* program to create a fully functional (motor and sensory) upper limb in ten years that responds to direct neural control. The goal is to create a prosthetic arm that has function almost identical to the natural limb in terms of motor control, dexterity, sensory feedback, and weight, for upper limb amputees (see Figure 2). The objective is to create a limb that is rugged and resilient to environmental factors to permit amputees to lead as normal a life as possible in spite of these severe injuries.

Blast-related injuries to the eye are also prevalent in today's battlefield and DARPA's *Neovision* program works to create the groundwork for synthetic visual

systems which will require viable neural/electrical interfaces to function. Advanced optical development is required to accomplish this ambitious goal, but proper functioning of this system will be dependent on a functional neurointerface between the brain and electrical components.

While restorative cellular repair of injury is not a specific target for this ECM project, this current line of research will also contribute to a comprehensive understanding of the wound environment. DARPA's *Restorative Injury Repair* program examines the cellular matrix, biofilm dynamics, growth factors and ultimately the process of morphogenesis leading with an eventual goal of complete anatomic and functional restoration of damaged tissues.

Neuro-Augmentation Devices

Research in neuro-prosthetics for amputees will also directly complement efforts to increase the capacity of healthy soldiers in the field. For instance, a high priority of the DoD is the utilization of a battlefield exoskeleton which would augment the natural biological capacity of individual soldiers. Equipped with this mobile to augment locomotion, a soldier could potentially outrun non-equipped aggressors and if necessary run for long periods of time without tiring (see Figure 3). More likely initial instantiations of this technology will permit an increase in the carried weight of personal battlefield protection without a corresponding decrease in soldier fatigue or mobility. These devices will likely utilize a force feedback, non-neural interface, which while functional, will

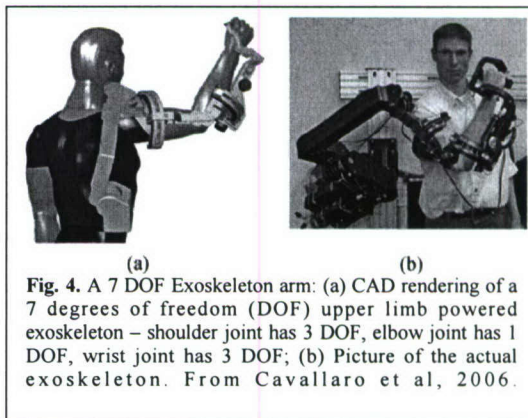


Figure 3. The Berkeley Lower Extremities Exoskeleton.

not support rapid agile movements typically required for soldiers in the field. It is speculated that this device would be advantageous when traveling on foot between battlefield environments, but would be cumbersome and slow during actual enemy engagement. Another expansion of this technology would create an exoskeleton attachment for the arm which would permit soldiers to carry heavy armaments in the field thereby increasing the ballistic force of handheld weapons (see Figure 4). The device would need to be rugged, lightweight, with minimal power requirements which all require further development.

III. Experimental Findings

In order to make progress toward functional neuro-prosthetic and neuro-augmentation devices (Rahman et al., 2006; Cavallaro et al., 2006), spinal cord

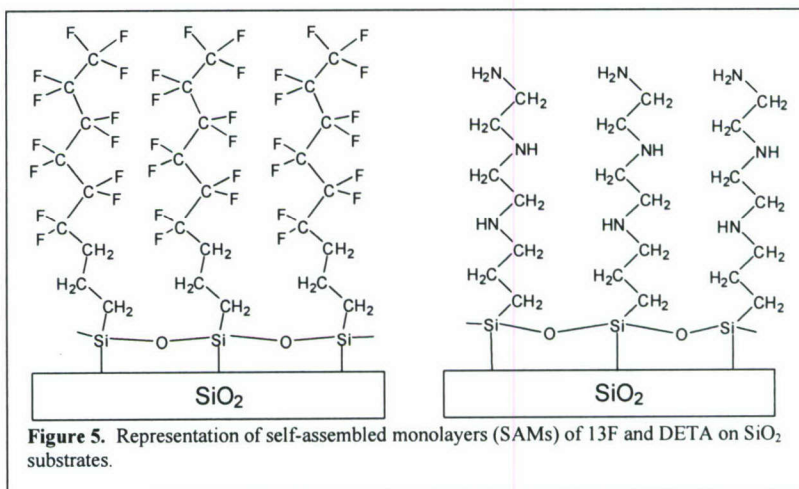


repair (Zhanga et al., 2004; Deumens et al., 2005), tissue reconstruction (Davis et al., 2000; Badylak, 2002, 2004), and regeneration of damaged neurons due to Alzheimer's disease (Greenberg and Jin, 2005; Yamaguchi, 2005), the interaction between neurons and their environment needs to be investigated. (Wright et al., 2002) The ECM of cultured cells on different surfaces has been studied in limited fashion. There are no detailed studies of the composition of neuronal ECM and the interactions of neurons and glial cells in a co-culture in a defined, serum-free system. Laminin and fibronectin produced by astrocytes cultured in serum (Price and Hynes, 1985; Laan et al., 1997) and fibronectin produced in mixed primary cerebellar cultures in serum (Gilad et al., 1990) have been investigated. Most of the attention to deposited ECM composition, however,

has been directed toward bone, vascular tissues and supportive mesangial cells. (Ishimura et al., 1993; Aarden et al., 1996; Davies, 1996; Kushida et al., 1999; Sottile and Hocking, 2002; Canavan et al., 2005a; Canavan et al., 2005b).

Understanding how a cell interacts with a certain surface, media component, growth factor, etc. is important in mimicking the *in vivo* conditions and can only be accomplished by controlling environmental variables. The laboratory has developed a system for cell culture that allows us to look at the growth and behavior of cultured neurons in a defined environment with a high degree of control over variability. (Schaffner et al., 1995; Das et al., 2003) The cells are cultured on a well-characterized and reproducible surface in a defined serum-free medium system. By controlling the number of variables the effect of a specific change in the system can be directly attributed to the cause. In this study, the ECM protein deposition of embryonic rat hippocampal (CNS neurons) was compared to embryonic rat dorsal root ganglion (PNS neurons) cultures using this established system, and we characterize and quantify the deposition of the extracellular matrix proteins, laminin, fibronectin, vitronectin and collagen on DETA. We compare the deposition on the cytophilic surface (DETA) to a cytophobic surface (13F), see Figure 5.

By studying the ECM deposition of cells in our system, we establish a baseline for cell behavior, which allows us to



evaluate the system's effectiveness at mimicking the *in vivo* environment. The goal is to make an *in vitro* system that is as close to *in vivo* conditions as possible and to determine which factors are essential for survival and function. After the baseline of cell function in this system is established, it can be used for biological study and to answer fundamental biological questions. The system can be made more complex by adding elements such as growth factors and proteins back into this model.

Materials and Methods

Embryonic hippocampal neurons were cultured with and without B27 in the media on DETA. Immunocytochemical experiments were performed at four, five or 11 days after culture to compare changes in the ECM deposition. Immunocytochemistry from the embryonic dorsal root ganglion culture was performed at day 4 or 5 and day 11 after culture. For the embryonic hippocampal (with B27) and DRG cultures, older cells (day 25 or day 30) were observed for comparison. Immunocytochemical experiments on cultures of embryonic hippocampal neurons and DRG cells on 13F surfaces were performed at day 4 after culture.

Surface Preparation. SAMs were prepared on SiO₂ substrates as detailed previously.(Ravenscroft et al., 1998) All silanes were used as received, and toluene (VWR) was distilled over sodium metal. Glass coverslips (VWR, 22 mm x 22 mm) were oxygen plasma cleaned for 20 minutes at 400-500 mmHg O₂ and reacted with a 0.1% trimethoxysilylpropyldiethylenetriamine (DETA) (United Chemical Technologies) solution in toluene. Coverslips with tridecafluoro-1, 1', 2, 2'-tetrahydrooctyl-1-trichlorosilane (13F) (Gelest) SAMs were prepared similarly. Representative coverslip

from each SAM preparation were analyzed by sessile water drop contact angle (Raméhart) and X-ray photoelectron spectroscopy (XPS) (Fissons Escalab 200i XL) prior to cell culture to verify surface composition.

Cell Cultures. Time mated Sprague-Dawley rats were housed in an animal facility at University of Central Florida. All research was approved by the Institutional Animal Care and Use Committee and conformed to NIH guidelines. Pregnant rats were anesthetized and non-survival caesarian sections were performed at embryonic day 15 (E15) or embryonic day 18 (E18) Embryos were surgically removed, and tissues obtained using standard dissection techniques under a stereomicroscope (Carl Zeiss, Stemi 2000).

Embryonic hippocampal neurons were isolated from E18 rat embryos. The embryos were removed from the mother rat and transferred to cold Hibernate E/ B27/ GlutaMAX™/ Antibiotic-antimycotic. The brain was removed from the decapitated embryo and transferred into a separate dish containing cold Hibernate E/ B27/ GlutaMAX™/ Antibiotic-antimycotic. The hippocampi were isolated by dissecting the hemispheres. Hippocampi were transferred into fresh Hibernate E/ B27/ GlutaMAX™/ Antibiotic-antimycotic. The tissue was mechanically dissociated in 1ml of Hibernate E/ B27/ Glutamax/ Antibiotic-antimycotic using a Pasteur pipette. The dissociated 1ml of cell suspension was layered over a 4 ml step gradient (Optiprep diluted 0.505: 0.495 (v/v%) with Hibernate E/ GlutaMAX™ / Antibiotic-antimycotic/ B27 and then made to 15%, 20%, 25% and 35% (v/v%) in Hibernate E/ GlutaMAX™/ antibiotic-antimycotic/ B27 followed by centrifugation for 15 min, using 800 g, at 4°C. One single dense band of cells was formed which was aspirated, collected, diluted in 5ml Hibernate E/ GlutaMAX™/ antibiotic-antimycotic/ B27, and centrifuged at 600 g for 2 minutes. The

pellet was re-suspended in culture medium (Neurobasal E/ B27/ GlutaMAX™/ Antibiotic-antimycotic). The cells were plated at a density of 100 cells/mm² on the DETA or 13F silane coated coverslips. The cells were maintained in a 5% CO₂ incubator with 95% relative humidity. Half of the medium was changed every 5 days. The same procedure for embryonic hippocampal cultures grown without B27 was followed except that B27 was omitted from the media.

Rat dorsal root ganglia (DRG) were isolated from E15 rat embryos and incubated in 0.05% trypsin-EDTA (Invitrogen) for 15 minutes in a 37°C water bath. After removal of the trypsin-EDTA, the cells were suspended in Hibernate E + 10% fetal bovine serum, and the tissue was gently triturated. The dissociated DRG tissue was pelleted by centrifugation, the Hibernate E + 10% FBS was removed, and the tissue was suspended in 400 L of Hibernate E. Next, the tissue was layered on a density gradient of Optiprep (0.505: 0.495 (v/v %)) solution and centrifuged at 1050g for 15 minutes at 4°C. After centrifugation, the resulting two bands were removed, and the pellet was discarded. The cells were pelleted by centrifugation, the supernatant removed, and the cells suspended in plating medium. DRGs were plated on DETA and 13F modified coverslips at a density of 300 cells / mm². The cells were maintained in a 5% CO₂ incubator with 95% relative humidity.

Immunocytochemistry. Primary IgG antibodies, mouse anti-human laminin, rabbit anti-rat fibronectin, chicken anti-rat neurofilament M, and rabbit anti-rat glial fibrillary acidic protein (GFAP), mouse anti-human vitronectin, and mouse anti-human collagen IV were obtained from Chemicon International and used as received. The secondary antibodies,

Alexa Fluor® 488, Alexa Fluor® 595, and Alexa Fluor® 647, were used as received from Invitrogen.

The neuronal and DRG cultures were fixed in 4°C 100% methanol for 5 minutes and rinsed twice with phosphate buffered saline (PBS). Next, cells were blocked for two hours with a solution of 0.05% saponin in PBS, 0.5% sodium azide, 5% bovine serum albumin (BSA), and 5% donkey serum. The cells were then incubated with primary antibodies diluted in the blocking solution overnight at 4°C. The next day, primary antibody solutions were aspirated, and the cells were rinsed three times with blocking solution. Then, secondary antibodies diluted 1:200 in blocking solution were added to the cells and incubated for 2 hours at room temperature in the dark. The secondary antibody solution was aspirated, the coverslips were rinsed three times in PBS, and allowed to dry. Finally, coverslips were mounted on glass slides using VectaShield mounting medium with DAPI (H-1200, Vector Labs) and fixed using clear nail polish. Scanning confocal microscopy was performed using a Carl Zeiss Axioskop2 and imaged with UltraView Imaging Suite v 5.5 (Perkin-Elmer). Multiple regions of each coverslip were imaged and immunostaining experiments were performed multiple times over different cell cultures.

Protein Isolation and Western Blot Analysis. ECM proteins were isolated by scraping the cultured coverslips with a cell scraper into a solution of 50 mM Tris, 150 mM NaCl, 1% Triton, and 0.1% SDS. The volume of scraping solution varied from 750 to 1500 µL. The solution was centrifuged at 12,000g for 20 minutes, and the supernatant was collected and stored at -80°C until needed. MicroBCA total protein quantification was performed on each supernatant. Gel electrophoresis using 4% stacking and 10%

separating polyacrylimde gels was used to separate the proteins, and the gel was transferred to nitrocellulose paper. Known amounts of the analyte protein were run on each gel for calibration. Laminin (mouse) protein was obtained from BioSource International and fibronectin (rat) protein was obtained from Biomedical Technologies. Rabbit anti-rat fibronectin or mouse anti-human laminin primary antibodies were used either with goat anti-rabbit IgG or goat anti-mouse IgG HRP-conjugated secondaries (Zymed) and developed with the Bio-Rad Opti-4CN kit. Western blots were read on a SynGene ChemiGeniusQ BioImaging System with GeneSnap (version 6.03.01) and GeneTools (version 3.05.03) software. The relative amounts of protein on the surfaces were normalized to the total protein as determined by MicroBCA analysis.

Results

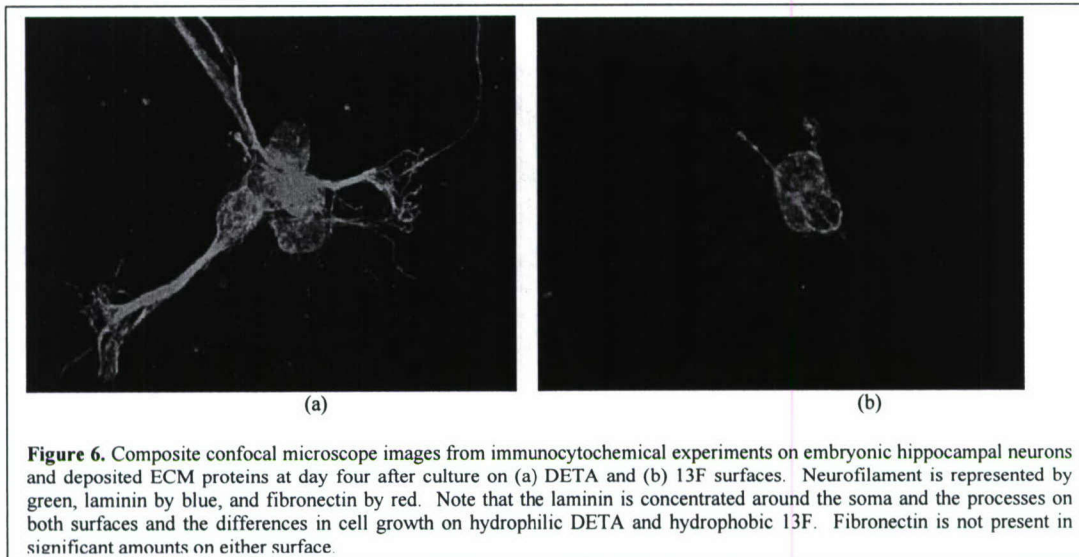
The embryonic rat hippocampal and DRG cells were cultured on self-assembled monolayers (SAMs) of DETA and 13F on SiO₂ substrates. The cells were grown in a defined, serum-free medium on a well characterized inorganic surface so that the deposited proteins were generated by the cells and not due to non-specific adsorption of protein from the medium. DETA is a cytophilic, permissive surface, while 13F is a cytophobic, non-permissive surface. The ECM deposition of the different cell cultures was compared on both DETA and 13F surfaces. See Table 1 for a comparison of deposited proteins for the experiments performed. The hippocampal neurons cultured on DETA spread out over the surface and form well defined processes; when on the hydrophobic 13F surface, neurons tend to clump together and processes are not well formed. The DRG cells on 13F spread out over the surface to a greater extent than in the

hippocampal culture, but the cells were clearly more clumped together as when compared to the DRG culture on DETA.

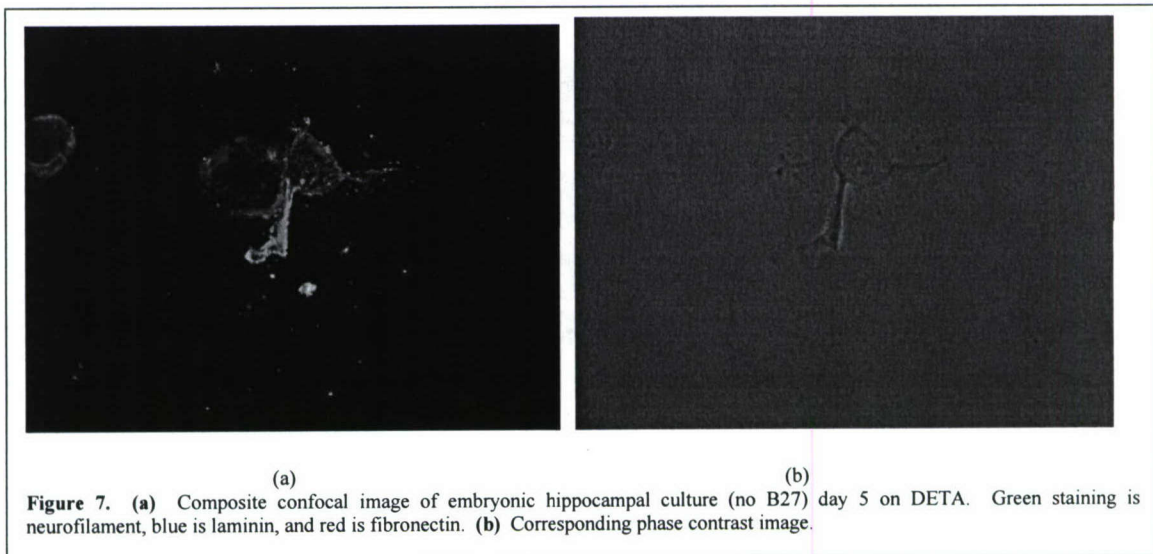
	Surface	Collagen IV	Fibronectin	Laminin	Vitronectin
Embryonic Hippocampal Day 4	DETA		-	++	
Embryonic Hippocampal Day 11	DETA	+	-	++	+ (only found near soma)
Embryonic Hippocampal Day 20+	DETA	++	-	++	+ (only found near soma)
Embryonic Hippocampal Day 4	13F		-	++	
Embryonic Hippocampal Day 7 w/o B27	DETA		+ scattered protein on surface	++ scattered protein on surface	
Dorsal Root Ganglion Day 4/5	DETA		++	++	
Dorsal Root Ganglion Day 11	DETA	+++	+++	+++	++
Dorsal Root Ganglion Day 25	DETA		+++	+++	
Dorsal Root Ganglion Day 4	13F		++	++	

Table 1. Comparison of immunocytochemical staining results for ECM proteins for different cultures and surfaces. +++ represents a very significant amount of staining for a protein; ++ represents a significant amount; + represents a small amount; and – represents a minimal or not detected amount. A box with no symbol indicates that the protein staining experiment was not performed.

ECM Dynamics in Embryonic Hippocampal Culture Immunocytochemical experiments were performed on embryonic hippocampal neurons at day 4 or 5 after culture, staining for combinations of neurofilament, laminin, fibronectin, vitronectin, and collagen. These cultures were triple stained for neurofilament, laminin and fibronectin and they revealed minimal or non-existent fibronectin staining (see Figure 6a.) The laminin appeared on and under the cell body, but it was not colocalized with neurofilament. When 0.7 μm slices were observed in the z-axis of the images generated by confocal microscopy, it was revealed that the laminin resided on the outer surfaces of the cell body and that the neuronal processes were lying on a laminin layer. Similar



results were obtained for embryonic hippocampal neurons on 13F surfaces, as shown in Figure 6b. Laminin covered the cell body with minimal or undetectable amounts of fibronectin present. On DETA surfaces, the neurons lie on collagen in a similar fashion to laminin (see Figure 7). Vitronectin is minimal and is closely associated with the soma. By day 11 after culture, laminin appears to cover the cell body more extensively, and we see no increase in fibronectin. After day 20, we stained for combinations of neurofilament, vitronectin, laminin, collagen, and fibronectin. Laminin and collagen are still closely associated with the soma and the processes; ECM proteins do not cover the



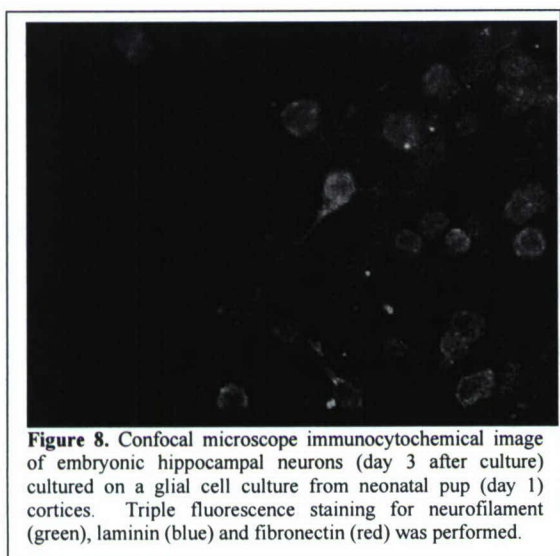


Figure 8. Confocal microscope immunocytochemical image of embryonic hippocampal neurons (day 3 after culture) cultured on a glial cell culture from neonatal pup (day 1) cortices. Triple fluorescence staining for neurofilament (green), laminin (blue) and fibronectin (red) was performed.

The embryonic hippocampal cultures on DETA surfaces grown in the absence of B27 deposited displayed different immunocytochemical results (see Figure 8).

These cultures were immunostained for combinations of neurofilament, GFAP, laminin and fibronectin (see Figure 9).

There was more laminin and fibronectin in the vicinity of the surviving neurons, but since the cell survival is low, the overall amount of protein is much lower when compared to a culture with B27 in the media. In these

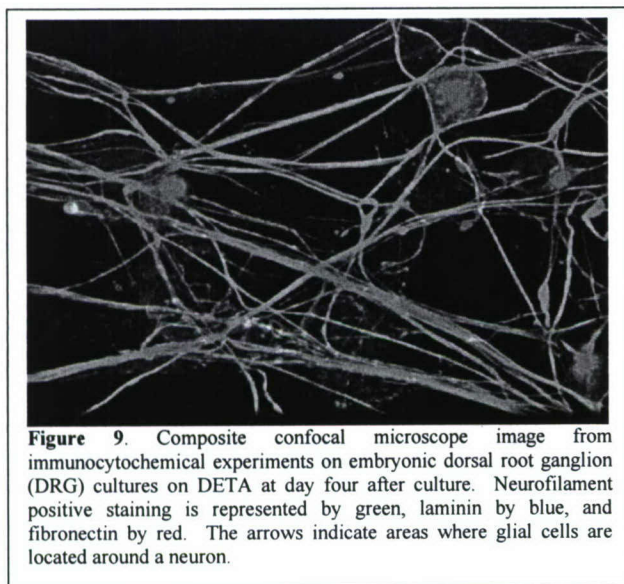


Figure 9. Composite confocal microscope image from immunocytochemical experiments on embryonic dorsal root ganglion (DRG) cultures on DETA at day four after culture. Neurofilament positive staining is represented by green, laminin by blue, and fibronectin by red. The arrows indicate areas where glial cells are located around a neuron.

cultures, the observed cells present did not stain positively for neurofilament but were positive for GFAP, indicating that glial cells were present in the culture. These cells have laminin and fibronectin associated with them on the surface and on the soma. Neurons in the cultures without B27 have laminin

associated with the soma, similar to neurons in the cultures containing B27 in the medium.

ECM Dynamics in Embryonic Dorsal Root Ganglion Cultures

The ECM deposition in DRG cultures was more extensive than in the hippocampal cultures. These cultures were immunostained for combinations of neurofilament, laminin, fibronectin, collagen, and vitronectin. Most of the neurons in these cultures at day four were associated with or surrounded by a cell type that did not stain positively for neurofilament. These other, non-neurofilament positive cells had laminin, fibronectin, or collagen around them that extended under and around the nearby neuron or neurons, often forming a “bed” of ECM for the neuron. This phenomenon could be seen upon inspection of the z-stacked images (see Figures 10 and 11). Vitronectin was present minimally. The extent of

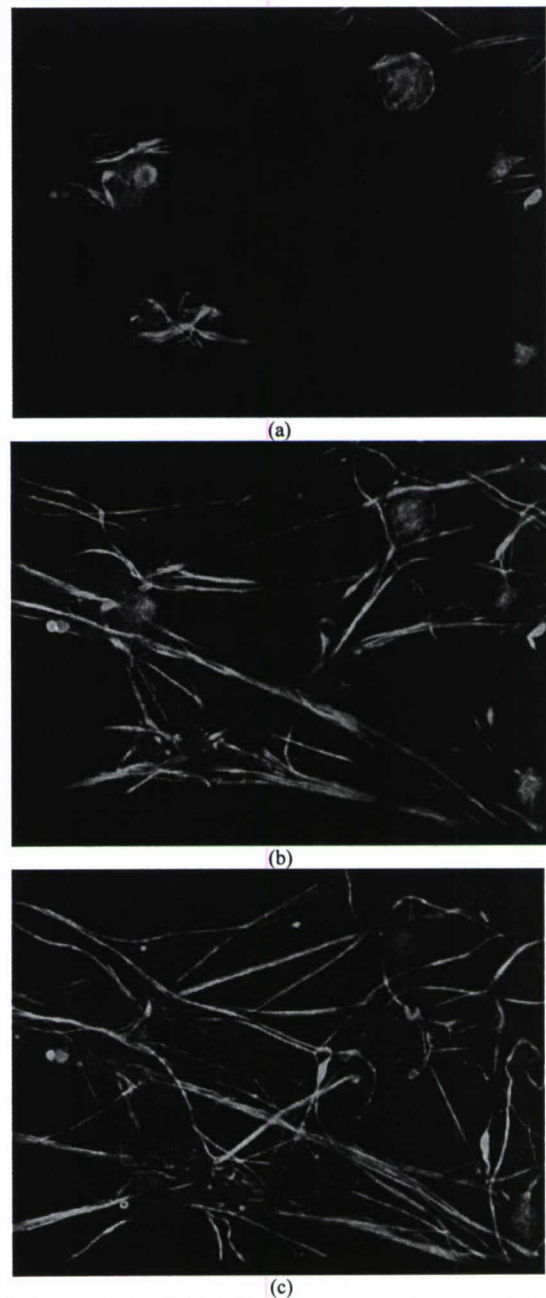


Figure 10. Confocal microscope images from immunocytochemical experiments on embryonic dorsal root ganglion (DRG) cultures on DETA at day four after culture. Neurofilament positive staining is represented by green, laminin by blue, and fibronectin by red. Each picture represents a $0.7\ \mu\text{m}$ wide slice of the image shown in Figure III. Representative slices of the 20 slices generated are shown: (a) Top of the cells image; (b) Middle image (c) Image at the surface of the SAM coverslip.

fibronectin, laminin, and collagen deposition on the surface was much greater in these

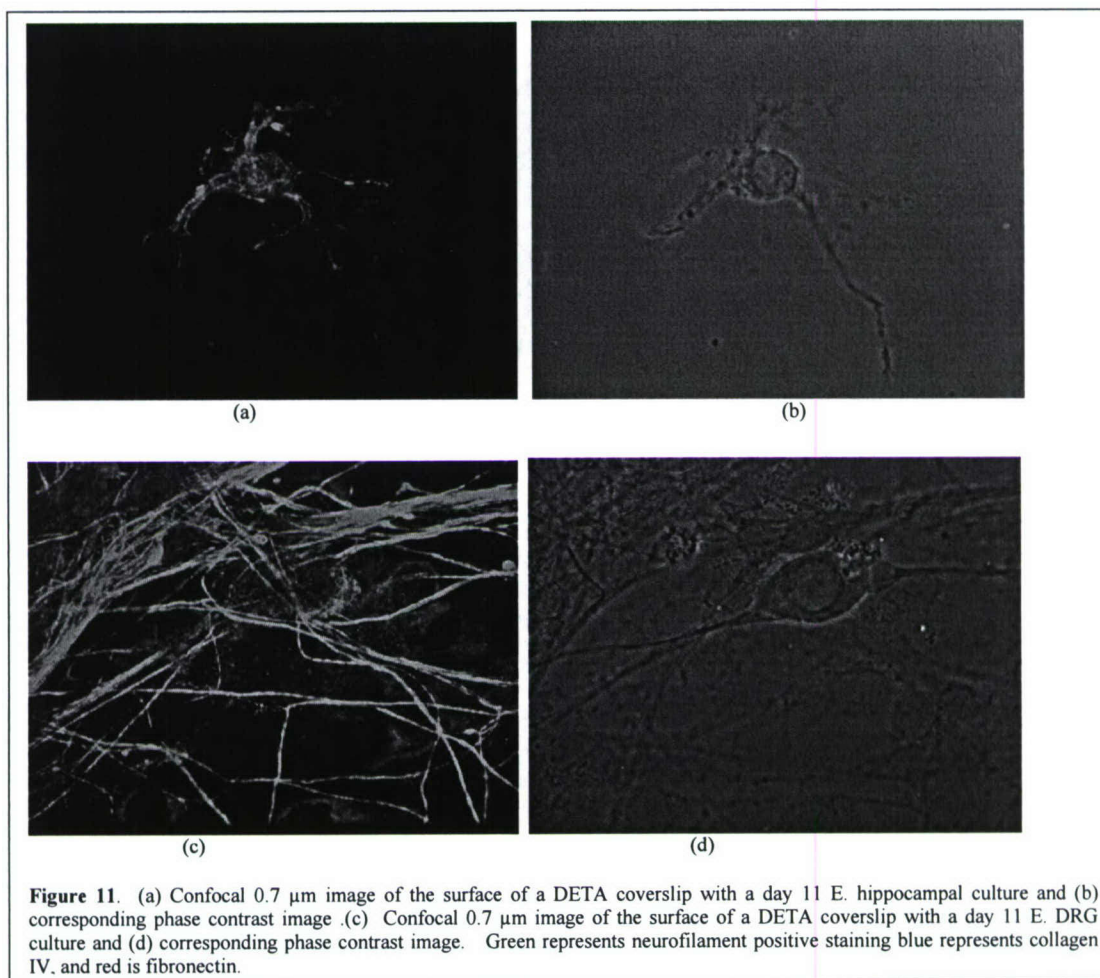


Figure 11. (a) Confocal 0.7 μm image of the surface of a DETA coverslip with a day 11 E. hippocampal culture and (b) corresponding phase contrast image. (c) Confocal 0.7 μm image of the surface of a DETA coverslip with a day 11 E. DRG culture and (d) corresponding phase contrast image. Green represents neurofilament positive staining blue represents collagen IV, and red is fibronectin.

cultures than what was observed in the embryonic hippocampal culture, and fibrillar aggregates of fibronectin could be seen on the surface. The non-neuronal cells seem to deposit mainly one type of protein instead of a mixture of the ECM proteins studied, as evidenced by immunocytochemical staining (see Figure 12). The older cultures (day 25) did not show increased amounts of proteins when compared to day 11, indicating that the protein deposition had stabilized as observed by immunocytochemistry.

Western blot analysis (see Figure 13) was used to quantify the percentage of laminin deposited by the hippocampal and DRG cultures and the percentage of fibronectin deposited by the DRG cultures on DETA surfaces. The amount of ECM protein detected was normalized to the total amount of protein on the coverslip as determined by

MicroBCA analysis. Laminin comprised $9.3 (\pm 1.9) \%$ of the protein deposited by the embryonic hippocampal cultures (with B27). Fibronectin comprised $23.6 (\pm 3.5) \%$ of the protein deposited by the embryonic DRG cultures. The hippocampal cultures (with B27) did not show a positive result for fibronectin on the western blots. It was discovered that the embryonic hippocampal culture without B27 had very low cell survival, and we were unable to detect laminin on the surface by western blot analysis. Occasionally, but not consistently, we were able to detect a fibronectin band from these cultures.

Discussion

It has been shown previously that glial cells produce laminin and fibronectin. (Price and Hynes, 1985; Gilad et al., 1990; Chintala et al., 1996; Thomas et al., 1996; Laan et al., 1997). Until now, there have

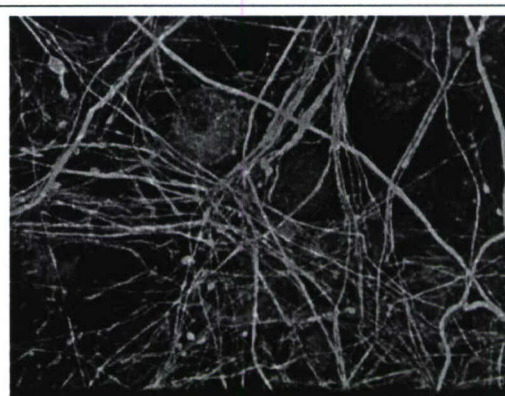


Figure 12. Confocal microscope images from immunocytochemical experiments on embryonic dorsal root ganglion (DRG) cultures on DETA at day 11 after culture. Neurofilament positive staining is represented by green, laminin by blue, and fibronectin by red. The amount of ECM protein on the surface is greater than at day 4 after culture. The arrows indicate cells that are secreting either laminin or fibronectin but do not appear to be secreting both.

been no studies of the ECM production and deposition of neurons both in pure and co-cultures in a defined, serum-free system. We have found that pure cultures of embryonic hippocampal neurons do not produce large areas of ECM spread over the coverslip. The cells deposit ECM proteins as a barrier to the inorganic DETA surface, possibly as a way to control their microenvironment, especially since the protein composition did not differ as a function of surface hydrophilicity or hydrophobicity. The neurons secrete laminin and collagen, but it is mostly concentrated around the soma and under the processes.

These results indicate the importance of a laminin matrix for hippocampal neuronal survival (Chen et al., 2003) even on a permissive surface such as DETA. A remaining question to answer is whether laminin and collagen are necessary for outgrowth of processes since the processes lie on these proteins

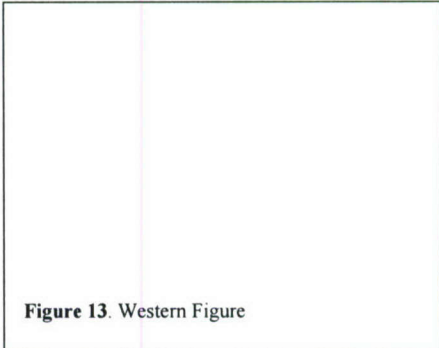
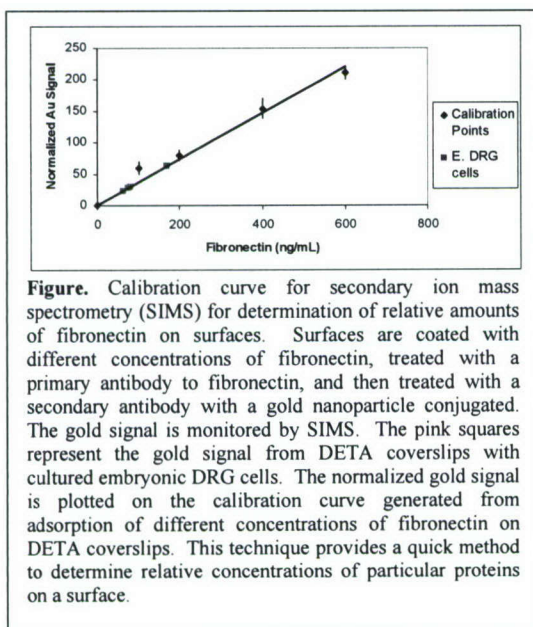


Figure 13. Western Figure

or if the neurite outgrowth occurs and as a result proteins are deposited. The amount of fibronectin and vitronectin present on the surface is minimal. An extensive network of ECM proteins does not cover the surface of the coverslip in hippocampal cultures, indicating that the primary function of the neuron is not the formation of an ECM network. In contrast, a much more extensive ECM network of laminin, collagen and fibronectin is present in the co-culture (DRG) at day 4 or 5 after culture. Vitronectin is associated with some of the neurons, but not deposited over the surface to the extent of the other proteins studied. ECM production is the responsibility of other cell types that associate themselves on and around the neurons, and the primary function of the neuron is not ECM production. The glial cells tend to congregate around, on, and under neurons and are responsible for deposition of the ECM proteins laminin, fibronectin, collagen and vitronectin, to a more limited extent. The glial cells present in the hippocampal cultures without B27 also congregated near neurons. The glial cells in the DRG culture seem to concentrate on the production of one type of protein; we did not observe these cells individually depositing multiple proteins. For instance, individual cells were observed to secrete laminin or fibronectin, but not both. Pure embryonic hippocampal neurons make a limited amount of ECM on both the cytophobic and the cytophilic surfaces, and there is

not a network of proteins due to the absence of glial cells. In the absence of B27, these cultures produce additional proteins due to the presence of glial cells. The limited ECM deposition in the pure culture as compared to the co-culture indicates a limited role for neurons in ECM deposition. The purpose of the glial cells, especially as seen in our system, may not be limited to ECM production but may extend to protection of the neurons and in facilitating cell signaling.

Understanding the roles of different cell types in the deposition of ECM and the interaction between these cells and artificial surfaces is crucial for progress in tissue engineering, the design of either neuro-prosthetic or neuro-augmentative devices, and regeneration of damaged neurons due to disease or injury. Our defined *in vitro* system minimizes the number of variables in the system, and simplifies the problem to make it more manageable. The cells cultured using our systems behave similarly to *in vivo* conditions; the cells secrete ECM proteins and interact. We can now use the system to



evaluate whether these secreted proteins are essential to function and to change the composition and amount of protein secreted. The system has a broader potential in providing a way to answer fundamental biological questions by controlling variables and by the ability to control the level of complexity. As greater understanding of a biological system is achieved using a highly defined and controlled system, factors can be

added back into the system to more closely mimic *in vivo* conditions.

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OBJECTIVE

Alteration of the ECM plays an important role in angiogenesis and the inflammatory response, processes that occur during tissue injury, wound healing and tumor formation. Cryptic (matricryptic) sites are exposed in collagens, fibronectin, laminins, tenascin, vitronectin, osteopontin, and elastin upon tissue injury or angiogenesis. The exposure of these sites may be involved in the regulation and signaling at the injury site, act as a scaffold for tissue repair, and result in the release of ECM components and growth factors from the ECM. [Davis, Bayless 2000] These sites are not active or exposed in normal, mature ECM. The ability to locate and characterize these sites will provide information about wound healing, infection, the inflammatory response, angiogenesis, and tumor formation and growth. The goal of the proposed research is to specifically target the matricryptic sites with derivatized magnetic nanoparticles and image them by magnetic resonance (MRI). The nanoparticles will be highly specific to areas of injury, inflammation, and tumor growth because the binding sites in the ECM targeted are only exposed under these conditions.

The ECM controls the functions associated with wound healing. In its native state, the ECM serves a structural role in cellular organization and as a barrier between tissues. When the ECM is altered or damaged, cryptic sites are exposed. These cryptic sites are responsible for directing the wound healing process in part by providing different binding motifs for ligands that do not bind in the native state. As the ECM and the wound repair, the cryptic sites disappear and the ECM returns to its native state.

The capability to image the exposed matricryptic sites in altered ECM will allow the existence of the sites to be verified, particularly *in vivo*, and visualized. The number of cryptic sites can then be quantified, providing insight into the mechanism of wound healing and the inflammatory response, angiogenesis and other processes involved with ECM degradation. Different types of cryptic sites can be simultaneously targeted by the use of differently functionalized nanoparticles. The nanoparticles will provide a method to determine the extent of the inflammatory response in a wound and to image the wound at different stages of healing to track healing progression. Specific binding to cryptic sites provides a method for a targeted drug delivery system directly to the injury site.

We propose to generate cryptic sites in collagen IV using conditioned media from HUVEC cultures, verify their existence by integrin binding affinity, utilize aptamer libraries to identify candidate molecules that bind specifically to cryptic sites, and derivatize magnetic nanoparticles with the candidate molecules. Additional experiments will include using ECM generated from human endothelial cells, generating cryptic sites, and testing derivatized nanoparticle binding. After identification of candidate derivatized nanoparticles, we will conduct *in vivo* studies to image the nanoparticles with magnetic resonance imaging (MRI). We can use this system to deliver drug therapy directly and specifically to the site of interest.

ADDENDUM

Technical report on military relevant applications of the research.

The ECM consists of a variety of proteins such as collagens, laminin, heparan-sulphate proteoglycans (HSPGs), fibulins agrin and SPARC.[Kallutri 2003] The ECM is crucial to the function and structure of blood vessels and capillaries. It also has non-structural functions such as sequestering of growth factors, regulating cell migration, and cell signaling at an injury site. The ECM is thought to play an important role in the signaling of endothelial cells and the regulation of soluble proteins. During the remodeling process, the endothelial cells proliferate and migrate. The cell interactions with the exposed cryptic sites may signal the formation of new blood vessels (neovascularization). [Hangai 2002, Hocking 2003] When the ECM is degraded or injured, cryptic sites in the proteins are exposed. Cryptic sites have been identified in fibronectin, vitronectin, collagens, laminins, osteopontin, tenascin, elastin, and hyaluronic acid. Cryptic sites are exposed by enzymatic degradation (wounds, burns), cell mediated mechanical forces, denaturation (burns), heterotypic binding (inflammation), and multimerization. Upon tissue injury, plasma-derived proteins, such as fibrinogen, fibronectin, vitronectin, are recruited to ECM of the injury site. Osteopontin, SPARC, thrombospondins, tenascins are released by cells in the area, and the ECM is altered to expose cryptic regions of proteins. Processes such as angiogenesis remodel the ECM through the actions of proteolytic enzymes such as matrix metalloproteinases (MMPs). [Rundhaug 2005] At a wound site, laminin-5, collagens I and IV, and tenascin-C are present. Also present are MMPs 1, 2, and 9, which degrade the ECM. In addition, epithelial growth factor (EGF) repeats are exposed and sometimes released. Sequestered growth factors can also be released. When intact, these ECM proteins present certain binding domains, and when altered, different binding domains are exposed. For example, collagen IV in mature, intact ECM binds $\alpha 1\beta 1$ integrin, but upon degradation and exposure of cryptic sites, collagen IV presents $\alpha \nu\beta 3$ binding domains. Native collagen binds $\alpha 2\beta 1$ integrin, but this integrin is not a ligand for altered or denatured collagen.

The ECM is a dynamic structure involved in tissue repair. It can be thought of as a history of events in the process of healing at an injury site. We can look at using the ECM to determine what cell types and molecules have been and are present at the injury site over time. By targeting the ECM and not particular cells or cell surface molecule, we can gain new insight into the wound healing process by observing the process from start to finish because the presence of the ECM is the one consistent part of the process; the ECM is stationary.

PROPOSED STUDY

We propose to study collagen IV, a major ECM component, and to generate cryptic sites in the collagen by using conditioned medium from human umbilical vein endothelial cells (HUVECs), which contains high levels of proteolytic enzymes. We will also study cryptic sites in thermally denatured collagen IV. Methods for generating the collagen IV cryptic sites and integrin binding studies have been reported previously in the literature. We will

focus first on a collagen IV cryptic site since there is a monoclonal antibody (HUIV 26) that has been reported. [Xu, Rodriguez 2001] We propose to quantify cryptic site exposure by using the primary antibody to the site and a secondary antibody with either a fluorophore or a gold nanoparticle attached. (See Preliminary Results below.) Fluorescence quantification can be achieved using NIH Image software [J.Comp.Neurol. 399: 424–426, 1998]; the gold quantification can be imaged using SEM and secondary ion mass spectrometry (SIMS) can be used to detect relative amounts of gold. Development of methods for cryptic site quantification will be useful for monitoring tissue injury and for identification of drugs to aid in healing. Once the cryptic sites have been generated, we will attach the monoclonal antibody for the site to a fluorescently labeled magnetic nanoparticle for imaging. [Perez, Simeone 2003]

We propose to identify target molecules (aptamers) that will selectively fit into and bind to the cryptic sites. Aptamers are synthetic oligonucleotides of RNA or DNA that have a variety of three-dimensional structures. The aptamer can bind to a target, such as a protein active site, peptides, and small molecules, by complementary shape interactions. The binding is highly specific, and there is a high affinity for the aptamer to the target. [Blank and Blind 2005] The aptamers that bind to the cryptic sites would be identified via SELEX (systematic evolution of ligands by exponential enrichment), an automated process in which single stranded nucleic acid sequences ($\sim 10^{15}$) are incubated with the target. Sequences that do not bind to the target are removed by washing and the target-aptamer complexes are recovered and amplified via RT-PCR. The process is repeated 12 to 15 times in order to select the aptamers with high affinity binding to the cryptic sites. Aptamers can be modified in certain positions on the base or on the 3' or 5'-end for immobilization or functionalization purposes or to increase the stability in the presence of nucleases. Key features of aptamers are that they are not toxic or immunogenic, making them ideal candidates to target areas of tissue injury in the body. The cryptic sites can be quantified using SIMS and selected aptamer candidates attached to gold nanoparticles. Development of this method will be useful in quantifying cryptic sites for which no antibodies exist presently. The aptamer nanoparticles are expected to be more stable than monoclonal antibody nanoparticles.

We propose to attach the candidate aptamers to paramagnetic or superparamagnetic nanoparticles labeled with a fluorescent molecule such as FITC or Rhodamine to allow for *in vitro* detection. A variety of chemical approaches can be used to attach the aptamers, including the use of the 3'-end of the aptamer, attachment of an $-NH_2$ functionality to the 3'- or 5'-end or attachment of biotin to the 3'- or 5'-end. [Blank and Blind 2005] Magnetic nanoparticles have been functionalized with small molecules by utilization of sulfhydryl, carboxyl, amine, and anhydride chemistries [Weissleder, Kelly 2005], and we can use these techniques to attach aptamers. Previously, we have successfully attached oligonucleotides to magnetic nanoparticles. [Perez, Josephson 2002] The decorated nanoparticles should now be specific to the cryptic sites and can be imaged using fluorescence.

The next set of experiments is to verify that aptamer nanoparticles target the cryptic site

in ECM derived from human endothelial cells and to conduct *in vivo* studies to image the derivatized nanoparticles bound to cryptic sites using magnetic resonance imaging (MRI). Nanoparticle MRI agents have high spatial resolution, and superparamagnetic nanoparticles, via T2 relaxation, generate high contrast that is not dependent on the water flux in the immediate region. [Lanza 2004, Weissleder, Frank]

In the first year, we expect to be able to generate the cryptic sites in collagen IV, quantify the number of sites, attach the monoclonal antibody to the magnetic nanoparticles, conduct the aptamer screening, attach the aptamers to the magnetic nanoparticles, and image the sites fluorescently. *In vivo* studies imaging magnetically and human endothelial cell ECM experiments would be part of follow on studies.

These highly specific nanoparticles will provide new insight into the process of healing, inflammation, and angiogenesis. A wound can be imaged in a non-invasive manner to monitor healing as it occurs. As a wound heals, we expect that the number of nanoparticles binding would decrease as cryptic sites disappear and the ECM returns to a normal, mature state. Conditions such as infection could be identified at an early stage. The nanoparticles could be functionalized with both aptamers and drugs for highly specific delivery of drugs directly to the wound site. This methodology would change the way in which tissue injury is dealt; currently, a wound is treated at the outermost layer, i.e. stitched, and the body is left to take care of the rest of the process. The ability to monitor and image the wound site will not only allow for new insight into the healing process, but also it might lead to new therapies that will make healing faster and better. The imaging would not be limited to MRI because bismuth nanoparticles can be used with candidate aptamers to image via X-ray computed tomography. [Rabin, Perez 2006] In addition, the methodology could be useful to monitor the integration of implanted devices and prosthetics in the body. Further steps would include identification of aptamers specific to the many other types of cryptic sites and attaching them to nanoparticles.

PRELIMINARY RESULTS

We have used Secondary Ion Mass Spectrometry (SIMS) as a quick method to compare relative amounts of proteins on a surface. We can use this method as a quick way to characterize the cryptic sites generated *in vitro*. In our previous work, we have built a calibration curve for the extracellular matrix protein, fibronectin, by adsorbing different concentrations of protein solution on to coverslips with a self-assembled monolayer of an aminosilane. The coverslips with the adsorbed protein are reacted with a primary antibody specific to fibronectin, and then they are reacted with a secondary antibody, IgG conjugated with a 20nm gold nanoparticle. The gold signal is monitored by SIMS and multiple points are taken and averaged on each surface. Figure I shows the calibration curve for fibronectin. The cryptic sites that we generate in the ECM can be relatively quantified by using the monoclonal antibody HUIV 26 and a secondary antibody with a gold nanoparticle attached.

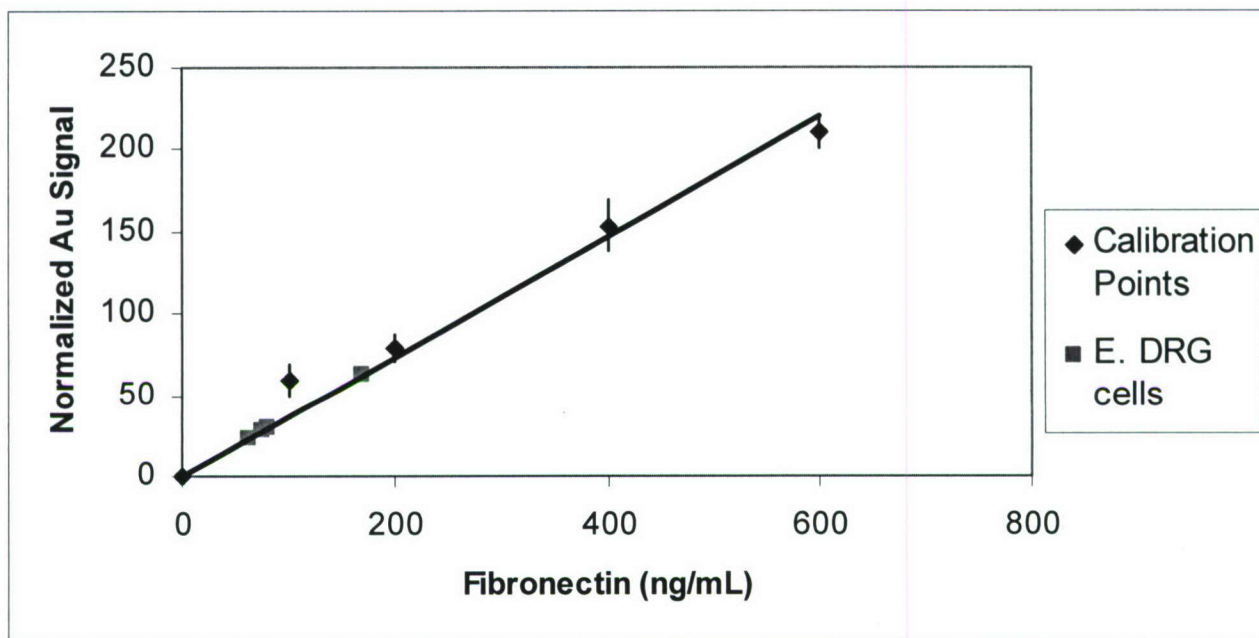


Figure I: SIMS Calibration Curve of Fibronectin Adsorbed on an Aminosilane Surface. The calibration curve from the gold signal from the secondary antibody is shown.