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# Molecular and Cellular Approaches for Evaluation of Ligand Binging to Vitronectin

Abdelhamid Mohamed Alsharif
*University of Tennessee - Knoxville*

# Molecular and Cellular Approaches for Evaluation of Ligand Binding to Vitronectin

Abdelhamid Alsharif
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Dr. Cynthia Peterson

**Abstract**

The blood protein vitronectin has been found to perform a variety of functions in the human body. Among these is its role in blood clot formation, the immune system, and tissue remodeling. By binding to the urokinase plasminogen activator receptor (uPAR), vitronectin facilitates cell adhesion and regulates tissue remodeling. This is a process that takes place in the formation of tumors and the progression of cancer cells. Vitronectin has also been found to play a key role in such pathogenic processes as invasion by *Candida albicans* yeast, which cause infections in immunocompromised hosts. This project aims to develop a better understanding of the molecular and cellular interactions of vitronectin binding to ligands. It aims to test the binding of vitronectin to the urokinase receptor and the effects of various factors on this binding. Among these factors are the interaction of vitronectin with Plasminogen Activator Inhibitor-1 (PAI-1) and the Extracellular Matrix (ECM) and the formation of multimeric vitronectin complexes. To determine the effects of these factors on vitronectin-urokinase receptor interaction, studies were conducted *in vitro* using isolated uPAR. These studies were performed by using the enzyme linked immuno-sorbent assays (ELISA) methods. The results of these experiments show a competition of PAI-1 with the urokinase receptor for binding to vitronectin. While these results agree with recent structural studies of the receptors that have been pursued, they contrast with previous observations of the effects of PAI-1 on the binding of vitronectin to another class of receptors, integrins. ELISA methods were also employed to develop appropriate conditions for the study of the binding of vitronectin to *C. albicans* cells. These assays showed increased binding to ECM with increased concentration. The aspects that were evaluated included

determination appropriate cell growth conditions, assay blocking agents, and cell growth media. The ability to measure the competitive and additive effects of ligand interactions on vitronectin can lead to a better understanding of such physiological processes as coagulation and angiogenesis, as well as lead to the development of therapeutic agents for certain diseases.

**Introduction**

Vitronectin is a plasma glycoprotein that has been found to participate in a variety of functions in the human body. As it is present both in circulation and in the extra cellular matrices of cells, vitronectin plays an important role is such processes as coagulation and cell adhesion. It thus leads to many effects dealing with the immune system, tissue remodeling, and angiogensis. Many of the functions of vitronectin are mediated by interactions with other proteins in the body. These include binding to cell-surface receptors such as integrins and the urokinase-type plasminogen activator receptor (uPAR). This binding interaction, which affects cell properties and functions, is affected by vitronectin binding to substrates like the type 1 plasminogen activator inhibitor (PAI-1).

Vitronectin is an adhesive protein that is found in human plasma, in circulation and in associations with matrices of connective tissues and fibers. It is produced mainly by the liver, but other tissues such as platelets and macrophages contain an immunochemically identical protein (Preissner and Jenne (2)). Vitronectin has been found to exist in three main forms. Full length vitronectin exits as a folded monomer in plasma, whereas it exists as a multimer in associations with ECM and other ligands. Also, vitronectin has been identified in a two-chain form in the blood, where cleavage of vitronectin by an unkown protease results in two chains joined by a disulfide bond; this has shown very little difference in function from full length vitronectin (Schroeck, et al.) While the detailed overall structure of vitronectin has not yet been fully elucidated, general features of the structure have been determined. The molecular structure was determined by sequencing of human and rabbit vitronectin cDNA and by analysis of exon-intron organization of the human gene. Amino acid residues 3-42 of the amino terminal of the

polypeptide chain are termed the "somatomedin B" domain. This domain contains eight interlinked cysteines and exhibits an overall acidic character. In the amino terminal region of the protein, four functionally important features are found. These include an arginine-glycine-aspartate (RGD) sequence that mediates binding of vitronectin to certain integrin receptors, a highly acidic region, putative crosslinking sites, and a binding site for the ECM component collagen. With its various binding domains, that include ones for associations with the glycosaminoglycan heparin and thrombin, vitronectin also has the ability to maintain different conformations. These properties allow vitronectin to be involved as an adhesive component and function in multiple haemostatic processes that include coagulation, fibrinolysis, and the plasminogen activation system (Preissner and Jenne (1)).

Vitronectin plays an important role in thrombosis and haemostasis through its associations with serine proteinase inhibitors (serpins) like anti-thrombin and plasminogen activator inhibitor type 1 (PAI-1). This association affects binding of vitronectin in the extracellular matrix to cell surface receptors like the urokinase plasminogen activator receptor, thus affecting cell adhesion, attachment and spreading, and migration. PAI-1 plays a key regulatory function in proteolytic processes that affect both tissue remodeling and tumor metastasis (Minor and Peterson). These effects of PAI-1 are mediated by its complex-formation with vitronectin and by its role in being the main inhibitor of both plasminogen activators, the urokinase type and the tissue type. A unique feature of this protein among serpins is its ability to persist in an active form that makes its reactive center loop accessible as well as a more stable inactive latent conformation that inserts the reactive center loop into the middle of the structure. When PAI-1 is bound to vitronectin, it remains in its active form for an extended period of time and its half-life is

significantly increased. This binding changes the conformation of vitronectin, leading to mulitmerization, affecting its functions in the body. The main PAI-1 binding region of vitronectin has been shown to be localized within the amino terminal somatomedin B (SMB) domain; another lower affinity binding site in the carboxy terminal region has been implied and seems to be involved in the formation of larger complexes (Schroeck, et al.). Formation of these complexes directly affects the binding of cells to the extracellular matrix through such cell surface receptors as the urokinase plasminogen activator receptor.

The plasminogen system has been found to play an important role in the remodelling of the extracellular matrix, cellular adhesion and migration; it is thus involved in inflammation, wound repair, angiogenesis, and tumor development. These effects take place by the generation of pericellular plasmin by urokinase plasminogen activator (uPA) followed by direct or indirect proteolysis of the extracellular matrix. uPA proteolytically activates plasmin by cleaving its zymogen, plasmonigen. The urokinase receptor (uPAR), which is a glycosylphosphatidylinositol (GPI) glycolipid-anchored receptor, binds urokinase and coordinates and focuses this plasmin-mediated cell-surface proteolysis. uPAR interacts with uPA in both its two-chain active form and its inactive single chain pro-enzyme form (pro-uPA) (Waltz, et al.). Although the uPAR/uPA complex is relatively stable on the cell-surface, binding of protease inhibitors like PAI-1 to urokinase causes the complex to be internalized from the cell surface and leads to degradation of uPA/PAI-1 thus inhibiting uPA activity (Cubellis, et al.). This process allows uPAR to be recycled to the cell surface and thus affects its distribution on the plasma membrane (Mondino, et al.).

By binding to vitronectin, uPAR has also been found to play a role in cellular adhesion, migration, and signaling. The roles of uPA and uPAR have been exhibited by the development and use of uPA and uPAR deficient mice; one such study has shown that uPA deficient mice are unable to support growth and progression of certain tumors (Mondino, et al.). The urokinase receptor contains a binding site for vitronectin that is distinct from the one for urokinase. The strength of the uPAR and vitronectin interaction is increased with the binding of uPAR to uPA in any of its forms; this shows that the uPAR and vitronectin binding is not dependent on proteolytic activity (Waltz, et al.). The role of uPAR in adhesion has been shown to be dependent upon functional and physical associations with another class of cell surface receptors, the integrins. Integrins promote cell adhesion, migration, and signalling by their connections to the cytoskeleton, which uPAR is presumed to utilize. Furthermore, as vitronectin is the major high affinity PAI-1 binding protein, uPAR binding to it allows for regulation of the proteolytic process that leads to cell migration and thus links this process to cell adhesion. In order for cell migration to occur, it must be coordinated with cell adhesion to the underlying cellular or extracellular matrix. It is affected by adhesion strength and reversibility, which may occur by certain proeolytic processes that include the urokinase plasminogen system (Chapman and Wei). As discussed above, the major high affinity binding site of PAI-1 on vitronectin is in the SMB domain. Similarly, research has shown that uPAR binding sequence of vitronectin is also located within the central region of the SMB domain. Another possible binding region on vitronectin is in the heparin binding domain, and it is being investigated (Deng, et al.). Data has also shown that integrins bind to vitronectin within the SMB region.

As seen, the blood glycoprotein vitronectin plays a key role in many processes through the human body ranging from wound healing to tissue remodelling and tumor progression. Studies have also shown that vitronectin has an effect on certain infectious organisms that can enter the human body. One such organism is the opportunistic fungal pathogen *Candida albicans*. It causes infections in immunocompromised hosts, such as those undergoing chemotherapy for tumor reduction in cancer patients and the prolonged use of immunosuppressive agents in organ transplant patients (Imbert et al.). It has also been found with increased frequency in patients with human immunodeficiency virus (HIV)-associated acquired immune deficiency syndrome (AIDS). *C. albicans* is a dimorphic fungus that shows morphologic switching, being able to grow in both budding yeast and filamentous hyphal form. The ability to germinate and form hyphae is thought to be essential for virulence of the organism. It disseminates within the vasculature of the host and penetrates tissues by adhering to the endothelial basement membrane and/or subendothelial extracellular matrix components. One such host ECM component that interacts with *C. albicans* is vitronectin. Several experiments have shown that microbial invasion and attachment is mediated by interacting with vitronectin; in the case of *C. albicans*, binding of vitronectin mediates fungus adherence to a macrophage cell line. Evidence has shown that the fungal cells may bind to vitronectin through certain integrin receptors (Santoni, et al.). One thus sees that, with its function as an adhesive molecule and its role in the immune system, vitronectin affects the invasion and infection of the pathogen *C. albicans*. This is also supported by recent studies which have shown that mice deficient in the vitronectin gene live longer with *C. albicans* infection than wild type mice with the infection. This has lead to the current interest in performing vitronectin binding assays with *C. albicans* cells.

Because of the many roles and functions that are carried out by vitronectin and their vast implications in the human body and on the medical field, it has been highly studied in relatively recent years. Details of the structural and mechanistic aspects of this protein will give insight into such areas as wound healing, tumor metastasis, and fungal infection. In my research, studies have been performed to determine the effects of PAI-1 on vitronectin with regard to the binding of vitronectin to the urokinase receptor. These studies were conducted *in vitro* and involved such factors as the presence of extracellular matrix extractions and the usage of the multimeric vitronectin conformation. Furthermore, experiments were conducted to develop a method to qualitatively measure the binding of *C. albicans* fungal cells to vitronectin with the presence of such factors as PAI-1 and extracellular matrix components.

**Materials and Methods**

These studies, which focused on developing a better understand of the binding of vitronectin to mammalian cells through the urokinase receptor as well as the binding of Candida Albicans cells to virtonectin, were conducted using similar methods.

In order to test the effects of PAI-1 on the binding of vitronectin to the urokinase receptor, *in vitro* experiments were performed using the methods of enzyme linked immuno-sorbent assays (ELISAs). For these experiments, vitronectin was purified from plasma in our laboratory and multimeric vitronectin was made by incubating it with 6 molar urea. Soluble uPAR was purchased from CalBiochem, and uPA was purchased from R & D Systems. Wild type and stable (14-1B) PAI-1 were purchased from Molecular Innovations. The rabbit anti-uPAR primary antibody was also purchased from Molecular Innovations. The general protocol for ELISAs are as follows. A 96-well ELISA plate was coated with vitronectin or isolated extracellular matrix overnight. The next day, 200 ul of 3% BSA in 1x PBS was added to each well for 1 hour to block the wells. For wells coated with extracellular matrix, vitronectin or a vitronectin and PAI-1 mixture was added to the wells after blocking and left for 1 hour. Both stable and wild type PAI-1 were used in these experiments. After rinsing, soluble uPAR was added to the wells along with isolated uPA (urokinase plasminogen activator) and incubated for 1 hour. The uPA/uPAR complex was formed by incubating 30 nM of each in 0.1% BSA in 1x PBS for 1 hour. Then a primary antibody was added for 1 hour. Determination of the most suitable anitbody for the experiments was conducted by performing an ELISA by binding various primary antibodies to serially diluted uPAR bound wells. Following incubation with the primary antibody, 100 ul of Peroxidase-labelled Rabbit IgG was added to each well and

incubated for 1 hour. At each step in the process, the plate was rinsed 3 times with a 1x PBS solution. Then 100 ul of a developing solution was added to each well for 1 hour. The developing solution consisted of 5 mg ABTS, 20 ul 30% Peroxide, and 25 mls 50 mM sodium citrate at pH 5.5. Finally, the wells turned a light green color and the binding was measured by reading the absorbance at 405 nm. The experiments were conducted using varying concentrations of vitronectin and PAI-1. To analyze the results, non-specific binding of the proteins was accounted for by subtracting the background binding of the compounds to the blocking agent. The majority of the experiments were carried out at room temperature, though a few mixtures were allowed to incubate at 37 C.

An ELISA based assay was also employed to test the binding of *Candida albicans* yeast cells to vitronectin. The wild type *C. albicans* SC5314 strain was used. Yeast cultures were grown for each experiment by isolating a colony of *C. albicans* from a YEPD plate stored at 4C and placing it in 50 ml solution of media. A new plate was streaked each week. Two different media solutions were used: a galactose and yeast nitrogen base solution shows greater adhesion of cells, while a YEPD solution of yeast extract, peptone, and glucose shows less adhesion properties. The cells are allowed to grow overnight at 30 C while shaking. The cells were counted and their viability determined by using a trypan blue stain and a hemacytometer. For the ELISA assay, plates were coated with extracellular matrix or vitronectin overnight. The next day, 200 ul of a 0.2% Carrageenan solution in 1x PBS was added to each well as a blocking agent and incubated for 1 hour at room temperature. When extracellular matrix was used, varying concentrations of vitronectin were incubated for 1 hour at room temperature following blocking. At this point, 100 ul of a 3x10^7 cells/ml solution of cells was added to each well, and

the plate was incubated at 37 C for 2 hours to ensure binding. After the cells were incubated on the plate for 2 hours at 37C, 200 ul of a developing solution of 0.3 mg/ml XXT, 1.3 mM Menadione, and 1x PBS was added to each well. As done with the uPAR assays, the wells were rinsed 3 times with 1x PBS at each step in the process. After the developing solution was incubated on the plate at 37 C for 1 hour, the plate was read by spectrometry at an absorbance of 490 nm. Again, background binding of the cells is accounted for by subtracting the measurements of binding to the blocking agent.

## Results

### uPAR binding assays:

Urokinase receptor (uPAR) binding assays, which are used to give qualitative measures, were performed to develop a better understanding of the binding of the urokinase receptor to vitronectin, multimeric vitronectin, and vitronectin in the presence and absence of extracellular matrix (ECM). Studies were also performed to determine the effects of PAI-1 on the interactions between uPAR and vitronectin. All assays were repeated until results were consistent. Figure 1 shows the effect of the binding affinities of the urokinase receptor to vitronectin when in the presence and absence of ECM and in the multimeric and monomeric forms. One clearly sees that the multimeric vitronectin form binds to uPAR with very little affinity both in the presence and absence of ECM. The presence of ECM increases the binding of monomeric vitronectin to uPAR. The association between uPAR and multimeric vitronectin is so low that assays with it and PAI-1 were not pursued. Figure 2 shows the effects of wild type and stable PAI-1 on the binding of uPAR to monomeric vitronectin in the absence of ECM. This assay illustrates that both types of PAI-1 competitively inhibit uPAR from binding to monomeric vitronectin by a similar degree. Figure 3 shows similar results for the effects of wild type and stable PAI-1 on the binding of uPAR to monomeric vitronectin in the presence of ECM. From looking at the general numbers of the assays in figures 2 and 3, one again sees the enhanced interaction of uPAR with vitronectin in the presence of ECM. Overall, the data shows that the interaction between uPAR and vitronectin is negatively affected by the addition of PAI-1. The highest amount of binding of vitronectin to the urokinase receptor is seen in the presence of ECM, the use of monomeric VN, and the absence of PAI-1. These results were shown to be consistent at

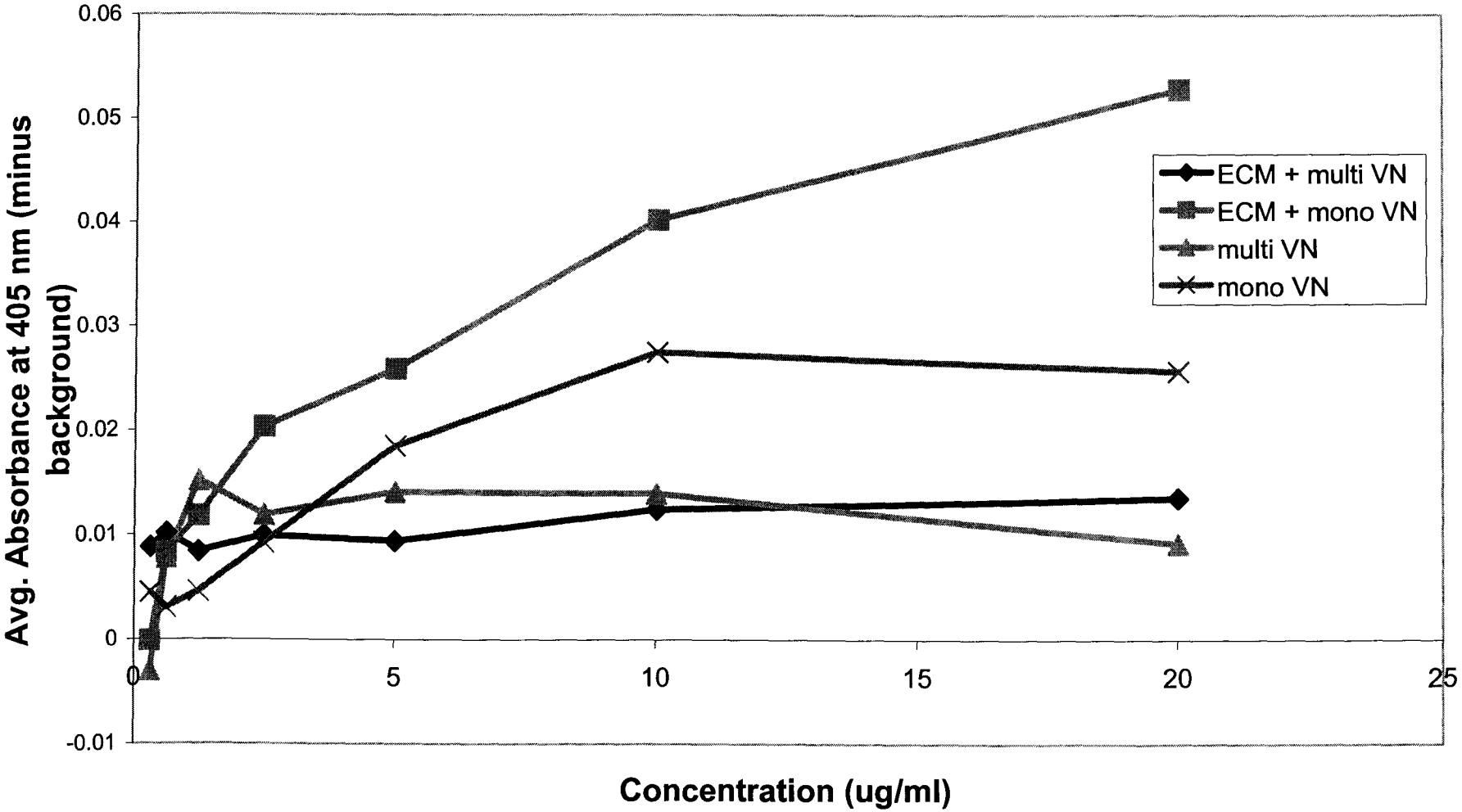
various concentrations of the substrates. A linear relationship is also seen in all figures between the substrate concentrations and the amount of binding.

***Candida albicans* binding assays:**

In order to test the binding of *C. albicans* cells to vitronectin and the effects of certain factors including PAI-1 on this binding, certain experimental conditions needed to be determined. Assays were performed to determine the optimal growing conditions for the C. albicans cells, the optimal blocking agent for the assays, and the optimum growth media for the cells. The binding of the cells to ECM was also tested. As with the uPAR assays, experiments were repeated to show consistency in data. Figure 4 shows the effects of two growing methods on the binding of different concentrations of cells to the ECM. The conditions that were altered included temperature, time allotment for incubation, and amount of shaking for the growth of the cells. Cells were grown at 30 C overnight with shaking and at 37 C for 36 hours without shaking. Cells grown under these two conditions showed similar amounts of growth and hyphal formation, and as seen in figure 4 , they showed similar amounts of binding to ECM. The conditions chosen for the assays involved cell growth at 30 C overnight with shaking. Figure 5 shows the data from assays that were performed to determine which type of blocking agent should be used in the ELISA method to block the cells from binding to the vacant plastic regions of the wells. From these experiments, a .2% carrageenan solution was found to be the solution to which the least amount of cells bound. This was shown in comparison with Super block, Casein block, and BSA; blank wells with no addition of a blocking agent were used as a control. Figure 6 shows the effects of different growth media on the adhesion properties of the *C. albicans* cells to ECM. As seen in the figure, growth of cells in galactose media caused the fungal cells to

become very adhesive, while the use of YEPD media caused cells to exhibit too little adhesive properties. Because of the linear binding relationship cells grown in the galactose media to ECM at the lower concentrations, this media was chosen for further assays. Also, as seen in figure 6, assays showed that the fungal cells do exhibit increased binding to ECM with increased ECM concentration. Figure 7 shows the binding of the fungal cells to various concentrations of vitronectin at constant ECM concentrations. Few differences between vitronectin concentrations were observed, though a linear relationship seems to be present at low vitronectin concentrations at the 2.5 ug ECM concentration. Thus, assays with vitronectin have been inconclusive, and addition of PAI-1 has not yet been performed.

**Figure 1: uPAR Binding Assay showing decreased binding with use of multimeric VN form and increased binding with presence of ECM**



# Figure 2: uPAR Biding Assay showing competitive binding of PAI-1 to monomeric VN in absence of ECM

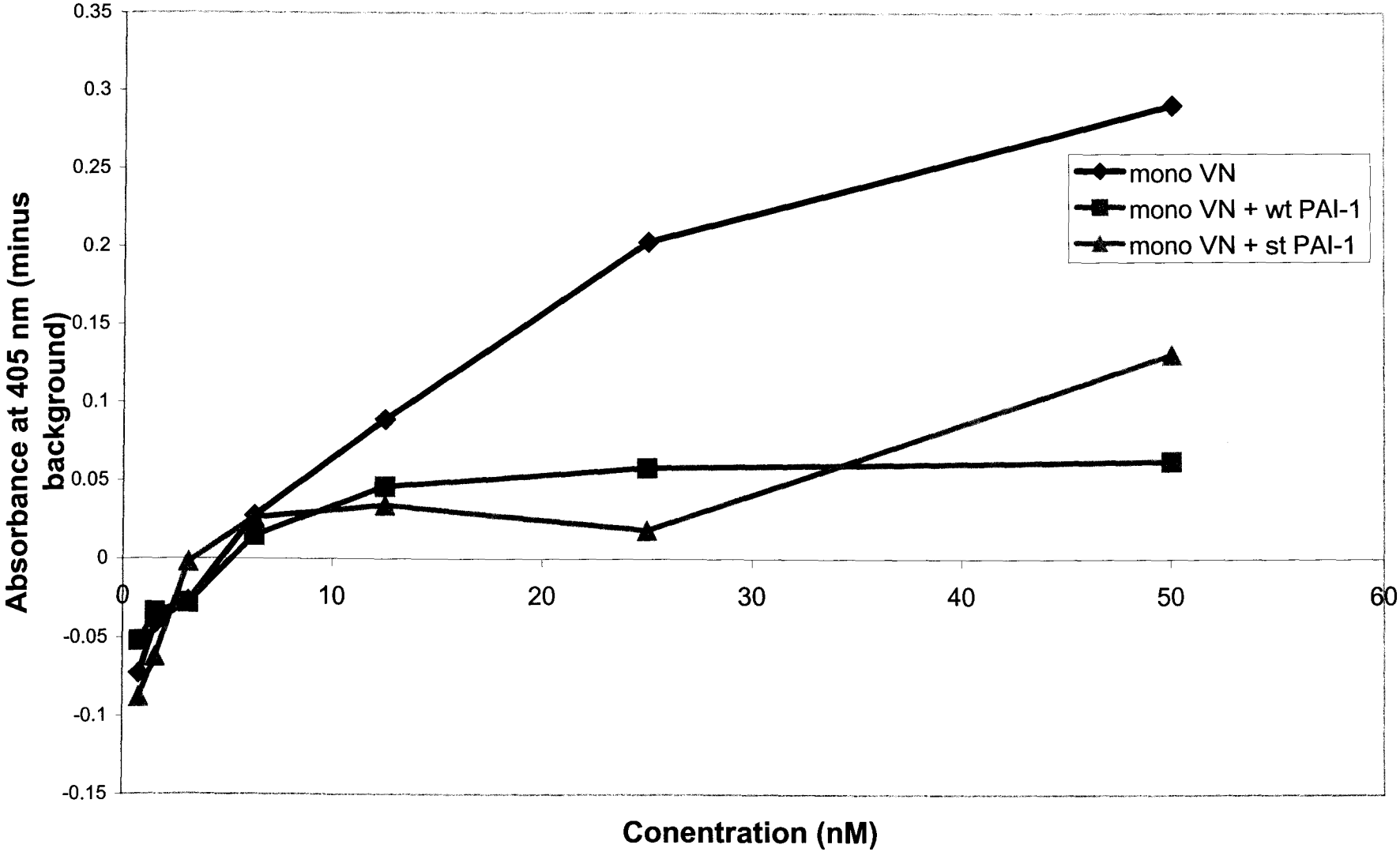


**Figure 3: uPAR Binding Assay showing competitive binding of PAI-1 to monomeric VN in presence of ECM**

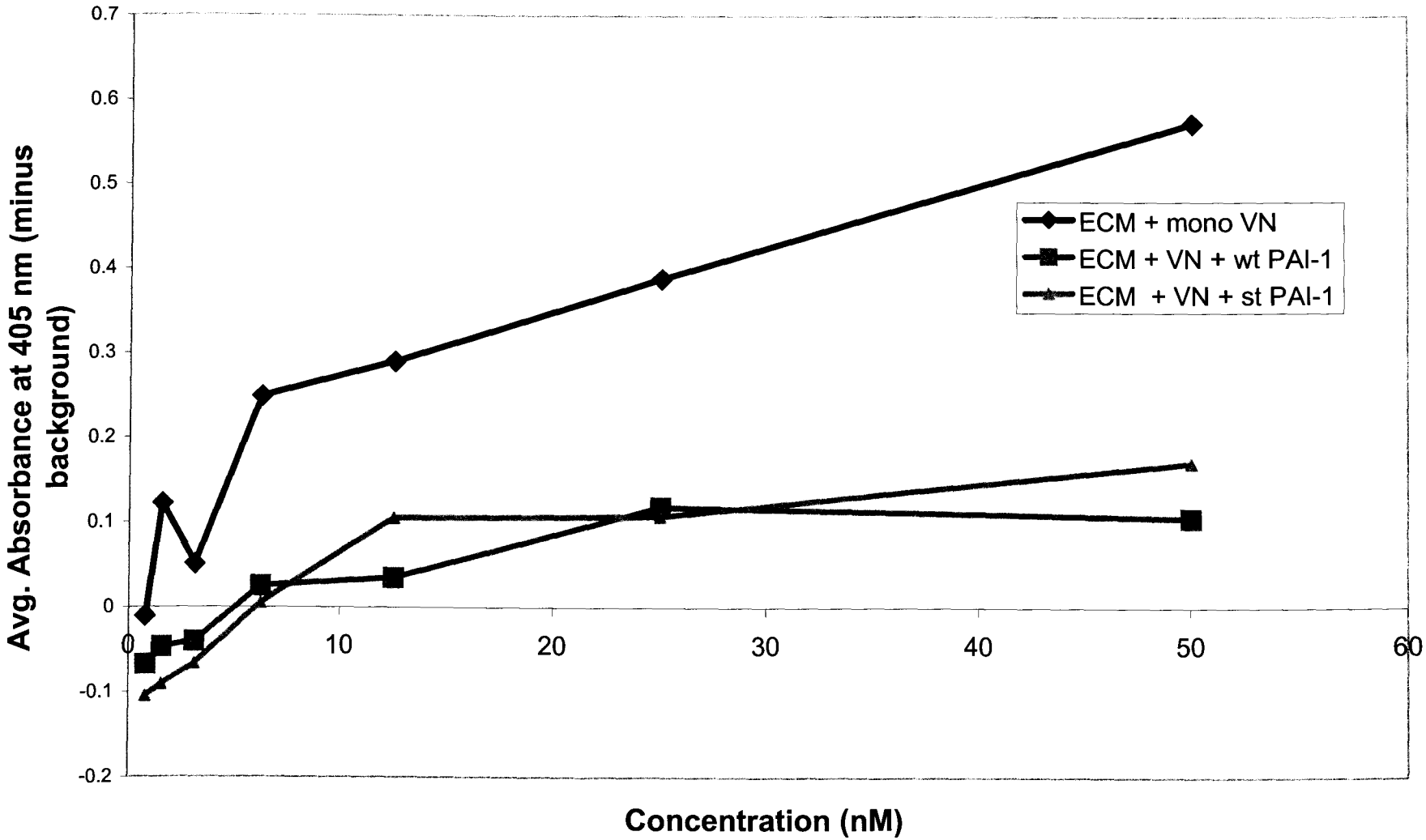
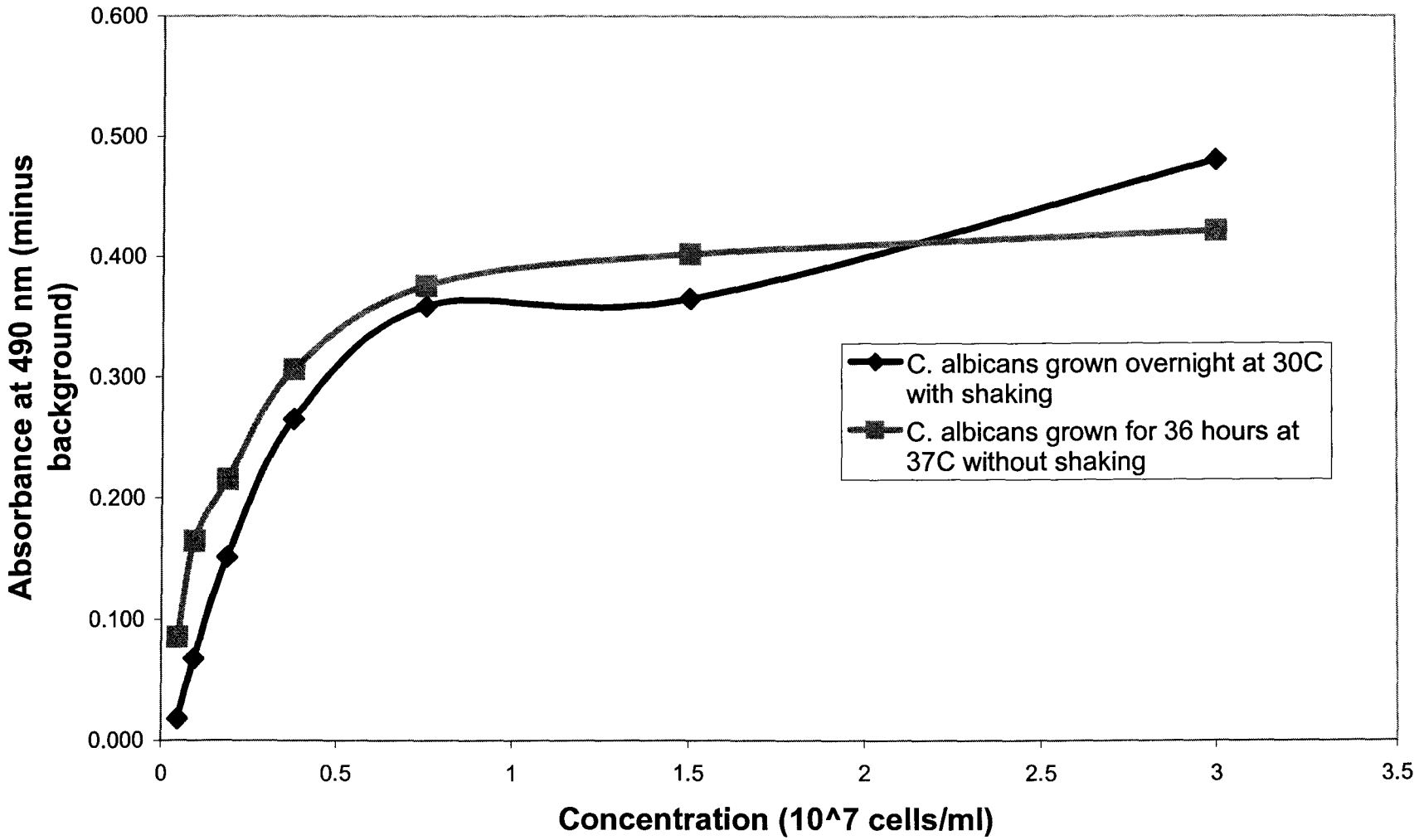


**Figure 4: C. albicans assay showing little difference in binding to 5 ug/ml ECM due to listed growth conditions**



# Figure 5: C. albicans assay showing least cell adhesion to Carrageenan blocker

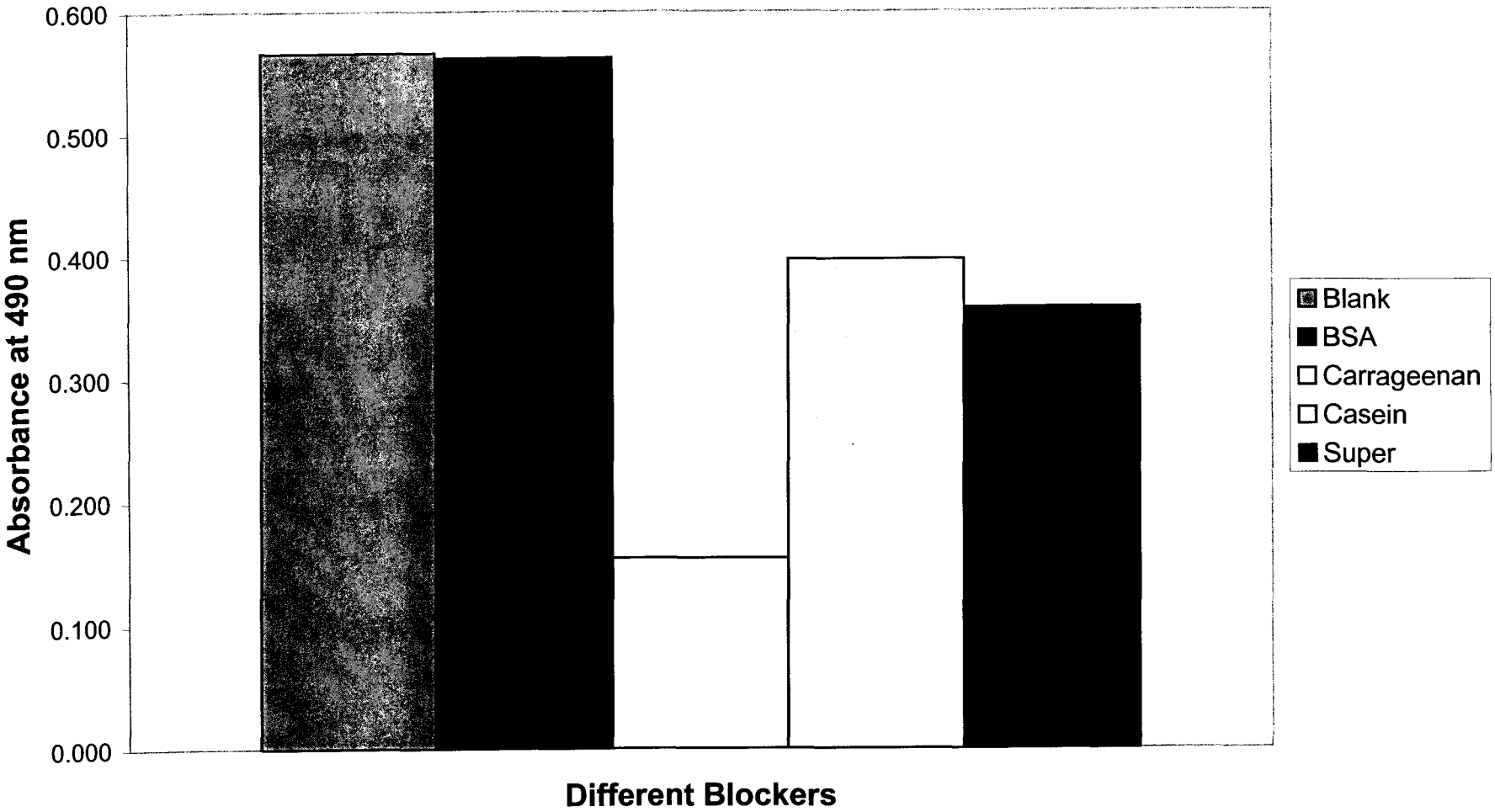


**Figure 6: C. albicans assay showing adhesive nature of cells by type of growth media**

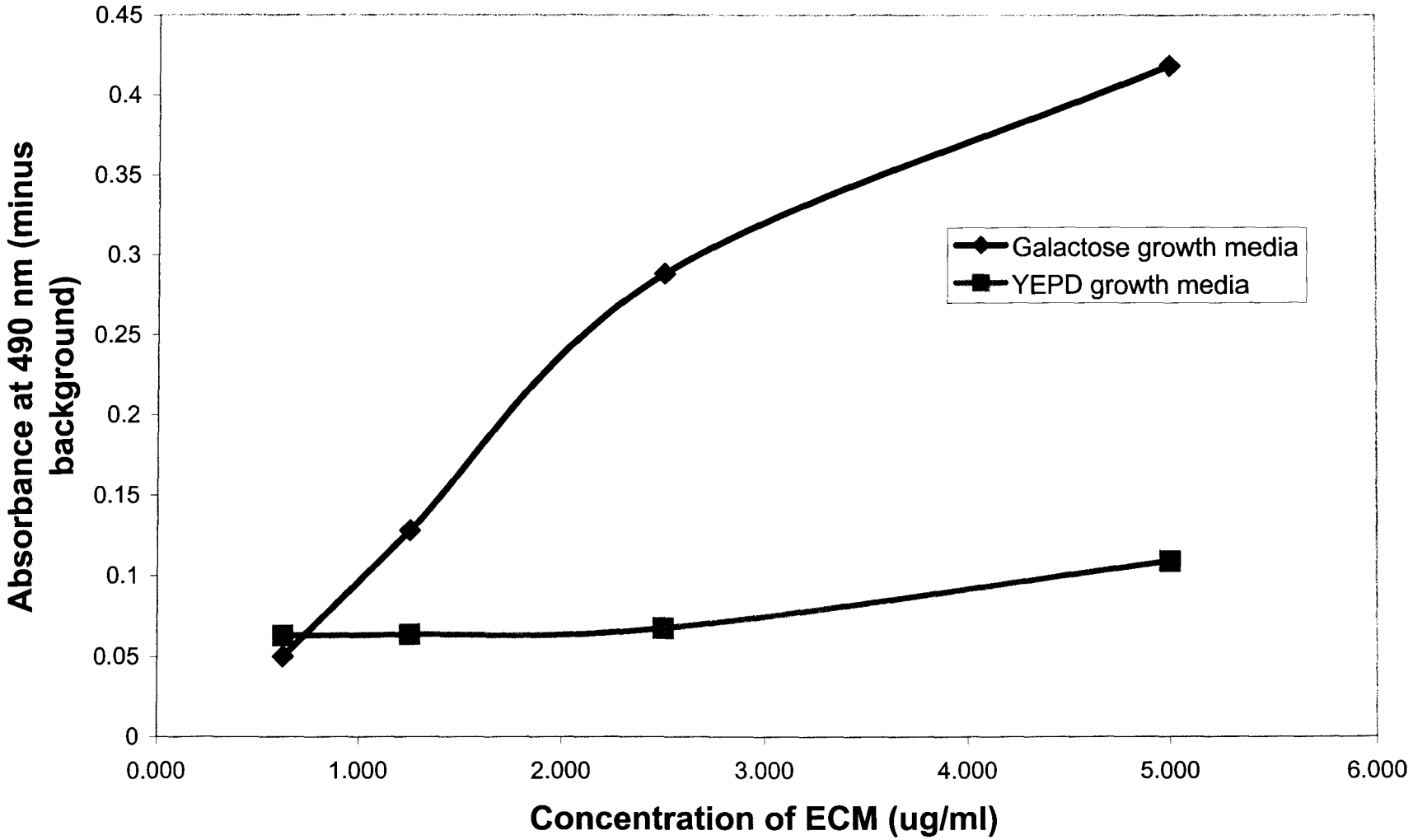
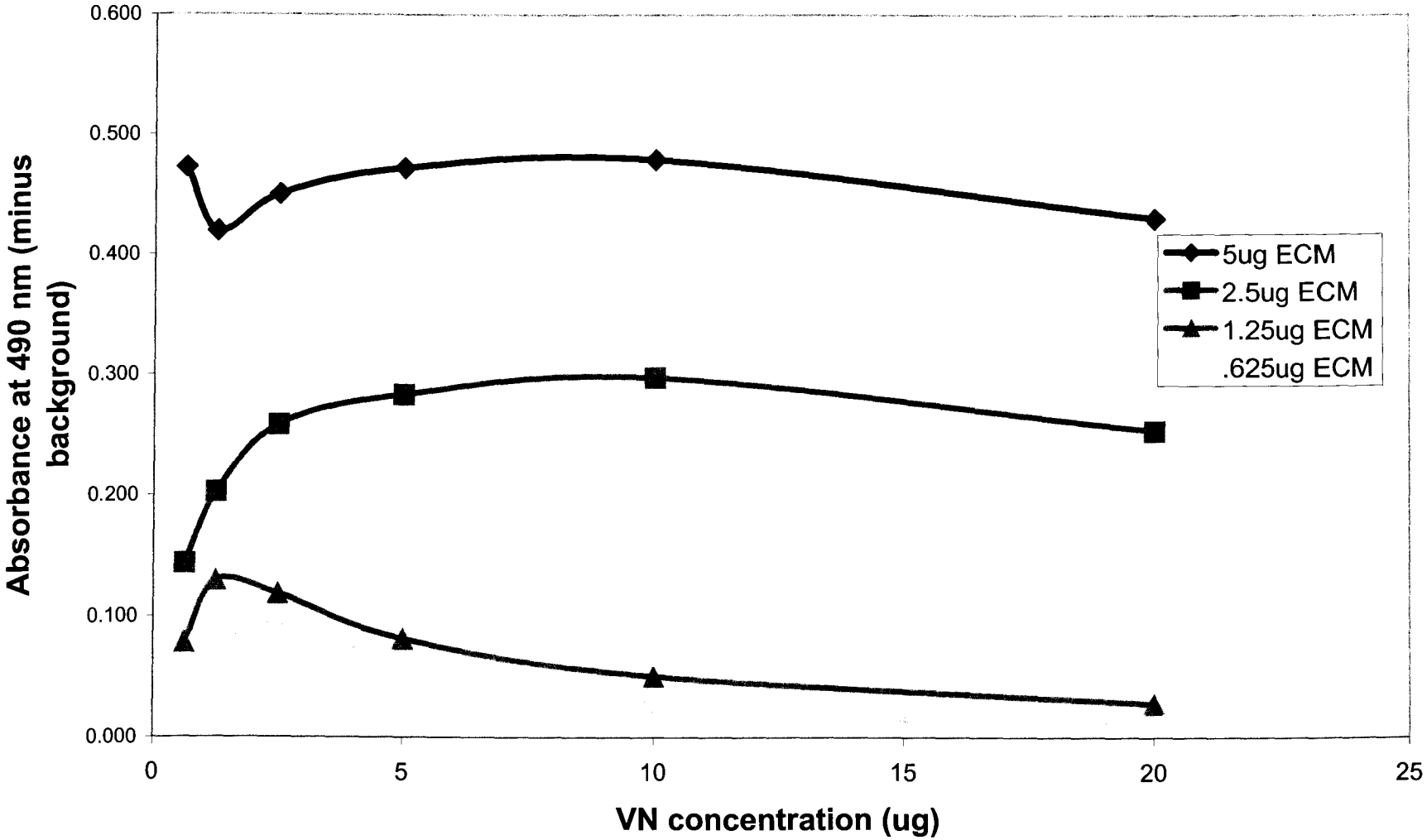


**Figure 7: C. albicans assay showing few differences in binding with varying vitronectin concentrations at constant ECM conentrations**



## Discussion

The urokinase receptor binding assays showed that PAI-1 competitively inhibits the binding of vitronectin to the cell surface receptor uPAR. This is opposite to observations made on the interactions of PAI-1 and vitronectin with the integrin class of receptors. The effects of the presence of ECM and the use of the multimeric vitronectin form were also presented; ECM presence enhanced the binding while the use of the multimeric form lessened it. These findings can give insight into the mechanisms and functions of the urokinase plasminogen activator system, which is involved in many important processes in the human body. This system is comprised of the serine protease urokinase (uPA), uPAR, PAI-1, and the proenzyme plasminogen which is cleaved by uPA to form the proteolytic enzyme plasmin. This system interacts with vitronectin to promote cell adhesion, migration, and signaling. Two schemes have been developed to explain their role in these processes. In one scheme, by binding uPAR to vitronectin, cell adhesion is promoted; when PAI-1 is present and binds to vitronectin, it is released from uPAR and the cell is free to migrate. In the other scheme when PAI-1 concentration is low, uPA is active and cleaves plasminogen to form plasmin, which cleaves the ECM and allows the cell to migrate (Preissner, et al.). The results of this study support the idea that PAI-1 binds to vitronectin and releases it from uPAR.

This plasminogen system has been shown to be involved in many processes that affect normal human development and health such as wound healing, tissue remodeling, and cancer. While the mechanistic functions of the urokinase receptor have been widely researched in terms of its role as a proteinase receptor in the plasminogen system, evidence has also shown that it is involved in signaling. This is largely due to its association with such proteins as vitronectin, members of the

integrin adhesion receptor superfamily, and a G-protein coupled receptor. These interactions allow for the transduction of intracellular signaling pathways that involve cytosolic and transmembrane kinases, cytoskeletal components and components of the cytoskeleton. These pathways include effects on cytoskeletal reorganization, intracellular calcium mobilization, and transcription factor activation (Blasi and Carmeliet).

Increasing evidence has shown that the urokinase plasminogen system, and specifically uPAR, is an important component in the progression of cancer, in tumor invasion, metastasis and angiogenesesis. Many clinical studies have shown that high levels of components of this system are found in tumors and lead to poor patient outcomes. Many of the growth factors needed for neovascularization, such as vascular endothelial growth factor (VEGF) and interleukins, have been found to upregulate the levels of uPA and uPAR in multiple cell types, such as macrophages and tumor stromal fibroblasts, which are involved in tumor progression. Other experimental studies have demonstrated that regulation of the expression of such plasminogen system components is beneficial to patient prognosis and outcome; this was shown in both animal and human systems (Mazar, et al.). As angiogenesis is a process that is necessary for normal development, the urokinase plasminogen system has also been implicated to play a role in cardiovascular function due to its interactions with integrins, extracellular matrix, and vitronectin (Preissner, et al.). This evidence supports the idea that inhibitors of uPAR and uPA may serve as potential therapeutic agents against angiogenesis and metastasis. In order to be able to develop such therapeutic agents, the mechanistic processes of this system and its relation to other factors in the human body must be better understood. The results of this study are a step in this direction.

Developing a method to test the binding of *Candida albicans* cells to vitronectin in the presence of extracellular matrix and PAI-1 is increasingly important due to the growing evidence of certain integrin like vitronectin receptors on the fungal cells. These studies would allow for the development of the mechanistic aspects of *C. albicans* invasion and pathogenesis as vitronectin has been hypothesized to be involved. This is supported by the evidence showing that the distribution of vitronectin in circulation and in the ECM overlaps with *C. albicans* tissue dissemination. Vitronectin has been found to mediate interactions of the fungal cells with phagocytic cells and to human endothelial cells. As *C. albicans* is an opportunistic pathogen that is capable of causing serious diseases, a better understanding of its mechanistic function may lead to beneficial outcomes in the development of anti-fungal therapies (Spreghini, et al.).

As seen, the results of this research are important to understanding human health and diseases and may lead to the development of therapeutic agents. Further research must be performed, though, to provide a better understanding of the detailed processes and interactions involved in the urokinase plasminogen system and the *C. albicans* infection system. With regard to urokinase receptor binding, future studies should include the use of high uPAR expression mammalian cells to demonstrate the binding interactions. With regard to the C. albicans assays, a more appropriate growth medium should be investigated for adhesive properties and assays testing the effects of PAI-1 on binding should be performed.

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