The Role of Mammary Epithelial Cells in the Adhesion of Breast Carcinoma Cells to Basement Membrane

Amit Gupta ~
Biological Sciences and Chemistry

Author

Amit discovered that he wants to continue doing research in the future, as well as attend medical school. As he explored different fields, he was able to envision himself doing cancer research beyond graduation. Amit describes his favorite research experience as the time he worked with three different types of snake venom in relation to cancer cell lines, which helped him to realize "how broad the applicability of science is." Amit strongly encourages all students to participate in research and feels that research has enhanced his education: "Due to the practicality of research, I have actually relearned my past knowledge, but this time with clearer definitions and better understanding."

Key Terms:

- Adhesion
- Basement membrane
- Breast cancer
- Conditioned medium
- Integrin
- Mammary epithelial
- Metastasis

Abstract

The inception of host invasion by tumor cells is characterized by the adhesion, or the physical attachment of the cancer cell to the basement membrane (BM). This process is mediated by integrins, which may be up-regulated by various transmembrane receptor proteins and ligands called cytokines. This study was performed to determine whether normal epithelial mammary cells (clone 184A1) increase cancer cell adhesion. We found that the MCF-7 breast cancer cell line binding to reconstituted BM increased in the presence of 184A1 conditioned medium (CM). Furthermore, 184A1 CM increased adhesion of three breast cancer cell lines (MCF-7, SK-BR-3, and BT-20) to the specific proteins of the BM (fibronectin, vitronectin, and laminin) but not to the extracellular matrix protein collagen I. The effect was partially reversed by peptides known to inhibit binding between the cell and the BM by blocking integrin-binding sites. These results suggest that normal mammary cells play a role in the progression of human breast cancer.

Faculty Mentor

Amit Gupta has shown that normal and cancer cells may interact in ways that promote the most dangerous behavior of cancer cells, namely their ability to spread throughout the body. Cancer spread begins when the tumor cells bind to the basement membrane, which is normally a barrier to cancer cell invasion. This research has shown that secretions of normal breast cells increase this binding process in breast cancer cells in culture. These findings raise the possibility that inhibiting this interaction may provide a way of blocking breast cancer spread. The opportunity to do biomedical research as an undergraduate not only allows the student to learn how to gain understanding of disease processes, but it also allows the student to make a direct contribution to the body of scientific knowledge.

~ Philip Carpenter
College of Medicine

Introduction

One of the major reasons cancer results in death is because of its ability to invade various tissues throughout the body. The spreading, or metastasis, of the tumor cell is at the heart of the malignant nature of the disease. In order to become malignant, the cancer cell must progress through three major stages: 1) adhesion, 2) invasion, and 3) metastasis. First, it separates from the adjacent cells. Then, adhesion occurs when the precancerous cell binds to the basement membrane, composed of a thin layer of structural proteins including fibronectin, vitronectin, laminin, Collagen IV, and others (Kumar et al., 1992). Next, it degrades the membrane proteins by a process called proteolysis. Finally, it migrates through the BM into the extracellular matrix, which is composed of collagen I, carbohydrate ground substances, fibroblasts, and other connective tissue cells (Kumar et al., 1992). Hence, adhesion is the pivotal step that occurs early in tumor metastasis. Two lines of evidence support the notion that adhesion is important for invasion (Kumar et al., 1992; Pignatelli and Vessey, 1994). For example, complex and coordinated reductions and increases in adhesion, mediated by cell-cell adhesion molecules (CAMs) and substratum adhesion molecules (SAMs), are necessary for tumor invasion (Hynes and Lander, 1992; Hynes, 1992).

An important group of proteins that mediate, or up-regulate, adhesion are integrins. Integrins are cell surface transmembrane glycoproteins which exist as noncovalently associated $\alpha\beta$ heterodimers (Hynes, 1992). They are expressed by the epithelial cells, as well as other cell types (Hynes, 1992). They show alternative specificity in binding to the BM when expressed in different cell types (Elices and Hemler, 1990; Languine et al., 1989). These integrins primarily bind to the arginine-glycine-aspartic acid (RGD) sequence, which is present in the amino acid sequence of the BM proteins fibronectin and vitronectin. Laminin, on the other hand, binds through the tyrosine-isoleucine-glycine-serine-aspartic acid (YIGSR) sites. Recent studies show that the integrin-dependent interaction of melanoma cells with extracellular matrix (ECM) components can regulate key biological processes, such as cancer cell proliferation and invasion (Hart, 1991). However, the addition of RGD-containing synthetic peptides, or naturally occurring RGD-containing proteins (known as disintegrins), disrupts integrin-mediated binding of cells to the BM. Hence, these peptides act as competitive inhibitors of integrin binding.

Cytokines also play distinctive roles in regulating cell-matrix and cell-cell interaction by influencing the expression of integrins during inflammation and remodeling of damaged tissue (Heymann et al., 1995). Previous studies have shown that the inflammatory cytokines IL-1β and TNF-α increase the expression of $\alpha1\beta1$ integrins on a human osteosarcoma (bone cancer) cell line, thereby increasing its adhesion to laminin (Santala and Heino, 1991). Cytokines have been shown to enhance the expression of $\alpha5\beta1$ on a melanoma cell line, increasing cell adhesion to fibronectin. These cytokines can also decrease the $\alpha6\beta1$ integrin in human endothelial cells (Defilippi et al., 1992). Therefore, cytokines can promote or repress cell adhesion.

Other cytokines and growth factors are also known to enhance cell-BM interaction by increasing integrin expression. For example, the expression of $\alpha2\beta1$- and $\alpha3\beta1$-integrins on BT-20 and $\alpha2\beta1$-integrin on SK-BR-3 breast cancer cells (which strongly express epidermal growth factor (EGF) receptors) was markedly increased by an addition of the heparin-binding EGF-like growth factor (HB-EGF) (Marita et al., 1996). Similarly, Ignotz et al. (1987) showed that transforming growth factor-β (TGF-β) elevates the expression of cell adhesion protein receptors (Ignotz and Massague, 1987; Ignotz et al., 1989). Hieno and his colleagues (1989) further observed that TGF-$\beta1$ regulates the expression of individual integrin subunits by a parallel, but independent, mechanism that results in the modulation of cell migration, arrangement, and development – all of which are guided by adhesion to the extracellular matrix (Heino et al., 1989).

The primary interest of this investigation is the role of normal breast cells in cancer progression. Previously, our research team observed that normal mammary duct cells (clone 184A1) secrete one or more proteins which enhance the migration and/or invasion of breast cancer cell lines (MCF-7, SKBR-3, and BT-20). We now show that in the presence of a 184A1 mammary epithelial cell conditioned medium, the adhesion of the cancer cell to fibronectin, vitronectin, and laminin increased four-fold. Furthermore, we demonstrate that the addition of peptides containing arginine-glycine-aspartic acid-serine (RGDS) or YIGSR sequences, which block the integrin-mediated binding of cells to the extracellular proteins (Ruoslahti and Pierschbacher, 1987), partially block 184A1-induced adhesion of the cancer cells. These results support the hypothesis that there is an induction of tumor cells binding to the basement membranes by proteins secreted into the conditioned medium of 184A1 cells. This correlation is significant in that it raises the possibility that normal breast epithelium contributes to breast cancer progression by increasing integrin-mediated binding of cancer cells to the basement membrane. This blocking mechanism may provide a potential target for novel cancer therapeutic interventions.

Material and Methods

Tissue culture

Tissue culture medium components were obtained from Sigma (St. Louis, Missouri) except where otherwise indicated. MCF-7 cells were donated by Dr. D. Mercola (Research Scientist at Sidney Kimmel in San Diego, Califor-

nia). SK-BR-3, BT-20, and 184A1 normal breast cells came from American Type Culture Collection (Rockville Pike, Maryland). MCF-7 cells medium consisted of RPMI 1640 mammalian cell culture medium containing 4mM glutamine, 0.2 U/mL bovine insulin, 10 U/mL penicillin, 10 µg/mL streptomycin, 5% newborn calf serum (Irvine Scientific in Santa Ana, California), and 10 nM estradiol. BT-20 and SK-BR-3 were cultured in RPMI 1640 with 10% fetal bovine serum (Gemini in Calabasas, California), 4 mM glutamine, 0.5 mg/mL bovine insulin, and antibiotics. Finally, 184A1 normal breast cells were grown in mammary epithelial growth medium, an immortal but not transformed cell line derived from normal mammary ducts (Clonetics in San Diego, California).

Collection of conditioned medium

A conditioned medium (CM) is a medium in which cells have been maintained and contain a substrate secreted by that cell type. CM was collected from 70-100% of the confluent plates of 184A1 cells. In order to minimize the amount of exogenous protein in the CM, the mammary epithelial growth medium (MEGM) used to establish the plate was discarded. The plate was rinsed with phosphate-buffered saline (PBS) and the medium was replaced with RPMI without serum. After 24-72 hr, the CM was passed through a filter with 0.2 µm pores to remove cell debris and possible bacterial contaminants. The number of viable cells producing the CM were counted with a hemocytometer. Under these conditions, the cells remained viable and continued to secrete proteins into the medium. The daily secretion products of a given number of cells could be determined for each batch of CM. The medium typically contained the products of $5x10^5$ cells per mL.

MCF-7 binding to Matrigel

Matrigel (Collaborative Biosystem in Dedham, Massachusetts) was diluted 1:9 with RPMI 1640. In wells of a 24-well plate, 100 µL of the Matrigel was added and incubated for 30 min at 37 °C.

After 30 min, either 100 µL of BCM or 184A1 CM was added to triplicate Matrigel-coated wells. Then, 100,000 MCF-7 cells were added to each well and incubated for 90 min at 37 °C.

After 90 min, the solution with non-adherent cells was carefully removed and the wells were washed with 500 µL of PBS. The plates were then stained with 2% crystal violet for 30 min. They were then washed with water and dried. All washes were performed gently to prevent dislodging of adherent cells.

Breast Cancer cell binding to basement membrane proteins

In a sterile 96-well plate, 20 µL of each of the following were added to the individual wells: fibronectin solution (1 mg/mL), vitronectin solution (5 µg/mL), laminin solution (1 mg/mL), or collagen I solution (5 mg/mL). The plates were then incubated at 37 °C for 24 hr to allow the proteins to bind.

After 24 hrs, one of the following of the fibronectin-coated wells was added to each well: 10 µL of RPMI, 14 µL of 184A1 CM, 10 µL of RGDS solution (1 mg/mL), or a combination of 10 µL of RGDS and 14 µL of 184A1 CM. This was repeated for the vitronectin and collagen I coated wells. Collagen I is an extracellular matrix protein included to compare the extent of cell binding to it with cell binding to the basement membrane proteins, such as vitronectin, fibronectin, and laminin. For the laminin-coated wells, all variables were held constant except RGDS, which was substituted with YIGSR peptide solution (1 mg/mL). RGDS peptide functions as an inhibitor of integrin-mediated cell binding to fibronectin, vitronectin, laminin, and collagen, but YIGSR is more efficient in inhibiting adhesion to laminin. To each combination of protein-coated well and its subsequent addition, 10,000 MCF-7 cells in 100 µL were added. The experiment was repeated using 10,000 BT-20 cells or 10,000 SK-BR-3 cells in 100 µL. Each cell type, 184A1 CM, RGDS, YIGSR (peptides which inhibit adhesion), or a combination of two, was examined for its effect on the adhesion of these cells to four different proteins. The amount of 184A1 CM added was equivalent to the daily secretory products of 10,000 184A1 cells.

After 90 min at 37 °C, the medium was gently removed and followed by a rinse of 50 µL of PBS. The wells were stained with 2% crystal violet for 30 min and then were rinsed with water. Finally, the cells were counted per mm^2 from under a 1 mm grid. Again, all the rinses were done gently in order to prevent dislodging of adherent cells.

Results

MCF-7 binding to the Matrigel

The main objective of this project was to determine whether 184A1 CM increases cell adhesion to the Matrigel, a mixture of basement membrane proteins. In this experiment, 100,000 MCF-7 cells in 1 mL of media were allowed to settle on Matrigel-coated wells for 90 min. The adherent cells were then stained and counted. The data shows that in the presence of 184A1 CM, MCF-7 binding to Matrigel increased by 50%. Specifically, in the absence of 184A1 CM, 102 cells per mm^2 bound to the Matrigel after 90 min. In the presence of 184A1 CM, 152 cells per mm^2 were bound.

To determine if this was due to an interaction with a specific protein and if it was common to other breast cancer cell lines, the experiment was repeated using purified BM proteins and two other cell types.

Breast cancer cells binding to basement membrane proteins

The main purpose of this experiment was to determine whether the cells would adhere more efficiently to purified BM proteins in the presence of 184A1 CM. In this experiment, 10,000 MCF-7, BT-20, or SK-BR-3 cells in 100 mL of media were allowed to settle on either fibronectin, vitronectin, laminin, or collagen I for 90 min. The adherent cells were then stained and counted (see Figure 1).

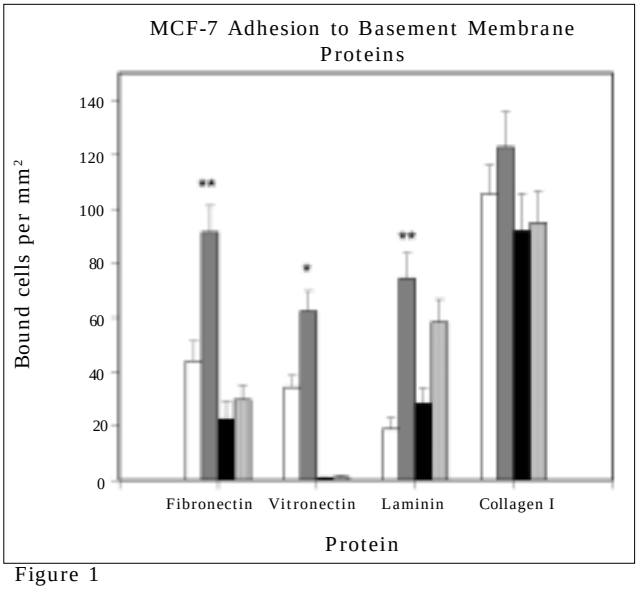

Figure 1

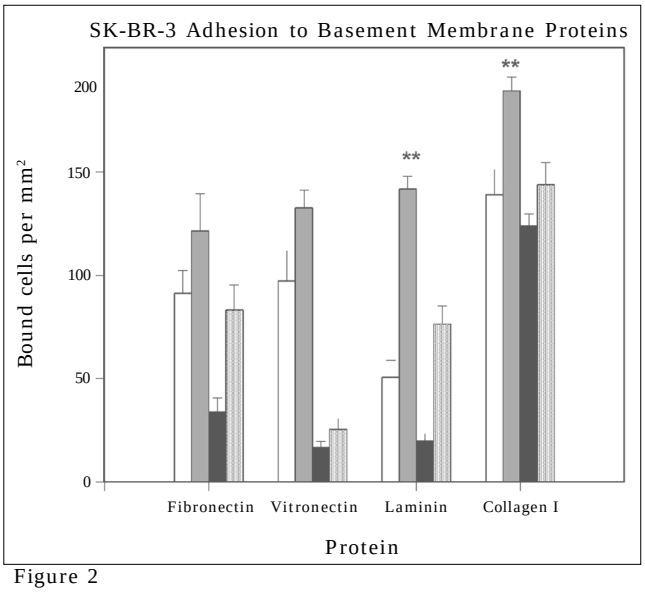

Figure 2

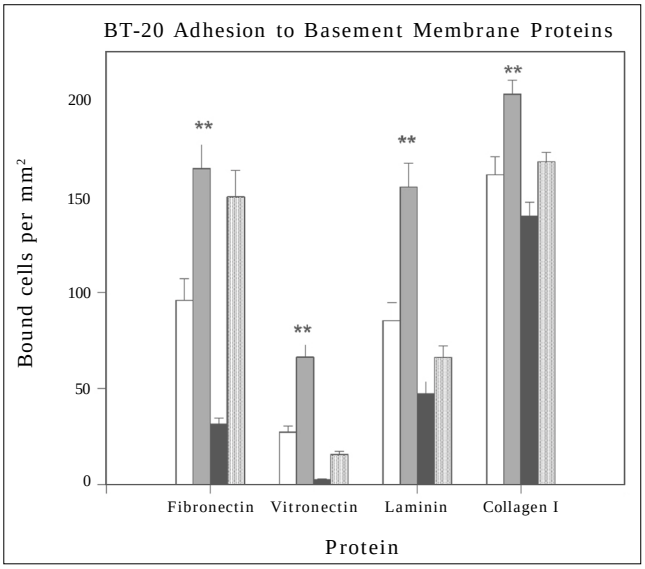

Figure 3

Figures 1, 2, 3. 10,000 MCF-7, BT-20 or SK-BR-3 cells in 100µL of media were allowed to settle on fibronectin, vitronectin, laminin, or collagen I for 90 min. The adherent cells were then stained and counted. In Figure 1, 2, and 3, each bar shows the number of bound cells per mm² for each of the proteins. The bars are defined as follows: 1) white is RPMI (control), 2) gray is the addition of 10% 184A1 CM, 3) black is 0.1 mg/mL RGDS (except for laminin, it is 0.1 mg/mL YIGSR), and 4) hatched lines are 10% 184A1 CM and 0.1 mg/mL of the appropriate inhibitor peptide. * and ** indicate $p < 0.05$ and 0.005, respectively, compared to RPMI-treated controls.

For fibronectin and vitronectin, secretion products of 184A1 normal breast cells increased binding in all breast cancer cell types but was less pronounced in SK-BR-3 cells. Although increased binding was observed in SK-BR-3 cells, it was not statistically significant ($p<0.05$ by Student's unpaired t-test). A significant increase in laminin binding was observed in the presence of 184A1 CM for all cell types ($p<0.001$ for each cell line). All cancer cell types bound strongly to collagen I, but little or no increase in binding to collagen I was induced by 184A1 CM.

The inhibitory peptides decreased binding of all cell types to fibronectin, vitronectin, and laminin. One exception was MCF-7 cells, which showed a low level of laminin binding (i.e., it was not decreased by YIGSR). In addition, 184A1-induced binding to laminin was not greatly decreased by YIGSR in MCF-7 cells. Therefore, the induced binding does not appear to be integrin-mediated. Similarly, for BT-20 and SK-BR-3, 184A1-induced binding to fibronectin was not integrin-mediated.

It was consistently observed throughout these two experiments that adhesion was increased with the addition of 184A1 mammary epithelium CM. Even in the presence of RGDS or YIGSR, 184A1 CM increased the adhesion of cells to fibronectin, vitronectin, and laminin. The exception was collagen I, for which there was no significant increase in adhesion.

Discussion

Our findings demonstrate that the normal epithelial mammary cell line-184A1 CM-secrete substances that increase cancer cell adhesion to three of the proteins located in the basement membrane. In the untreated condition, breast cancer cells adhered to reconstituted BM material. In addition, the presence of 184A1 CM increased adhesion by 50%. Furthermore, untreated cancer cells adhered to specific BM proteins (i.e., fibronectin, vitronectin, and laminin). Adhesion of the MCF-7 ($p<0.003$) and BT-20 cells ($p<0.001$) to these BM proteins was significantly increased in the presence of 184A1 CM. In SK-BR-3 cells, adhesion to only laminin was increased ($p<0.0001$). In the presence of the integrin binding inhibitors, RGDS or YIGSR, adhesion of all three cell lines to the BM proteins was decreased in the absence of 184A1 CM. When the 184A1 CM was introduced to the latter system, the cell adhesion increased slightly in most cases. This suggests that 184A1-induced adhesion to these BM proteins is mediated by integrin binding inhibitors.

Several cytokines and growth factors are known to increase adhesion to the BM proteins by over-expressing integrin in variety of cell lines (Santala and Heino, 1991; Defilippi et al., 1992; Marita et al., 1996). Our observations raise the possibility that 184A1 CM increases cell adhesion to the BM proteins by producing cytokines that induce integrin expression. Alternatively, the 184A1 normal breast cells may secrete proteins that directly bind the cells to the substrate (i.e., a glue-like molecule). When RGDS or YIGSR was added to 184A1 CM, adhesion decreased compared to 184A1 CM alone in at least two BM proteins for each cell line. These findings suggest that 184A1 CM may have induced integrin expression on the cell line. Binding to collagen I was not increased, however, nor was it greatly inhibited by RGDS. Since collagen I is an extracellular matrix (ECM) protein not found in BM, the adhesion induced by 184A1 CM is more selective towards BM rather than ECM proteins.

Conclusion

Adhesion plays a major role in cancer progression because it is the first step of invasion (tumor spread). Invasion causes the clinical effects of cancer and metastasis and ultimately results in the death of the patient. Our study shows that 184A1 normal breast cells are involved in mediating induction of adhesion of cancer cells, suggesting that factors present in normal breast cells contribute to the malignancy. This possibility is supported by other work in the laboratory that suggests 184A1 CM increases invasion through Matrigel. This interaction between normal cells to increase cancer cell aggressiveness may also occur *in vivo*. For example, previous studies have shown that when normal mammary epithelial cells are co-injected with breast tumor cells into mice, the tumors are more likely to metastasize than when the cancer cells are injected alone (Price et al., 1990; Miller et al., 1989). These results suggest that the mammary epithelial cells secrete factors that promote breast cancer metastasis. This correlates with our observation that substances secreted by normal breast cells enhance cancer cell adhesion and invasion *in vivo*. Furthermore, the inhibition of adhesion by RGDS and YIGSR peptides provides evidence for the involvement of integrins. Further work will be needed to determine whether this mechanism is important in patients with breast cancer. By understanding what proteins are involved in tumor progression, a target can be identified and possibly blocked in order to more effectively treat cancer.

Acknowledgments

First of all, I wish to convey my sincere gratitude to my supervisor, Dr. Philip M. Carpenter, for his most generous help, because without him this paper would not have been possible. I also want to thank our grant support, UC Cancer Research Coordinating Committee. Last but not least, I want to thank Dr. Zahida Arain, who was always willing to help me in the lab whenever I was in need.

Works Cited

Defilippi, P., Silengo, L. and Tarone, G. "α6β1 integrin (laminin) is down-regulated by tumor necrosis factor-α and interleukin-1β in human endothelial cells." Journal of Biological Chemistry 267 (1992): 18303-18307.

Elices, M.J. and Hemler, M.E. "The human integrin VLA-2 is a collagen receptor on some cells and a collagen/laminin receptor on others." Proceedings of the National Academy of Science, USA 86 (1990): 9906-9910.

Hart, I.R., Birch, M. and Marshall, J.F. "Cell adhesion receptor expression during melanoma progression and metastasis." Cancer Metastasis Review 10 (1991): 115-128.

Heino, J., Ignotz, R.A., Helmer, M.E., Crouse, C. and Massague, J. "Regulation of cell adhesion receptors by transforming growth factor-β." Journal of Biological Chemistry 264 (1989): 380-388.

Heymann, D., Harb, J., Ringeard, S., Blanchard, F., Lassort, D., Raher, S. and Godard, A. "Upmodulation of αvβ1 integrin expression on human tumor cells by human interleukin for DA Cells/leukemia inhibitor factor and oncostatin M on fibronectin." Journal of Biological Chemistry 58 (1995): 305-314.

Hynes, R.O. and Lander, A.D. "Contact and adhesive specificities in the associations, migrations, and targeting of cells and axons." Cell 68 (1992): 303-322.

Hynes, R.O. "Integrins: versatility, modulation, and signaling in cell adhesion." Cell 69 (1992): 11-25.

Ignotz, R.A. and Massague, J. "Cell adhesion protein receptors as targets for transforming growth factor-b action." Cell 51(1987): 189-197.

Ignotz, R.A., Heino, J. and Massague, J. "Regulation of cell adhesion receptors by transforming growth factor-β." Journal of Biological Chemistry 261(1989): 389-392.

Kumar, K., Cortan, R. and Robbins, S.L. Neoplasia. Basic Pathology. 5th ed. (1992): 171-214.

Languine, L.R., Gehlsen, K.R. and Wayner, E. "Endothelial cells use α2β1 integrins as a laminin receptors." Journal of Biological Chemistry 109 (1989): 2455-2462.

Marita, T., Kawakami-Kimura, N., Sato, M., Matsuura, N., Higashiyama, S., Taniguchi, N. and Kannagi, R. "Alteration of integrins by heparin-binding EGF-like growth factor in human breast cancer cells." Oncology 53 (1996): 374-381.

Miller, F.R., McEachern, D. and Miller, B.E. "Growth regulation of mouse mammary tumor cells in collagen gel cultures by diffusible factors produced by normal mammary gland epithelium and stromal fibroblasts." Cancer Research 49 (1989): 6091-6097.

Pignatelli, M. and Vessey, C.J. "Adhesion molecules: novel molecular tools in tumor pathology. Progress in Pathology." Human Pathology 25 (1994): 849-856.

Price, J.E., Polyzos, A., Zhang, R.D. and Danies, L.M. "Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice." Cancer Research 50 (1990): 717-721.

Ruoslahti, E. and Pierschbacher, M.D. "New perspectives in cell adhesion: RGD and integrins." Science 238 (1987): 491-497.

Santala, P. and Heino, J. "Regulation of integrin-type cell adhesion receptors by cytokines." Journal of Biological Chemistry 34 (1991): 23505-23509.