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QUANTIFICATION OF HUMAN TUMOR CELL LOCOMOTORY BEHAVIOR IN A THREE-DIMENSIONAL COLLAGEN GEL MATRIX

Yola Boulos

Department of Dental Sciences
McGill University, Montreal

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Abstract

Conventional two-dimensional glass and plastic in vitro cell culture systems potentially lack an accurate representation of the in vivo reality of three-dimensional cell locomotory processes; an important loss of valuable information or even misinformation in understanding and interpreting underlying cell behavioral mechanisms could result. This thesis uses a dynamic quantification approach of human tumor cell locomotory behavior in a three-dimensional collagen gel matrix in conjunction with time-lapse videomicroscopy and computer digitization. This approach better approximates physiological in vivo cell behavior by allowing cell trajectory analysis and expression of phenotypic characteristics previously not observed in two-dimensional culture systems or end point measurements. The main objective of this thesis is to determine whether there are quantifiable differences between benign, malignant, and normal cell locomotory behavior that can be measured in a significant and useful manner. The second objective of this thesis is to determine whether quantifying cell locomotory behavior can potentially reveal the existence of functional subpopulations within a heterogeneous tumor population. Further study of such a three-dimensional system can expand our insight into the metastatic and invasive processes of cancer and potentially contribute in assessing neoplasm aggressiveness and even patient morbidity.

Résumé

Les méthodes conventionnelles de cultures cellulaires à deux dimensions in vitro (sur vitre et plastique), manquent de représentation préçise des processus physiologiques de locomotions cellulaires. En conséquence, une perte d'informations importantes ou même une fausse représentation peut s'ensuivre lors de l'interprétation des mécanismes cellulaires. Cette thèse utilise une approche dynamique de quantification des comportements cellulaires des tumeurs humaines dans une matrice en trois dimensions de collagène en gelée. En plus, l'enregistrement microscopique accéléré et la digitalisation assistée par l'ordinateur sont employés. Cette approche d'analyse de trajectoire cellulaire pourrait probablement mieux estimer le comportement physiologique cellulaire en permettant l'expression phénotypique qui n'a pas été observée avec les méthodes résultantes ou à deux dimensions. L'objectif principal de cette thèse est de déterminer s'il y a des différences quantifiées entre tumeurs bénignes, malines et tissus normaux et si elles peuvent être mesurées de manière significative et utile. Le deuxième objectif est de déterminer si la quantification du comportement cellulaire de locomotion des tumeurs peut révéler l'existence des sous-populations fonctionelles parmi une population hétérogène de cellules cancéreuses. Des études supplémentaires d'un tel système de culture en trois dimensions peuvent élargir nos connaissances des processus de métastase et nous renseigner sur l'invasion du cancer ou même d'évaluer la morbidité.

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Bottoms up!

Contents

List of Tables

List of Figures

Chapter 1

Introduction

Mammalian cell locomotion is important in physiological processes such as embryonic morphogenesis [1,2], nerve regeneration [2], wound healing [2,3], inflammatory reactions, and tumor cell invasion [4]. Among the cells that have been studied from a locomotive perspective are: amoebae [5], neural crest cells, fibroblasts, lymphocytes, epithelial, and cancer cells [6,7,8].

In human cancers, spread of cancer through migration- that is, metastasis- is usually what determines aggressiveness [9]. Therefore, knowledge of tumor cell migratory characteristics could be very important.

1.1 Problem Definition

Evaluating and quantifying in vivo human tumor cell locomotory behavior during invasion [5] is no simple task. In investigating cell motility and invasiveness, one must keep in mind the physiochemical complexity of the surrounding environment [10]. Therefore, representation of mammalian cell locomotory behavior as a 2-dimensional in vitro process greatly detracts from the reality of certain cellular events occurring in a 3-dimensional (3-D) in vivo environment.

Conventional and widely used in vitro systems such as glass and plastic, often fall short of providing an accurate representation of locomotory behavior of cells [11]. Problems that 2-dimensional in vitro systems exhibit include: poor or non existent cell-matrix interactions leading to misrepresentation of environment-induced cell behavior; and phenotypic behavior which is specific to that system and which will not necessarily be the same as that in vivo [12].

Another major problem includes the pre-existing conventional methods of quantification of cell locomotory behavior. These often involve end point methods which only take into account a resultant behavior such as net displacement of cells. An example is the Boyden chamber technique which is known as an end point method. Consequently, cellular events occurring in between the end points are discarded and a tremendous amount of valuable information is lost [6,13].

Evaluation of cell shape using conventional histological techniques, precludes analysis of locomotory behavior at different stages, therefore making quantification over time difficult. This is because locomotion is inferred from cell shape at the given instant of fixation [1]. Furthermore, the static nature of these methods will be less revealing of the variant behavior of some cells potentially exposing the existence of cell subpopulations than would be an approach using time-lapse recording.

These systems often fail to answer questions such as : by what mechanism are the cells locomoting? ; what are the physical and chemical reactions involved during tumor cell locomotion? ; what is the result of interactions of cells with the matrix? ; and examined in this thesis, how can we measure this?

This thesis does not deal with specific molecular mechanisms of tumor cell locomotion or cell-cell and cell-matrix interactions, use of various extracellular matrix (ECM) components other than collagen type I, nor molecular biology of cancer. Although cellular behavior is described at the gross level, further detail of molecular mechanisms, as well as other related work can be found in the relevant papers cited in later sections.

1.2 Thesis Area and Goals

This thesis deals with a mathematically oriented approach to dynamic quantification of human tumor single cell locomotory behavior. Head and neck cancers are studied and a 3-D collagen gel matrix system is used as the in vitro cell culture system. Malignant, benign and normal primary tissue explants from the head and neck region are used. This is combined with time-lapse videomicroscopy and computer digitization to produce detailed dynamic behavior of a cell's locomotory behavior in a 3D environment.

The first goal of this thesis was to determine whether there are quantifiable differences in locomotory behavior between malignant, benign, and normal cells within a 3-D collagen matrix. This thesis presents the result of this work using different parameters of measurement deemed relevant in evaluating behavioral differences and outcomes.

The aforementioned goal is significant because a direct quantitative approach to cellular behavior should include measurement of all events occurring during the process of

locomotion from one point to another. In addition, an effective cell behavior model - in this case, tumor invasion- should approximate its equivalent in vivo microenvironment. The 3-D collagen matrix achieves this by providing a biologically and physically similar substratum [8]. Since it is generally known that the extracellular environment has a direct instructive effect on cell migratory behavior [8,14,15] as well as phenotypic expression, one can begin to appreciate the importance of using 3-D substrata as the in vivo system of choice. A poor cell-matrix interface can modify the complexity of cellular events thus altering their behavior. This, combined with a quantitative approach to measuring behavior only at certain points in time would result in losing important information useful in understanding the very process of cell locomotion and tumor invasion.

The second goal of this thesis was to use different approaches of measurement to attempt to represent various aspects of single cell as well as cell population behavior.

This is significant because by approaching cell locomotory analyses from different dimensions might provide the cell biology of tumor invasion such as with the existence of functional subpopulations [8,15]. Again, one cannot get extensive tumor cell behavioral information by examining fixed tissue specimens or even conventional end point techniques such as the Boyden Chamber [16]. One can obtain tumor type and stage , but predicting the degree of aggressiveness of a tumor is more difficult. This is because current prognostic techniques are evaluated individually and depend on tumor grade, perineural, vascular, lymphatic invasion, or extracapsular spread of a tumor [17]. However, these techniques could be problematic since a given oral tumor for example, could be small yet behave more aggressively than a tumor that is larger in size [18] -such is the case with melanomas where the primary could be quite small yet it could have metastasized quite extensively. Thus, detecting certain types of locomotory behavior from a given tumor could be significant for patient diagnosis and prognosis.

This thesis presents a pilot study of single tumor cell measurement, however it does not provide a comprehensive study of a prognostic nature because of time and scope constraints. The intention is to show that cell behavior can be measured in a valid manner and that there exists a myriad of measures capable of representing different aspects of cell locomotion; these have the potential of being further analyzed by more complex mathematical techniques.

1.3 Contributions of the Thesis

The main contribution of this thesis lies within the approach of quantification of human tumor single cell behavior using a combination of techniques. These techniques are:

incorporation of cells within a 3-dimensional collagen gel matrix, time-lapse videomicroscopy, computer cell tracking, and digitization.The results obtained can be analyzed many different ways. Therefore, this shows that a mathematical approach to biological phenomenon can be relatively straightforward and easy to apply using readily available equipment and materials.

This thesis also demonstrates that we can obtain reproducible cell behavior seen with other 3-D studies [6,19,20,21]. This paves the way for further study of tumor cell behavior by more complex ECM components, addition of external stimulatory factors, interactions with other tumors, and more complex mathematical analyses.

1.4 Content Preview

Chapter 2 discusses background of cancer and related work on previous cell locomotory analyses and techniques as well as the cell culture system used. Chapter 3 presents the experimental protocol used in this thesis. Chapter 4 outlines the results obtained, their significance, and analysis. Chapter 5 evaluates the hypothesis and whether the goals of the thesis were attained. It also concludes the thesis.

Chapter 2

Background and Related Work

This thesis mostly deals with human tumor single cell locomotion in an in vivo 3-dimensional (3-D) collagen gel matrix system. Therefore, it is necessary to present the relevance of cell locomotion in cancer invasion and metastasis as well as previous work relating to measurement of tumor cell locomotion. This section examines the two main requirements that should be considered in a cell locomotory study. These are: the cell's trajectory to obtain a more sensitive quantification of locomotory behavior, and the importance of the extracellular matrix's (ECM) in regulating cell behavior.

2.1 Cancer - General Overview

This section presents a brief and general review of basic concepts in neoplasia relevant to the study of tumor cell locomotion examined in this thesis.

2.1.1 Incidence of Head and Neck Cancer

Head and neck cancers constitute 2.4% of new cancers in Canada [22]. Almost half these cases do not survive longer than five years and by the time the primary tumor is discovered it will usually have metastasized. Furthermore, the incidence and survival rate of head and neck cancers has not improved over the last thirty years [22]. The main reason is because of misconceptions and lack of knowledge of appropriate screening procedures and diagnosis, thus most oral lesions are not diagnosed until they reach an advanced stage [18].

2.1.2 Brief Definition of Cancer and its Origins

Neoplasia refers to uncoordinated, unregulated cell proliferation and differentiation. Cancer is the result of genetic damage of which unrestrained cell growth, invasion, and metastases ensues [23]. Tumors originate from different tissues, organs, and cells; this is taken into account when classifying a tumor. A common feature of neoplasms is their loss of responsiveness to normal regulatory growth controls. Neoplasms can either be benign or malignant.

Briefly, benign tumors remain localized and they do not spread, infiltrate, invade, or metastasize. Most, though not all, benign neoplasms are enclosed within a fibrous capsule. Malignant tumors can infiltrate, invade, and destroy surrounding tissue. In some instances, malignant tumors appear to be encapsulated. In order to reliably distinguish malignant from benign tumors, one characteristic commonly used is the development of metastases; another feature is local invasiveness. However, not all cancers can metastasize even if they are highly invasive in their primary site of origin.

2.1.3 Heterogeneity of Tumor Cell Population

As tumors progress over time, they usually become more aggressive and increasingly malignant [9]. So there is an incremental increase in growth, invasiveness, and metastasis. This is accounted for by the heterogeneity of a tumor cell population. Heterogeneity refers to the manifestation of cell subpopulations with different phenotypic attributes such as: growth rate, metastatic ability, drug responsiveness, invasiveness, and aggressiveness [10,24,25].

Although most tumor cells are monoclonal in origin, genetic instability can account for a heterogeneous population of constituent cells well before clinically evident. The fact that tumor cell populations are subjected to selection pressures from the body predisposes them to becoming resistant more with respect to host defense. Compounded to this, is the molecular and cellular heterogeneity in cancer cell populations which is yet another hindrance to common prognostic or diagnostic techniques. As a consequence, it would be of interest to determine whether existence of heterogeneity in a tumor cell population would have a role in the varying degrees of invasiveness of a cancer.

2.1.4 Current Diagnostic Methods and Problems

Clinical assessment of tumor aggressiveness and degree of malignancy can use cytological differentiation. Grading a tumor according to stage is based on increasingly complex and descriptive characterizations with no clear cut criteria [9]. Current diagnostic

systems use parameters such as primary tumor size, extent of node involvement, and metastases.

Sometimes it is even difficult to use clinical laboratory techniques to distinguish between border line cases. Common tumor diagnostic techniques include: hematoxylin and eosin staining, electron microscopy, immunohistochemistry, and flow cytometry [23]. These methods can indicate the presence of cell surface markers, certain ultrastructural features, as well as DNA abnormalities. In spite of this wealth of information, prediction of tumor aggressiveness can nonetheless involve some uncertainty. For example, cell marker detection can be problematic since it is not always reliable and can vary with the procedure of fixation and detection [23].

Furthermore, the size of the primary neoplasm does not necessarily correlate with aggressiveness and extent of metastatic spread. For example, a cancer can be quite large yet may not have spread and vice versa. An example of the former is a papillary carcinoma and of the latter, could be that of a melanoma. Therefore, prognosis of a cancer could depend on a myriad of diagnostic features such as: locomotion, size, and depth penetration.

2.2 Tumor Cell Locomotion in Invasion and Metastasis

The following examines briefly the actual propagation of cancer in invasion and metastasis as well as the role of single locomoting cells in contributing to the unpredictability of neoplasm behavior as well as outcome.

2.2.1 Progression of Cancer Via Invasion and Metastasis

Metastasis is an important cause of death for cancer patients. The dissemination of cancer as well as its heterogeneous cell make-up are the major difficulties in treating cancer. Many factors may be involved in the spread of neoplastic cells throughout the body. Extrinsic influences include growth factors, the immune system, and vascularization to name a few. Intrinsic factors depend on the individual cell's activity and may affect the metastatic potential [19]. It has therefore been realized the importance of being able to predict the metastatic aggressiveness of each patient's tumor in order to be able to decrease the risk of invasiveness [26] and improve accuracy of diagnosis. Since the metastatic process is so complex, it has been necessary to focus on one variable at a time- in this case tumor cell locomotion in order to understand the sum of the processes involved.

Tumor cell metastasis is a "complex multifactorial process involving many cellular activities" [27] from the gross to the molecular levels. Briefly, cell locomotion is important in metastasis in order that the tumor cells invade and extravasate from the vasculature and

the host tissue. Metastasis includes the following steps [28]: (i) invasion into the vasculature of the tumor, (ii) transportation of tumor cells in the bloodstream, (iii) arrest of tumor cells in the "vasculature of distant organs", and (iv) invasion of the tumor cells through the vasculature and into the organ at the secondary site. Tumor cell movement can be stimulated through production of autocrine motility factor, or presence of soluble factors (chemoattractants) present in the ECM. These chemoattractants can be matrix-derived and include fragments of fibronectin, collagen, and laminin [28].

According to Mareel [29], invasion refers to tumors occupying tissues that are not their own. Invasion occurs at two major steps during metastasis. First, tumor cells must cross a host tissue and penetrate blood or lymphatic vessels. Then, circulating tumor cells are arrested within a vessel lumen or may infiltrate adjacent tissue in which they proliferate. Several mechanisms of tumor cell invasion into host tissue have been proposed such as: increased translocative motility of malignant cells; degradation of surrounding matrix by tumor-derived lytic enzymes; and cellular proliferation of the neoplasm leading to increased mechanical pressure [30]. The exact nature of tumor cell invasion is not entirely known. Tumor invasion could also proceed by a state similar to that of an embryonic system [31].

2.2.2 Motility of Single Locomoting Tumor Cells

The study of tumor cell motility is important. In addition to the currently accepted concept of single locomoting cells (SLC) having a role in cancer cell invasion and metastasis dissemination [5,32] there are other structures thought to potentially have a role in cancer. These include: locomoting cell clusters (LCC) which are detached group of cells and outgrowths (OG) which are tissue structures protruding into the surrounding matrix [1,33,34] LCCs and OGs are discussed in further detail in other work [1,5,34,35].

Tumor cell motility is often used interchangeably with cell translation [27]. In addition to translational locomotion, motility also comprises the dynamic nature of cell processes such as stationary type of motility which leads to pronounced changes in cell shape but not to translocation. Stationary motility comprises: membrane ruffling, protrusion and retraction of cytoplasmic extensions of various configurations and sizes (pseudopodia, villi, ruffles, blebs, filopodia, and lamellopodia). It is quite possible that stationary motility can also contribute to invasion [5,36] and indicates the complexity of invasion. Each of these activities occurs in response to biological phenomena which can be hormonal, oncogenic, growth factor related, or ECM related. However, the focus here will be more on translational motility.

It is generally believed that single tumor cells invade and metastasize by developing a migratory invasive and metastatic phenotype and/or genotype [1]. Actual translocation of tumor cells occurs as a result of two major events. First, a driving force is generated by assembly and disassembly of an actomyosin system during polymerization and depolymerization [5]. Secondly, generation of a driving force results in translocation where adhesion and de-adhesion of cellular locomotory organs to the surrounding matrix occurs. Pseudopodia are the locomotory organs of a cell and are formed with the help of gelsolin which is an actin-binding protein. In the presence of Ca^{++}, gelsolin facilitates F-actin disassembly and the pseudopod is formed. On the other hand, pseudopod disassembly occurs when polyphosphoinositides derived from the cell membrane dissociate gelsolin/actin units thus facilitating actin polymerization. Cell locomotion during invasion requires attachment via cell surface receptors and detachment possibly through localized microdegradation of the substrate. Alternatively, tumor cell invasion can occur by shape transformation and migration through the 3-D network of the surrounding matrix.

An important determinant of metastatic capability is the motile ability of individual tumor cells. This even varies among the malignancies within the same tumor. Potential explanation of the heterogeneous behavior lies within the extent of genetic variability of transformation. This is further corroborated by differences in motility in established cell lines from histologically similar human carcinomas [5].

First evidence of tumor cell movement was obtained from histological observations where cells detached from the primary tumor. Time-lapse cinematography by Wood et al. [37] provided more definitive evidence of tumor cell locomotion.

2.3 Extracellular Matrix and its Role in Tumor Cell Locomotion

In order to comprehend tumor cell locomotion, one cannot only consider isolated molecular or cellular events but should also take into account the sum of the cascade of events as well as the role of the microenvironment.

2.3.1 ECM- Structure and Function

As a tumor metastasizes it must interact with the extracellular matrix (ECM) at several points. The ECM consists of either basement membrane or interstitial connective tissue. Basement membranes (BM) are largely composed of laminin, collagen type IV, and heparin sulfate proteoglycan. BMs form a physiological barrier between epithelial cells and the adjacent stroma and become permeable during tissue development and repair. They are resorbed during tumor invasion and it is believed that this step leads to metastasis. It has

been proposed that the events which occur when tumor cells invade the BM are: attachment to BM via cell surface receptors; degradation of adjacent BM by enzymes secreted by the tumor cells; directed migration in response to chemotactic stimuli [16,35,38].

Interstitial connective tissue is largely maintained by fibroblasts and its main structural components include: collagen type I and III, and fibronectin [39].

2.3.2 Instructive Effect of the ECM

The ECM is instructive. It can serve as a physio-chemical substratum allowing for cell-cell and cell-matrix interactions [40]. The ECM has important regulatory effects on cell differentiation, proliferation, and cell migratory behavior [12,13,15,20,36]. Physically, the ECM provides the scaffolding for tumor cells to locomote; cells can wrap their pseudopodial extensions around collagen fibrils of the matrix.[1] Chemically, the ECM is composed of ground substances (such as collagen, fibronectin, laminin, and various glycosaminoglycans) which interact with cells in the microenvironment [41]. Through a system of signaling molecules, the ECM plays a major regulatory role by influencing cell malignancy and resultant invasive growth [36]. These interactions occur by way of cell surface receptors such as integrins, cadherins , and other cell adhesion molecules which can modulate cytoskeletal changes and longer term changes in genetic expression.

2.3.3 ECM's Role in Tumor Cell Invasion and Metastasis

Traditional models of tumor cell invasion mechanisms usually include degradation of the surrounding matrix. However, extensive lysis of the ECM might in fact be counterproductive from the point of view of a locomoting tumor cell which probably does not have a swimming mechanism. Instead, tumor cell invasion may work through more subtle means by simply breaking local adhesion points of contact and generating motility stimulating ECM fragments such as laminin or fibronectin [39]. Schor et al. [20] suggested that degradation *per se* of the surrounding matrix -collagen in this case- may not necessarily accompany tumor cell invasion into that substratum [31]. Correlation of metastatic ability with enzymatic digestion of the matrix might indicate a far more complex relationship of tumor cell invasion into the ECM. For instance, tumor cell locomotion might occur through contact guidance where cells orient themselves along the collagen bundles

[1] Incidentally, Haston et al. [42], obtained evidence that lymphocytes may locomote through collagen gels by physically wrapping their pseudopodial extensions around the collagen fibrils instead of only migrating by using adhesion receptors.

within the ECM [43]. By adapting their shape, tumor cells might also locomote through the collagen fibers rather than lysing them.

2.4 Quantification of Tumor Cell Locomotory Behavior

One approach to understand invasion and metastasis is through quantification of SLC behavior. This comprehensive approach compares many aspects of tumor cell behavior. It is important to provide an overview of present quantification methods of tumor cell locomotion.

2.4.1 Basic Methods of Tumor Cell Locomotory Studies

A variety of experimental assays have been developed to study tumor cell locomotion and invasiveness [10,29]. Commonly used methods include: the Boyden chamber [44] and its variations [38,45]; 3-dimensional collagen matrices; biological tissues and membranes; cultures on glass or plastic plates ; and liquid media cultures. Some of these assays are used more with invasion in mind rather than locomotion per se. The prevalent Boyden assay shall be discussed in more detail as an example of the lack of correlation between the in vivo and in vitro situations. In addition, most of the migration cell assays have been done on artificial substrata such as: glass, plastic, or polycarbonate or nitrocellulose filters in the Boyden chamber assay [46]. These methods hardly take into account cell-matrix interactions.

Since cells in vivo exist within a 3-D environment, it should be apparent that differences could exist between cell behavior in 3-D and 2-D in vitro culture systems [14,47]. Most of our current knowledge of cell translocative locomotion is based on 2-dimensional (2-D) studies. The nature of the substrate on which cells grow can affect the extent of cell spreading. A large amount of this information has been derived from the use of end point methods such as the Boyden chamber. As for other studies of cell locomotory behavior, they have relied on assessing invasiveness into the gel by evaluating the furthest migratory capacity of cells after layering them on the 3-D matrices' surface[48]. This approach is essentially the equivalent of the end-point methods and has the same drawbacks such as not providing trajectory information of individual cells during their course of migration . More specifically, the simple distance traveled by a cell into a filter or a gel hardly provides detailed information of locomotory parameters of a given behavior, nor does it reflect molecular mechanisms. For example, if a cell's speed of invasion increases, one can ask whether the increase in speed is due to an increase in cell's velocity or to a decrease in the frequency and duration of stopping.

Prevailing knowledge of tumor cell locomotion during invasion has been obtained by indirect and direct methods [47]. Static morphology from tumor explants have provided indirect evidence of cancer locomotion by using light, electron, and scanning microscopy. Using the previous techniques, the position of single or clustered tumor cells relative to the main tissue explant, supposedly aids in inferring certain behavioral characteristics of invasion. For example, distance traveled and cell shape have been used to infer locomotory behavior. Whereas, according to Strauli [5] cells traveling within a tissue hardly maintain a characteristic shape which immediately suggests locomotion. Other indirect methods which also provide similar information, include in vitro culturing of tumor cells on biological and artificial membranes, inoculation into and layering on top of tissues and organs, and confluent cell layers. On the other hand, direct evidence of cell locomotion has been gathered by time-lapse filming of tumor cells within live and transparent membranes such as the mesentery [49] or within a rabbit ear chamber [37]. The time-lapse filming allowed direct observation of actual cell locomotion. However, these systems presented difficulties in recording and culturing the cells due to the thickness of the tissue and limited manipulation.

2.4.2 Quantitative Assays of Tumor Cell Behavior

This section will present an overview of existent and widely used tumor cell invasive and locomotory assessment techniques.

2.4.2.1 Simple 2-D Assays

Despite the obvious lack of correlation to the in vivo situation, cell locomotion is sometimes evaluated on two-dimensional surfaces. These include plastic or glass that can be uncoated or coated with various substrata. Locomotion on two-dimensional surfaces probably has different requirements than in a more complex three-dimensional environment; this would likely result in different physiological or even mechanical locomotory behaviors. Differences in morphological appearance between cells embedded in a collagen gel matrix and monolayer cultures on glass or plastic surfaces have been noted by Haramaki [11] and others [50]. Morphologically, cells on monolayer cultures are constrained and forced to develop on a foreign architectural environment; this does not reflect the in vivo environment [50].

Culturing cells in a liquid medium does not approximate the in vivo situation accurately enough since mammalian cells (such as epithelial or tumor cells) do not have the ability to locomote by swimming. A supporting matrix is probably required for cell

attachment and locomotion. The matrix should be surrounding the cells in all planes rather than on one side.

2.4.2.2 Boyden Chamber and its Variations

In attempting to identify cell chemotactic behavior, S. Boyden developed a series of experiments in 1962 which according to him, provided a quantitative means to measure relative chemotactic abilities of various soluble substances [44]. Cells used in this study were rabbit polymorphonuclear leukocytes. The substances tested for chemotactic activity included animal, plant, and bacterial proteins as well as non dialyzable macromolecules.

This basic assumption of his proposal centered around the chamber where if a soluble substance was chemotactic, the cell would actively migrate towards or away from it. To ensure that *active* migration was occurring and not a simple gradient or diffusive response, Boyden employed a polycarbonate filter with pores smaller than the size of the cells which must actively migrate to be able to pass through . The filter is placed between two chambers where a cell suspension with a known number of cells is placed on one side and the test solution is placed on the other side.

After incubation, the filter is removed, fixed, and then "invasive " properties of cells are quantitatively assessed by counting microscopically [44,45]. Using different concentrations of a given test solution, one could assess the relative chemotactic effect of various soluble substances. According to Boyden, this assay allows *quantitative* estimation of chemotaxis and ultimately tumor invasiveness [51] which could be applied to answer questions such as the mechanisms involved in leukocyte differentiation through secretion of soluble factors, between self and foreign cells. Chemotactic and chemokinetic cell behavior are distinguished by applying checkerboard analysis in this assay [16,41].

The aforementioned may be useful in assessing chemotactic properties of a cell. However the specific invasive behavior of a cell and the mechanisms involved cannot be determined from this end-point method. The experimental set-up differs significantly from the in vivo situation. First, the cells are surrounded by a liquid media suspension and placed on a filter on which cells locomote. The filter quite likely differs from an in vivo matrix or 3-D scaffolding on which to locomote. Moreover, the events that occur from the time of loading of the cells up until fixing the filter are completely disregarded so it is quite difficult to determine the molecular mechanisms involved in the invasive process. Another essential factor that is not accounted for is the ECM's effect on the cells. So lack of chemoinvasiveness could be attributed to a lower chemotactic response due to the absence of appropriate stimulating factors.

Also the process of cells going through the millipore filter is supposed to approximate the invasive process of tumor cells passing through a basement membrane. One could also ask the question of whether pores potentially limit locomotion in the horizontal plane and thus eliminate an important component of locomotory behavior. There is also the possibility that only the cells that initially happen to be placed over the pores migrate through. Under certain conditions, the proportion of cells found at the opposite side of the filter was found to be roughly equal to the number of pores per filter [16,52].

2.4.2.3 Modified Boyden Chamber

In a paper by Albini et al. [38], the Boyden chamber assay is taken one step further in order to quantify tumor cell invasiveness. This is done by placing matrigel which is reconstituted basement membrane onto the filter of the Boyden chamber. Tumor, as well as normal cells (fibroblasts, and epithelial cells) are used . Also, intrasplenic injection of the tumors cells into nude mice is carried out to confirm malignant or benign phenotype of the cells studied. Fibroblast-conditioned media which contains collagen, fibronectin, as well as other unknown chemotactic factors is used to promote increased penetration of the cells into the matrix.

By using different concentrations of matrigel, different degrees of invasiveness were obtained; the more concentrated, the longer it took for the cells to penetrate. It was also found that cells cultured on matrigel as opposed to plastic surfaces, displayed morphological differences [16,38] that indicated locomotion.

The use of a reconstituted BM is somewhat of an improvement to the use of a Boyden chamber since it is a relatively closer approximation to the in vivo situation. Matrigel is a urea extract of the Engelbreth-Holm-Swarm tumor [10,38]. This does not reflect the actual situation where the cells are already in the stroma or have invaded the BM which is *not* in a liquid state. Another factor not taken into account is the possibility of the BM being polarized where apical receptors might differ greatly from basal ones; this in turn could have an effect on locomotory behavior. Thirdly, there indeed is selection of a subpopulation as evidenced by back-seeding the cells that had succeeded in invading the filter the first time [29]. Cells penetrating the gel and the filter could simply have a phenotype that favors them in this respect, but it does not necessarily mean that the remaining cells might not be able to invade so this is not truly representative of the whole population. This could be true for any in vivo or in vitro environment. It may simply be that the experimental set up may not be relevant to the in vivo reality. Finally, given the complexity of biological tissue in the immediate vicinity as well as of the whole organism, there still may be an immense lack of appropriate chemotactic substances even if fibroblast-

conditioned media was used. Since fibroblasts are simply cultured in medium, it is highly probable that there is a lack of appropriate stimulus for in vitro production of factors that are made in vivo [45]. For example, some tumor cells have been found to secrete factors such as *autocrine motility factor* (AMF) which stimulates in vitro motility [53]. AMF in turn, could stimulate the production of other cytokines from normal cells which could have an effect on the tumor cells. In a paper by Simon et al [38], it was concluded that this modified model of the Boyden Chamber does not provide a universal test to predict tumor cell invasiveness; they found no correlation between chemoinvasion in vitro and metastatic potential in vivo.

2.4.2.4 Biological Tissues

Commonly used tissues and membranes include: amnion, chorioallantoic membrane, lens capsule, and epithelial cell cultures [10,38]. The membranes might be used as a diaphragm between a two-chamber compartment [29] to assess the confrontation of invasive cells with normal tissues. There is still the problem of selection of subpopulation of cells on the basis of their capacity to migrate across normal tissue. A prevalent problem with biological tissues is their lack of transparency which makes any quantitative analysis of invasiveness even more difficult [51].

Variability of the tissue within and among samples (for example thickness) is definitely an important factor to consider in an experimental set-up. Furthermore, biological tissues such as the amnion are usually much thicker than the BM [10].

Another assay involves injection of cancer cells into the tissue of a host (usually in the spleen) to verify whether the cells are benign or malignant. This procedure is commonly used in conjunction with other assays to confirm malignant or benign behavior. So by injection of a lab-cultured cell line which probably consists of a selected subpopulation, causes even further selection of the population. These cell lines are artificially separated cells which is not how tumor cells originate. This has its limits, however one can still use in vivo injection for qualitative rather than quantitative assessment of metastasis.

2.4.3 Quantification of Tumor Cell Behavior Within a 3-D Collagen Matrix

The following examines the assessment of cell trajectory using 3-D in vitro matrix environments. Attachment to the BM, its degradation, and locomotion are important events but may be insufficient on their own for invasion and metastasis [44]. This indicates that events occurring such as tumor single cell migration are more complex than simply starting

at a given point and ending at another. Instead, a whole cascade of reactions and interactions emphasize the complexity of cell invasion and its microenvironment in conjunction with the organism. Using the end-point methods versus cell-trajectory methods for example, neglects other events which occur in invasion. The cellular mechanisms of tumor cell locomotion are quite complex and have yet to be fully elucidated.

2.4.3.1 Review of 3-Dimensional Studies

It is important to note that the 3-D matrix environment can support and contain a whole population of heterogeneous cells such as fibroblasts and lymphocytes rather than a selection of only invasive phenotypes. These "support" cells which very likely interact with the tumor cells are not selected out of the population but are maintained in culture to carry out functions such as collagen secretion and receptor expression respectively. This is an important consideration since a complex cascade of cellular and stromal interactions is probably involved in tumor cell locomotion. Furthermore, placing cells *within* the matrix rather than layering on top and allowing them to settle or penetrate makes more sense because in vivo, cells are in an environment where they are surrounded by biological matrix in all planes. Also, layering the cells has the limitation of initially being a 2-D plane and might still select for invasive subpopulations where only a few cells can penetrate into the collagen [54]. The latter was noted by Schor [24] that only certain cells layered on top of the gel were able to migrate into it. One can therefore assume that suspending tumor cells within the collagen matrix allows analysis of a randomly selected heterogeneous cell population [55]. Likewise, the argument presented by Schor et al. [20] whereby layering cells on top of a matrix suggests penetration does not correspond with the fact that tumor cells exist within a tissue, surrounded by all planes, and are not neatly placed on top of a biological membrane or tissue.

Among the early work done where the importance using a 3-D substratum was recognized and used, was that of Leighton in the 1950s [56]. Using standard histologic techniques to microscopically examine cell cultures within a cellulose sponge matrix he came to the following conclusions: tumor cells of different origins and species (rat, mouse, chick embryo) grew well in a matrix of cellulose sponge surrounded by plasma clots within glass tubes; since morphology and function are often correlated, it could be that the qualities of the original tumor are also retained. The 3-D arrangement of the cells within the plasma-sponge matrix allowed greater surface area of cell-matrix contact thus enhancing growth as opposed to a simple 2-D plasma clot culture. It was observed that the pattern of cellular growth in the plasma-sponge matrix- as opposed to the simple 2D plasma clot culture- bore a greater resemblance to the cells in the original in vivo tissue. Many of the

characteristics of cell cultures in 3-D that were not observed on monolayer cultures could be attributed to the maintenance of the cells' shape [57]. Leighton also suggested that the physical structure of the sponge could provide a mechanical means by which cell locomotion could occur. The viability of the cells probably depended on the metabolic exchange occurring deep in the plasma clot. Leighton also observed and suggested that tumor cell aggregates as being the units of metastasis rather than simple single cells [56].

The use of hydrated collagen lattices as 2- or 3-D substrata was described by Elsdale et al. [43]. Normal as well as transformed fibroblasts were used to study cell morphology, motility, adhesion, and growth; these cells were cultured on plastic, on collagen, and within the collagen lattice. The use of a different substratum revealed different aspects of cell behavior. It was also found that fibroblast morphology in the 3-D collagen gel was the same as had been observed in histological sections of the connective tissue [43,58].

Further study of tumor cell invasion, lymphocyte, and fibroblast motility in a 3-D collagen type I gel matrix was carried out by Schor et al.[21,24,58]. It was recognized that the study of cell migratory behavior in vitro should be carried out on biological macromolecular matrices rather than artificial ones. A series of experiments was carried out where melanoma cells and fibroblasts were plated on a plastic surface, on top of, and within a collagen type I gel matrix. Interestingly, two patterns of tumor invasion based on histological data were noted by Strauli [47]: tumors of epithelial origin (e.g. adenocarcinoma) invaded the adjacent tissue as an organized cluster of cells; and tumors of mesenchymal origin (e.g. fibrosarcoma) and malignant melanoma usually invade surrounding tissue in the form of single migrating tumor cells [24]. So, not only is it important to take into consideration the nature of the heterogeneous nature of neoplasms and their origins, but it is also important to consider the surrounding substratum when using an in vitro model. It was also recognized that collagen gel substrata allow phenotypic expression of cell behavior that is different and more in-vivo like than conventional plastic tissue culture systems [20].

3-D gel matrix systems such as collagen, fibrin, and matrigel have been used with other types of tissue [11,15,46] in conjunction with time-lapse microscopy. However, some studies evaluated upward and downward migration [48,54,59] as a quantitative assay of extent of invasion into the collagen.

Strohmaier et al [60] used a 3-D collagen G (90% collagen type I and 10% type III) matrix as an interstitial invasion model and matrigel as a BM (collagen type IV) invasion model. These systems were used since they are the two important barriers tumor cells must cross to invade and metastasize. Each substrate was shown to have different effects on cell

invasive behavior. The 3-D aspect of the cell matrix was reconstructed in the xy and xz planes using inverse light and confocal laser scanning microscopy. However, the tumor cells were seeded on top and underwent extensive staining and fixation. It was also recognized that Matrigel does not always correlate with in vivo substrata.

2.4.3.2 Collagen as a 3-D Matrix Substrate

3-D collagen matrices have previously been used in cell motility and tumor invasion studies [24,25,61]. In the 3-D model represented in this thesis and previous work [6], it could potentially reflect the transition of cancer *in situ* to invasive cancer where the basement membrane is often lacking or is defective and the cancer cells invade the adjacent tissue matrix [1].

Compared to the previous assays mentioned, 3-D collagen type I matrices provide numerous advantages. Collagen type I is used since it is a principal component of interstitial matrix and the fibrils are arranged in a manner similar to that of the BM and interstitial matrix [24,43]. Collagen also provides architectural stability of the interstitial connective tissue [50] and was found to be the major constituent of peritumoral matrix [43,50,61,62]. As opposed to glass or plastic surfaces, collagen gels offers obvious advantages in terms of physical and chemical similarities to the in vivo interstitium of the tissue environment [8,15,62,63]. The physical nature of the collagen fibers could by itself stimulate the locomotory phenotype of tumor cells in collagen [64] but not in liquid culture. Also, polymerized collagen provides a supporting scaffold in which cells can anchor and be able to locomote [35] ; whereas in liquid media, mammalian cells do not have the means by which to propel themselves. Another advantage of collagen type I gel matrix, is that collagen can be alloyed with fibronectin, heparin sulfate, laminin, and other glycoproteins and proteoglycans [62]. In addition, collagen type I was found to have a role in modulating cell growth and differentiation [11,15,50].

Haramaki et al. [11] showed that cells cultured in 3-D collagen matrices have shown better developed organelles, increased morphological resemblance by electron microscopy to their physiological in vivo counterpart [11,15], reconstruction of glandular and tubular structures, as well as induction of cellular polarity. Studies done by Toda et al. [15] with cells embedded within a 3-D collagen gel matrix to reconstruct human thyroid follicles have shown a morphological and functional similarity to the physiological situation; this was in contrast to thyroid cells cultured as a monolayer culture.

Advantages of a 3-D collagen gel include : the use of a biological rather than an artificial substratum; cell behavior can be quantified due to the transparent nature of the gel; increasingly complex matrices may be constructed by addition of other ECM components;

and other normal cells may be incorporated into the gel in order to study cell-cell and cell-matrix interactions during neoplastic invasion [24].

According to Gutman et al. [59] optimum collagen concentration for the highest migratory rate was 1.59 mg/ml. Whereas one of the findings by Schor et al. (1982) was an optimal collagen concentration of 2 mg/ml for maximal cell migration of fibroblasts and melanoma cells. A higher collagen concentration would impede cell migration due to the increased density of fibers. Conversely, a lower concentration would render the gel unstable, fragile, and would not provide sufficient "traction" for cell migration [11,50,59]. It is important to consider the surrounding matrix. Simply migrating further into the gel might not necessarily indicate a more physiological environment.

2.4.3.3 Direct Assessment of Tumor Cell Trajectory

Cell locomotion was often assessed by measuring its average rate of progression in leading front methods. These cells are not representative of the whole cell population. Rather, individual cell analysis would provide a more accurate estimate of cell population behavior.

The transparency of the collagen allows the cells within it to be easily viewed with the light microscope. Most importantly, time-lapse videomicroscopy of the cells and subsequent cell tracking provide a wealth of information such as locomotory behavior, cell paths, cyclic cell activity, and dynamic morphology which can be measured and quantified to ultimately reflect molecular mechanisms. This information which is preserved as a dynamic means (on videotape) as well as by static means (histological fixation), is otherwise discarded or ignored by the end-point methods. For instance, fixed cells from an end-point assay could show a cell to be rounded; a false conclusion would be to say that it is definitely a non locomotory cell simply because of its shape. In contrast, time-lapsing cells within a collagen matrix could show it to exhibit ruffling behavior which could be a potential precursor to locomotion and invasiveness [5]. The ability to time-lapse also provides the ability to fix the cells at any desired moment of activity. Whereas with the Boyden chamber, only a limited portion of cells which have invaded the filter can be stained and fixed.

Direct evaluation of cell trajectory (lymphocytes in this case) was described by Noble [65] whereby quantitative parameters such as speed, velocity, percentage of active locomoting cells, and directionality could be measured. Cell trajectory was quantified using time-lapse videomicroscopy and ensuing cell tracking. This is in contrast to end point methods where much of this data is eliminated and only resultant behavior is taken in to consideration [55].

With 3-D tracking and digitization we obtain representation of the cells' translational nature but do not entirely incorporate the dynamic aspect of cell motility. Combination of more complex techniques of analysis have been proposed by Partin et al. [27] where time-lapse videomicroscopy was combined with Fourier analysis in order to provide mathematical representation of a cell's shape.

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Tissue Culture Preparation - Reagents and Equipment

Purified native type I dermal bovine collagen 3 mg/ml (Vitrogen 100, Collagen Corp., Palo Alto, USA) consisting of 99.9% pure collagen (95-98% type I collagen and 5-2% type III collagen) was purchased from Celtrix, Santa Clara, CA, USA.

Minimal Essential Eagle Medium (MEM) - 1X and 10X- were obtained from Flow Laboratories , McLean, VA, USA. Newborn Calf Serum (NCS) , Dulbecco's phosphate buffered saline (PBS), HCO_3^- sodium bicarbonate 7.5% , 50 U/ml penicillin- 50µM/ml streptomycin, and 2 mM L-glutamine (100x) were all obtained from Gibco, Grand Island, NY, USA. Dimethylsulfoxide (DMSO) was procured from Fisher Scientific. Formalin (4%) for fixing was purchased from Sigma, St. Louis, MO, USA.

Tissue culture plates (35 x 10 mm) and 96-well flat bottom tissue culture polystyrene microtiter plates were obtained from Corning Glassworks, Corning, NY, USA. The carbon dioxide concentration in the 37° C incubator was maintained at 5% with a 100 % bone dry CO_2 cylinder which was purchased from MEGS, Ville St. Laurent, Que., Canada. Parafilm "M" was obtained from American Natl. Cam., Greenwich, CT, USA.

MEM Culture Medium: Cell cultures were maintained in MEM 1X supplemented with 10% NCS , 1 ml per 100 ml MEM 1X of 50 U/ml penicillin- 50µM/ml streptomycin solution dissolved in 20 ml PBS, and 1 ml L-glutamine (also dissolved in PBS) per 100 ml MEM 1X . The pH was adjusted to 7.4.

Freezing Medium: Freezing media consisted of 10% DMSO , 20% NCS, and 70% MEM 1X culture medium. Tissue specimens were frozen down for later use with freezing medium and placed in 2 ml freezing vials from Nalgene, Rochester, NY, USA. The samples were maintained in a -80° C freezer (REVCO Scientific Inc., Asheville, NC, USA , Model #ULT1790-5A) from Fisher Scientific, Montreal, Canada.

3.1.2 Time-Lapse Videomicroscopy

Wild and Nikon microscopes were used (total magnification of 64x) with Panasonic AG-6730 and Panasonic AG-6720A time-lapse units from Matsushita Electric, Japan. A 5 % CO_2 - balanced air gas cylinder (MEGS) maintained the cultures during time-lapse videorecordings of the tissue cultures as well as a 37° C air curtain incubator from SAGE Instruments (subsidiary of Orion Research), Cambridge, Mass., USA.

3.1.3 Computer Cell Tracking and Data Analysis Tools

Data processing was achieved using a Mac IIvx personal computer (7.5.1 System Software, 20M of RAM) containing the following support software: a digitizing tablet for cell tracking (SM camera for Screen Machine, Version 2.5); Microsoft Excel version 5.0 permitted transformation and analysis of the digitized data into real values of pixels, microns, and angles; STATISTICA™ (release 4.1) was used for data analysis.

3.2 Methods

3.2.1 Collection of Tissue Specimens

Within 2-3 hours of surgery, unfixed and untreated freshly excised primary tumor specimens from the head and neck region were obtained from Dr. Tabah of the Department of Surgery, Montreal General Hospital, and from Dr. Black of the Department of Otolaryngology, Jewish General Hospital, Montreal, Canada. A pathologist localized the benign or malignant section of the tissue by frozen section whereby a neighboring fresh piece (5-20 mm^3) was excised for culture. Normal gingival or oral mucosal specimens from healthy individuals were obtained from Dr. Clokie, Department of Dentistry, Montreal General Hospital. This normal tissue served as the control in the experiments conducted. Each specimen was assigned a numerical code to reduce experimental bias. Each specimen was maintained for transport in a sterile 15 cc tube containing MEM culture medium. Table 1 provides patient and tissue culture data of the specimens used.

3.2.2 Preparation and In Vitro Cultivation of Tissue Explants

3.2.2.1 Preparation of Tissue Specimens

Under a sterile laminar flow hood (sterilization was achieved through overnight UV irradiation), each tissue specimen was placed in a 35 x 10 mm tissue culture plate with 1 ml MEM culture medium. It was then carefully cut with a scalpel into approximately 5 mm^3

	SEX/AGE	TUMOR CODE	PATHOLOGY	TOT. PERIOD OF CULTURE (days)	TOTAL # CELLS TRACKED	TOTAL TIME TRACKED (hrs)
Malignant	M/62	52	SCCA	10	25	14.5
	F/31	58	Pap. Carcinoma	9	33	19.2
	M/78	66	SCCA	13	53	18.2
	F/33	68	Pap. Carcinoma	12	5	2.3
	F/69	73	SCCA	25	34	29.5
	F/49	94	SCCA	24	149	65.6
Benign	F/48	62	Foll. adenoma	27	9	2.4
	F/43	67	Coll. nod. thy.	17	51	15.3
	F/25	76	Papillary thyroid	10	47	47
		93	Multinod. goiter	28	5	15.4
Normal	M/46	99	Normal gingiva	30	26	50.5

Table 1: Patient data and tissue specimens used for primary culture within a 3-D collagen matrix. Malignant samples included: squamous cell carcinoma (SCCA), papillary carcinoma (Pap. Carcinoma). Benign specimens included: follicular adenoma (Foll. adenoma), colloid nodular thyroidectomy (Coll. nod. thy.), and multinodular goiter (Multinodular goiter). Normal gingival tissue was used as the control.

pieces. The culture medium was exchanged three times to reduce risk of bacterial contamination. From one to three tissue sections were transferred to freezing vials containing 1 ml DMSO freezing medium; the vials were placed in a -80° C freezer for storage and further use.

For culture preparation, a 5 mm^3 piece of either fresh or previously frozen tissue explant in a 35 x 10 mm culture plate containing 1 ml of MEM culture medium was further sectioned into 20-25 pieces of less than 1 mm^3. Again, the specimens were rinsed 3 times in MEM culture medium. Figure 1 provides an outline of the preparation and cultivation of the cell culture for subsequent cell tracking and data analysis.

3.2.2.2 Preparation of 3-Dimensional Collagen Lattice

The collagen gel matrix was prepared according to the description of Noble (1987). Under a laminar flow hood, 125 µl of 10% NCS was added to 200 µl HCO_3^- (7.5%) until pH of 7.2-7.8 was attained. 1.5 ml type I native collagen (Vitrogen 100) was then added along with 1.0 ml MEM culture medium to obtain a final collagen concentration of

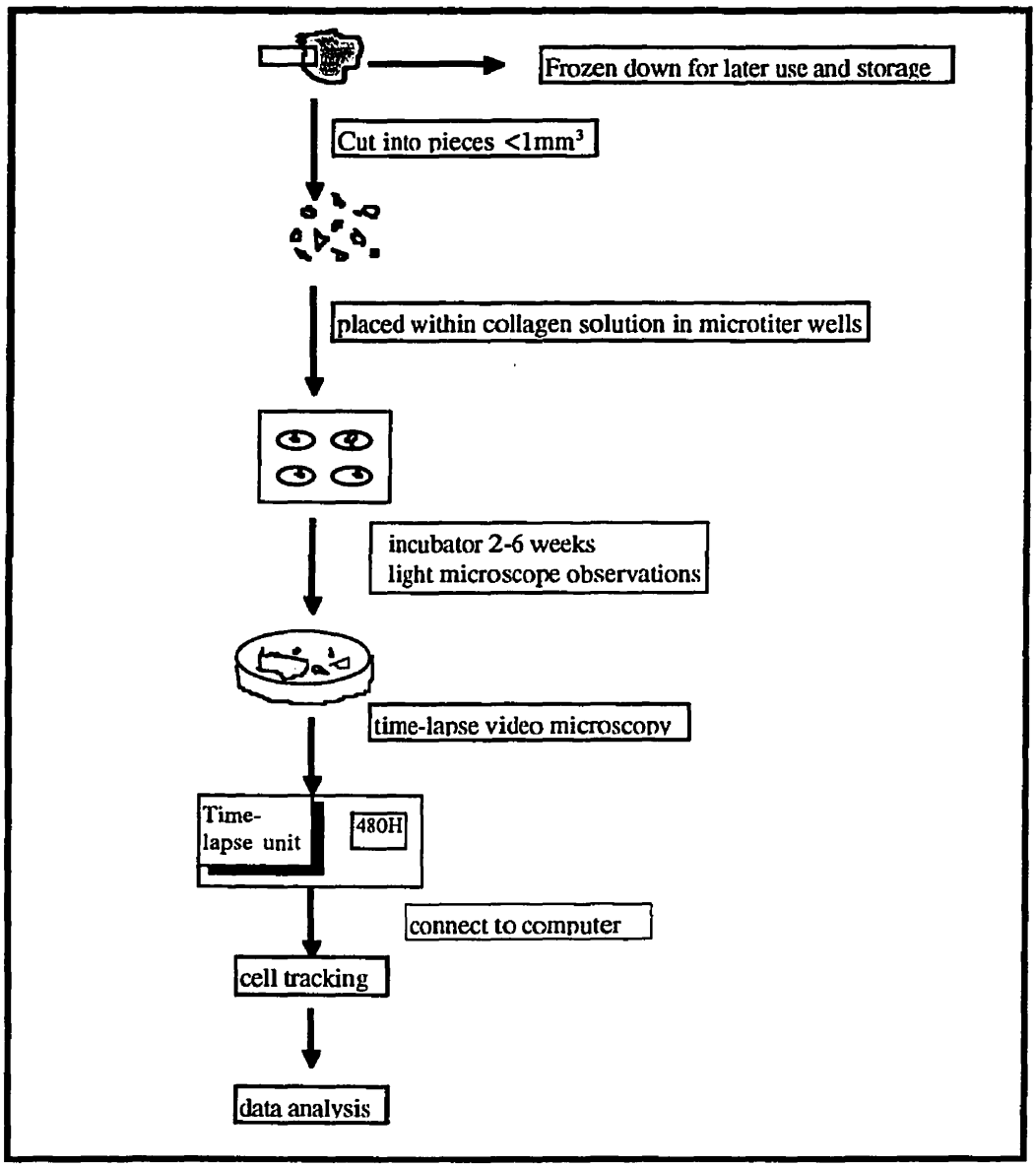


Figure 1 Preparation and cultivation of primary tissue samples in a 3-D collagen gel matrix for further analysis. Tissue samples cultivated within a collagen lattice were maintained over a period of 2-6 weeks.Tissue cultures were observed for changes in cell growth, recorded with time-lapse videomicroscopy, and computer analyzed

1.5 mg/ml with the pH of the solution stabilizing to 7.3 in the 37° C - 5% CO_2 incubator. With an eppendorf pipette (Sigma, St. Louis, MO), 100 µl of the collagen solution was added per well of a 96-well flat bottom tissue culture polystyrene microtiter plate.

Using a micropipette tip, each tissue section was then centered within the collagen suspension in each well . This suspension was incubated for 20-30 minutes at 37° C- 5%

CO_2 to allow the collagen to polymerize into a gel 6.4 mm in diameter and 3-4 mm in depth. Four drops of MEM overlay culture medium were added onto each collagen lattice. Also, autoclaved distilled water was added to the wells at the border of the plate in order to keep an H_2O saturated atmosphere for each plate. The culture preparations were maintained in the 37 ° C - 5% CO_2 incubator and the overlay culture medium was replaced every three days.

3.2.3 Time-Lapse Videomicroscopy and Computer Cell Tracking

3.2.3.1 Time-Lapse of Potential Tumor Single Locomoting Cells

Tissue explant behavior was monitored daily for single locomoting cells (SLC) using the bright field inverted light microscope. The set-up for time-lapsing and recording cell locomotory behavior is outlined in Figure 2. Whenever non lymphocytic or non fibroblastic cells were observed, the specimen was time-lapsed at a magnification of 64X. Furthermore, at the start of tracking the SLCs had to have no physical interactions with fibroblasts (FBL), lymphocytes (LY), outgrowths (OG), locomoting cell clusters (LCC), or exist within a collagen lysed area. LYs were characterized as lymphoid cells, 6-10 μm in diameter with a characteristic "tail" uropodal extension, as well as a usually higher velocity than other cells in the culture. OGs were characterized as an organization of cells still attached to the primary tissue sample protruding into the collagen matrix. LCCs were described as aggregates of cells unattached to the primary sample. Examples of these cellular structures are shown in Figures 3 - 8 (results, pages 30 - 32).

First, the microtiter plate was sealed with Parafilm (American Natl. Cam., Greenwich, CT) and placed within a transparent plastic chamber. In order to mimic the incubator environment, 5% CO_2 was introduced into the chamber using 2 mm diameter surgical tubing. An air curtain incubator maintained an ambient temperature of 37° C (Figure 2).

The sample was taped at a time-lapse setting of 480H which means that each 4 minutes of real time were compressed into 1 second of videotape. The time-lapsed sessions ranged from 3-24 hours.

3.2.3.2 Computer-Assisted Tracking of Selected SLCs

The Panasonic time-lapse unit was connected to the Macintosh IIvx personal computer with the graphics software. Single locomoting cell behavior was quantified using the digitizing tablet software. Each SLC was manually tracked by means of the computer's recording of the cursor position at a given sampling interval. The computer

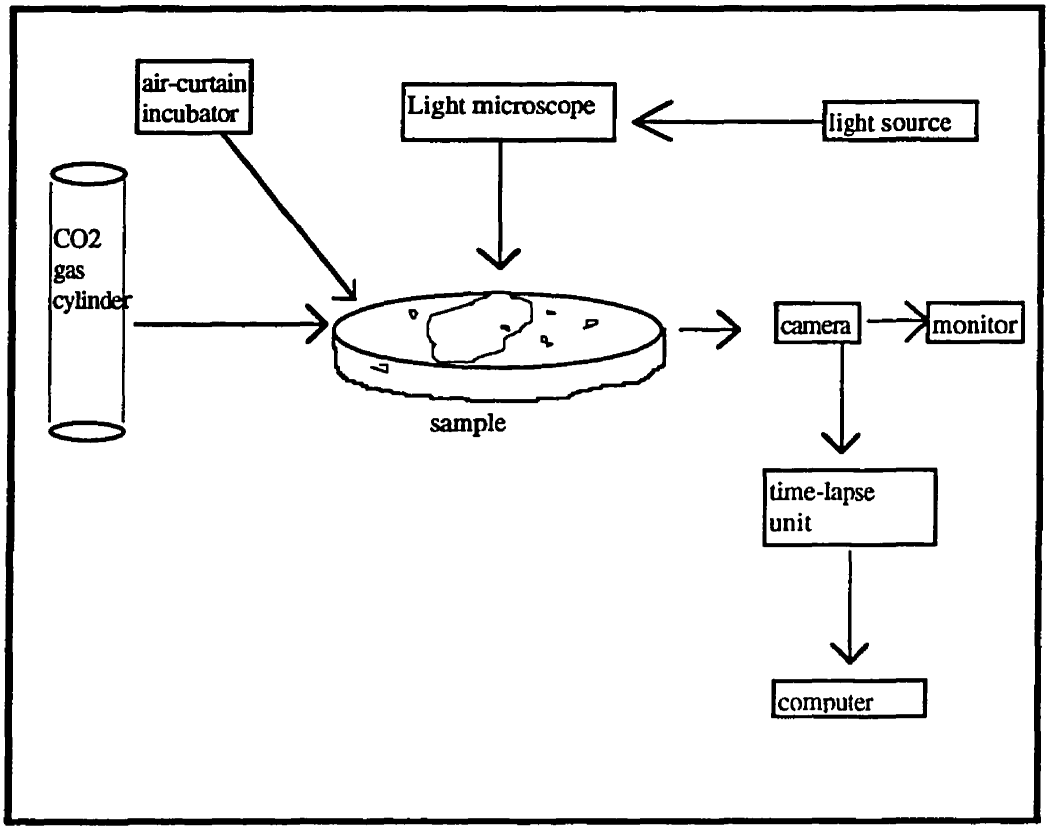


Figure 2: Tissue culture set-up for time-lapse recording and observation. Primary tissue cultures were monitored by light microscope every 2-3 days for cell growth behavior. The samples were maintained at 37 degrees centigrade with an air-curtain incubator, as well as 5% carbon dioxide-95% air from a gas cylinder which provided the incubator-like environment. Time-lapse recordings were carried out at a rate of 4 minutes of real time compressed into 1 second of video tape. The time-lapse unit was then attached to the computer for eventual data analysis.

sampling time interval for cell tracking was selected at 0.7 sec which is equivalent to 2.8 min. of real time. Given the speed of SLCs which is slower than that of LYs for example, a higher sampling rate would not necessarily increase resolution of cell trajectory parameters. Alternatively, a lower sampling rate would result in loss of trajectory data.

Selection criteria for tracking tumor SLCs (Figure 3, page 30) included: non fibroblastic, non lymphoid, and non monocytic cell diameter greater than 20 μm [1,66] irregular and frequently changing cell shape, manifestation of pseudopodial, ruffling or blebbing behavior. Cells that underwent mitosis were also excluded. Criteria for tracking normal single cells consisted of monocytic or epithelial-like cells; fibroblast and lymphocyte-like cells were easily excluded because of their typical morphology. Normal cell criteria differed from that of tumor cells since it was expected that normal locomoting cells would not show the same locomotory profiles as single tumor cells.

3.2.3.3 2-D Computer Cell Tracking of a 3-D Culture System

Tracking of SLCs was quantified in the x-y plane; cell locomotion along the z axis was minimal as was observed from time-lapsed recordings where the cells remained mainly in focus and therefore within the same plane. For a digitized representation of cells' path from a squamous cell carcinoma refer to Figure 9 (results, page 33). Single cell locomotion along the x-y axes was proposed to reflect the structural organization of collagen fibers within the gel [1].

3.2.4 Data Analysis and Statistics

After tracking the SLCs, the digitized data was exported into an Excel spreadsheet. Exported data included: distance migrated, x-y coordinates, angle, and absolute time. A stage micrometer was used to calculate the microscope-to computer screen correction factors used to convert the exported values to pixels, microns, and angles. The first and last three values for each cell tracked were discarded; this is in order to take into account the time required to place the crosshairs of the computer's cursor onto the center of the cell as well as to consider the time required to stop the computer recording at the end of tracking.

Data processing within each sample was carried out for single cell as well as cell populations analyses. In order to reduce the possibility of error due to manual digitization in locating the centers of the cells a motility correction factor was applied by assigning a 0 value to any distance traveled of 2 pixels or less. This correction factor was obtained by measuring the distance between the center and edge of the cell several times and taking the average. Evaluation of locomotive parameters for individual cells and cell populations included: the frequency and distribution of locomotive "stops" and "goes"; persistence (ratio of a cell's start-to-end point distance divided by its cumulative distance migrated over a cell's path); instantaneous velocity (velocity traveled in the smallest increment of time measurable - 2.8 minutes in this case); and average speed. Data were expressed as mean ± SD. Table 2 provides a summary of equations generated using an Excel spreadsheet to calculate cell locomotory parameters.

The null hypothesis was: the quantification of human tumor single locomoting cells appearing from tissue cultured within a 3-D collagen gel matrix will not show differences between malignant, benign, and normal specimens.

LOCOMOTOR PARAMETER	INDIVIDUAL CELL DATA[1]	CELL POPULATION DATA[2]
Sampling interval (seconds)	=n*(0.7), n=1,2,3...n+1	= n*(0.7), n=1,2,3...n+1
Real time (hours)	=n*(0.0467) [3], n=1,2,3...n+1	=n*(0.0467), n=1,2,3...n+1
Motility correction factor	Replace distances of 1&2 [4] with 0	Replace distances of 1&2 with 0
Corrected avg. inst. vel.(μm/hr)	=(sum distance/# non-0 cases)/ 0.0467 * correction factor [5]	= (sum distance/# non-0 cases)/ 0.0467 * correction factor
Corrected rate speed (μm/hr)	=(sum dist./tot. # cases)/ 0.0467	=(sum dist./tot. # cases)/ 0.0467
# locomoting cells	=countif distance > 0	=countif distance > 0
# non-locomoting cells	=countif distance = 0	=countif distance = 0
% time of migration	= (dist.>0)/(dist. >0+ dist=0) *100	= (dist.>0)/(dist. >0+ dist=0) *100
Start-to-end point dist.(μm)	=[$\sqrt{(x_1 - x_2)^2 + (y_1 - y_2)^2}$] * (correction factor)	not applicable
Cumulative dist. traveled (μm)	=sum distance * correction factor	not applicable
Persistence	=start-to-end point distance/ cum. distance	not applicable

Table 2: Standard spreadsheet calculations of locomotor parameters for single cell and cell population analysis. Data processing was carried out after discarding the first and last three values tracked in order to take into account the time required to place the computer cursor onto the center of the cell.

[1] Individual cell data consisted of calculations for each individual cell tracked within a given population. In this case, individual cell data were in column format therefore, calculations were done for each column.
[2] Cell population data consisted of combining individual cell data of all the constituent cells tracked within a given sample. In this case, population data was calculated in "row format."
[3] The computer sampling interval of 0.7 sec was calculated to be the equivalent of 2.8 minutes in real time i.e. 4 minutes of real time were compressed into 1 second of video taped recording which in turn was tracked at a sampling rate of 0.7 seconds. Then, 2.8 min./(60 min/hr) = 0.0467 hours.
[4] Distance values of 1 or 2 pixels were replaced with a "0" value in order to take into account unwanted cursor movements during cell tracking.
[5] A correction factor was used to take into account the field differences between video time-lapse units used and the computer screen. For example, a correction factor of 0.94 corresponded with the use of the AG6720A time-lapse unit.

Chapter 4

Results and Analysis

Cell motility analyses were carried out using an in vivo 3-dimensional collagen matrix system. Single cell locomotory behavior was recorded and analyzed using malignant, benign, and normal tissue samples from the head and neck region. This section illustrates the range of data that can be obtained from analysis of cell locomotory behavior.

4.1 General Representation of Initial Data

Time-lapse videomicroscopy recordings and subsequent computer digitization provided initial visual representation of the data. Snapshots of the time-lapse recordings as well as digitized representations of the cell trajectory provided an initial glimpse into the large amount of potential data that analysis of this type can produce.

4.1.1 Images of Relevant Cellular Structures

The cellular structures represented in Figures 3-8 were surmised after observation of the time-lapse recordings (magnification of 64X). Examining cells at a given instant in time such as simply looking at "snapshots" of cell cultures, proved to be difficult in terms of identifying the cells. However, time-lapse recording of cell migration behavior allowed the identification of dynamic locomotory morphology with a greater certainty. For example the single cells in Figures 3 and 7, have different dynamic locomotory profiles. The bottom-most cell in Figure 7 showed prominent pseudopodia whereas the topmost cell showed a rounded appearance; yet, over time they met the criteria for single locomoting tumor cells. Observed single locomoting tumor cell behavior included: irregular and frequent shape changes, a slower velocity than lymphocytes, non-leukocytic cells, a diameter greater than twenty microns, and pseudopodial, ruffling or blebbing behavior. As expected, normal epithelial or monocytic-like cells that were tracked showed different

locomotory profiles. Fibroblasts and lymphocytes were quite easy to identify because of their typical shape and locomotory profiles. As seen in Figures 3,6, and 7, fibroblasts exhibited an elongated bipolar morphology and very low velocity in the order of 10 μm/hr and less. On the other hand, lymphocytes were approximately 6-10 μms in size, and typically rounded with a characteristic uropodal tail extension; those that locomoted did so at much higher velocities than fibroblasts or single locomoting tumor cells.

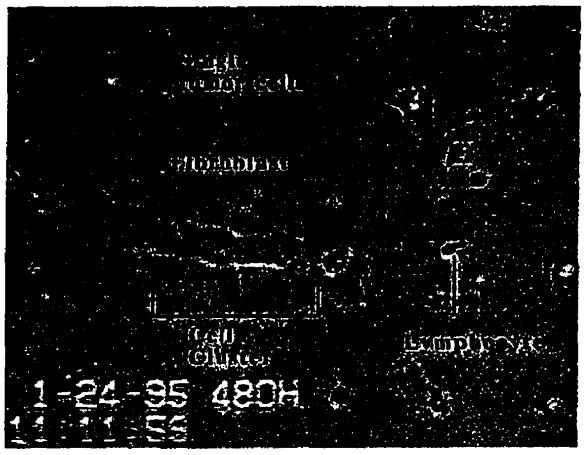


Figure 3: Typical morphology of single tumor cell, locomoting cell cluster, fibroblasts and lymphocytes of invasive squamous cell carcinoma in a 3-D collagen lattice. Individual cells can be seen in this cell cluster; pseudopodia can be seen in the single locomoting tumor cells; fibroblasts usually show an elongated shape; lymphocytes have a characteristic uropodal tail. Differences in cell speed and morphology were observed in the time-lapse recording for each sample. This image was digitized from a 20 hour time-lapse recording at day 7 of cell culture. This image represents a field of 600 μm x 420 μm.

Figure 4: Single tumor cells and outgrowth from a squamous cell carcinoma. Appearance of this outgrowth was after 6 days of culture within a 3-D collagen lattice. Large single locomoting tumor cells detached from the outgrowth. After 18 days of culture, the outgrowth had largely "disintegrated" into single tumor cells. This image was digitized from a field representing 600 μm x 420 μm .

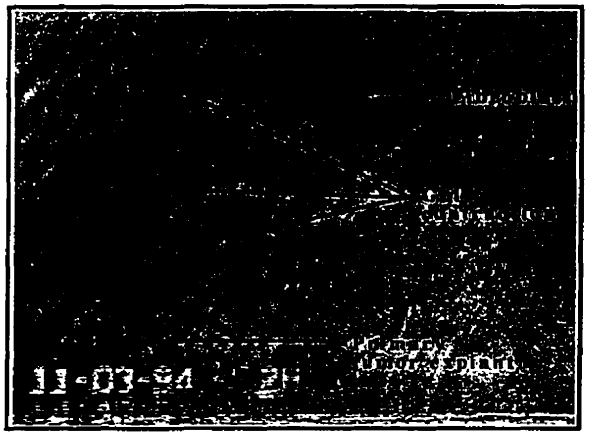


Figure 5: Gel contraction of collagen lattice. At day 14 of cultivation of a papillary carcinoma within a collagen matrix, gel contraction of the collagen fibers occurs. In this case, extensive contraction occurs since (not seen in image) half of the gel was contracted. The thick longitudinal fibers seen here are compressed collagen matrix fibers. Gel contraction was often observed with samples that contained many fibroblasts that seemed to be aligned in a given direction. The field represented here is 600 μm x 400 μm.

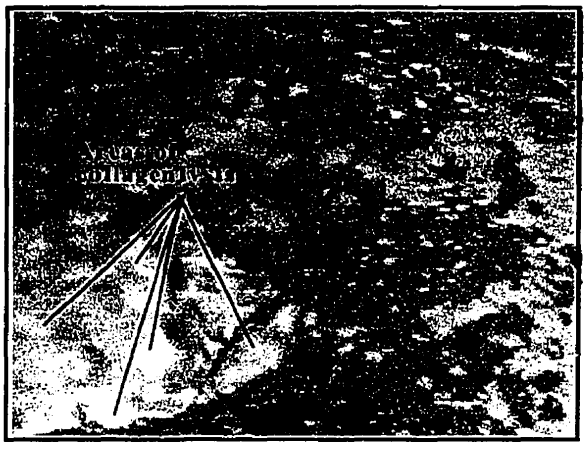


Figure 6: Lysis of the collagen matrix containing an invasive squamous cell carcinoma. Extensive collagen lysis occurred after 11 days of culture. The light-colored areas were clear of visible collagen matrix. Cellular debris was usually found at the bottom of the well. Collagenolysis was frequently noted as the cultures "aged" and observed cellular activity decreased. The field represented here is approximately 580 μm x 400 μm.

Figure 7: Single tumor cells at different stages of locomotion. This image illustrates quite well the different appearances a single locomoting tumor cell can have. The uppermost tumor cell is above the plane of observation. The single cell at the bottom of the screen has rather prominent pseudopodia. The single cells in this squamous cell carcinoma sample generally showed prominent pseudopodia and a high rate of locomotion. The field represented here was 600 μm x 420 μm.

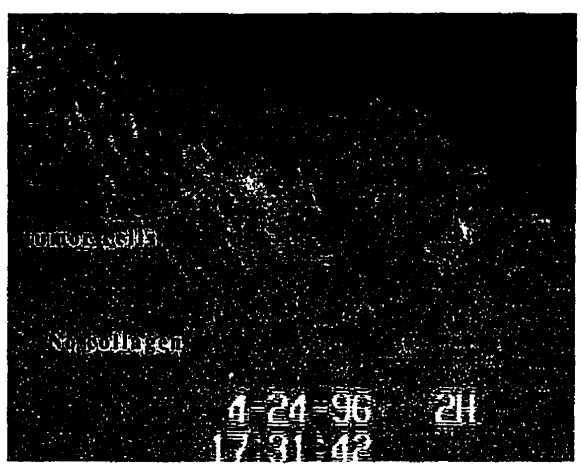


Figure 8: Tissue culture of a squamous cell carcinoma cultured in a well without a collagen matrix. Here, the cells were rounded and did not exhibit pseudopodia nor similar behavior to the same sample cultured within a 3-D collagen matrix. The field represented here was 600 μm x 420 μm.

4.1.2 Digitized Representations of Cell Trajectories

The trajectories of single cells that were tracked, were digitized into a 2-D representation of cell path. Plotting cell path as x-y coordinates provided a projection of cell trajectory that could be analyzed quantitatively. Figure 9 shows the coordinates of 8 locomoting cells. This plot visually shows the number of single locomoting cells; when compared to representative plots of benign and normal samples, malignant samples generally had a higher number of single locomoting cells. This in itself could be an indicator of cellular behavior.

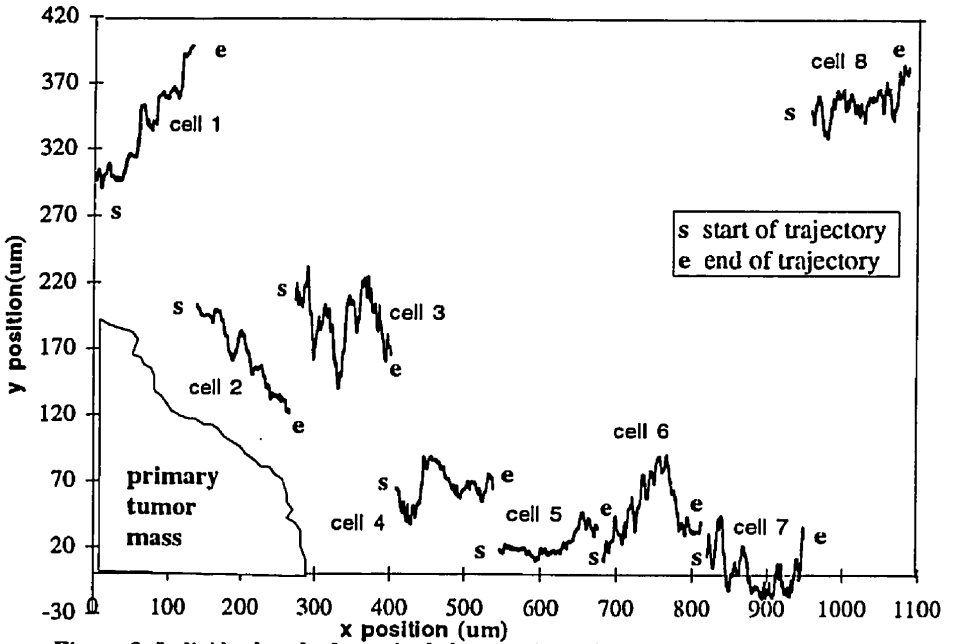


Figure 9: Individual paths from single locomoting cells within a given squamous cell carcinoma cell population. The trajectories (microns) of 8 single locomoting cells were digitized and reconstructed as a 2-D representation. The cells seem to have migrated in a directional manner since they were migrating out and away from the tumor mass. The observation period was approximately 6 hours.

Figure 10 shows in more detail a representative path of an individual locomoting tumor cell that was recorded by videomicroscopy and computer digitized. A comparison of the ratio of tumor cell displacement and path trajectory yielded the persistence (that is, directionality of a cell). The total path length was found to be 426.76 μm and the start-to-end displacement was 152.02 μm. The ratio of the latter over the former gave a persistence of 0.36. The closer the ratio was to 1, the higher the directionality of a migrating cell. From this representative example, it could be seen that start-to-end point displacement does

not necessarily correlate with time of observation. End point measurements only take into account the displacement of a cell, as a result, a large amount of information is discarded.

In this particular sample, 87.5% of the cells showed little or no persistence (< 0.5). The displacement /distance ratios for tracked malignant, benign, and normal cells ranged from 0.013 to 0.747. This indicates functional heterogeneity of locomotory cell behavior.

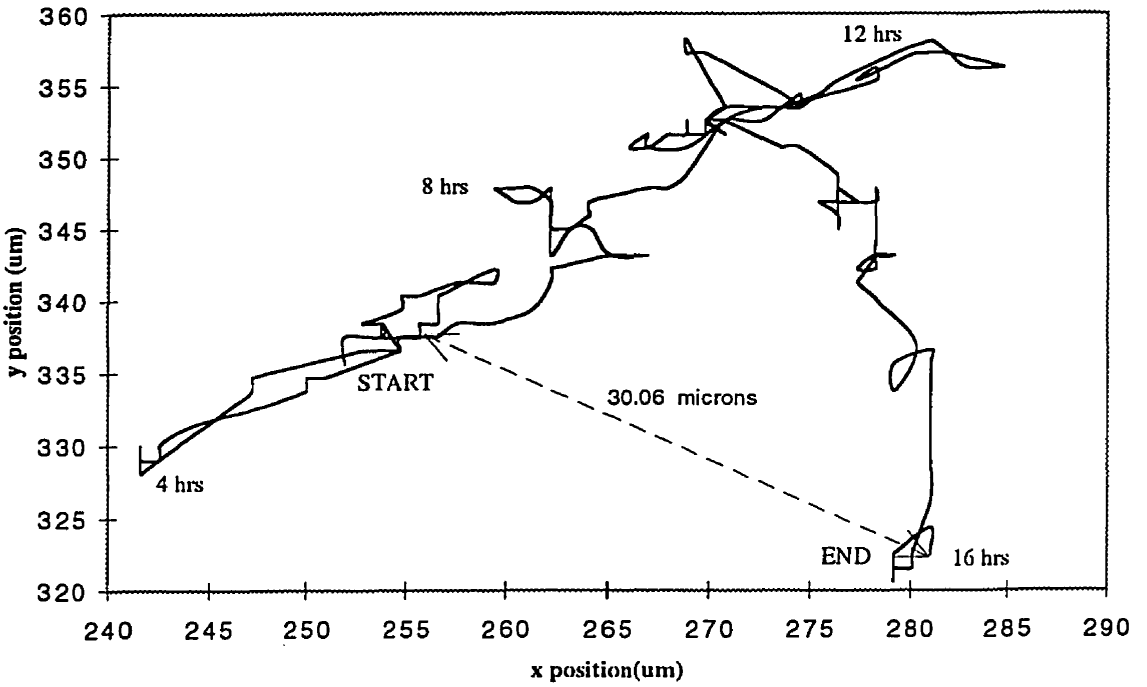


Figure 10: Single locomoting cell trajectory. Two-dimensional projection of trajectory (----) and displacement (- - -) of a locomoting tumor cell (squamous cell carcinoma) during a 16 hour observation period.

4.2 Functional Subpopulations Within the Same and Amongst Different Cell Populations

This section illustrates the cell locomotory analysis carried out for each specimen studied. Each sample was analyzed at two levels: individual cell data and cell population. Assessment at the cellular level would seem to provide a much more sensitive measure of cell behavior since individual locomotory profiles rather than resultant data were analyzed.

4.2.1 Analysis of Individual Cell Locomotory Parameters

Parameters used to represent individual cell data included: average instantaneous velocity, speed, % time that the cells migrated, and total distance traveled. Representative samples were used to illustrate data obtained for malignant, benign, and normal cultures.

4.2.1.1 Individual Cell Data

a) Malignant

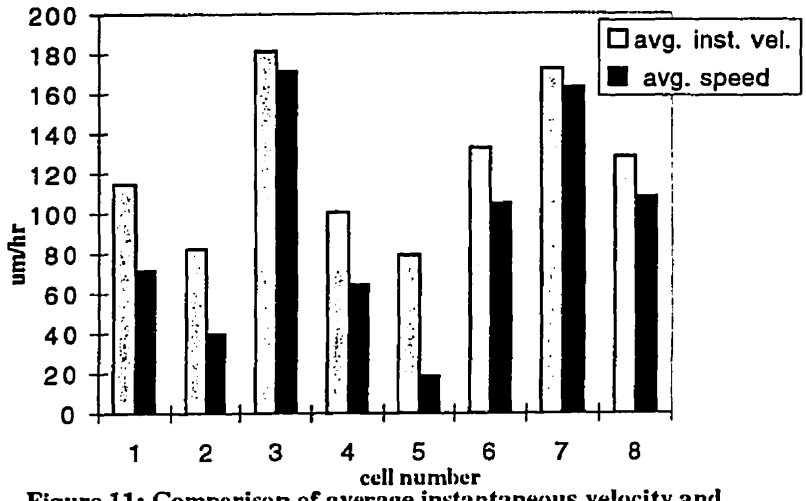


Figure 11: Comparison of average instantaneous velocity and speed of a squamous cell carcinoma. Average speed takes into account the frequency of distribution of "stops" and "goes" of a locomoting cell

b) Benign

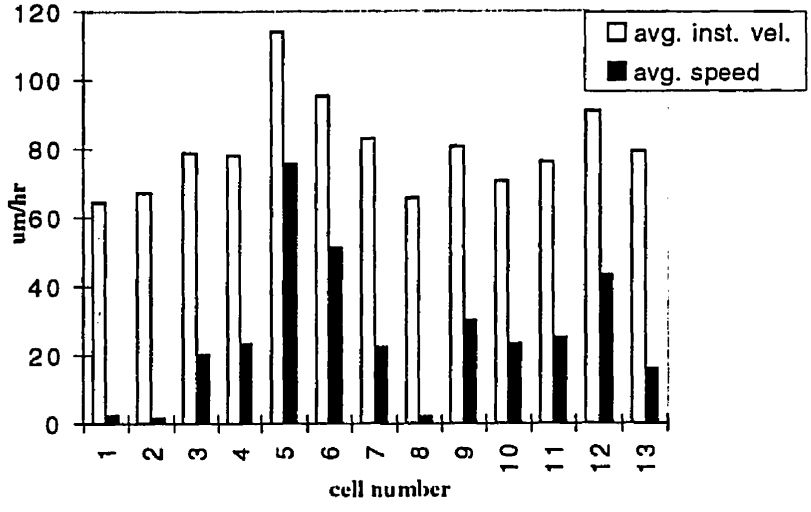


Figure 12: Comparison of average instantaneous velocity and average speed of a benign colloid nodular thyroidectomy

c) Normal

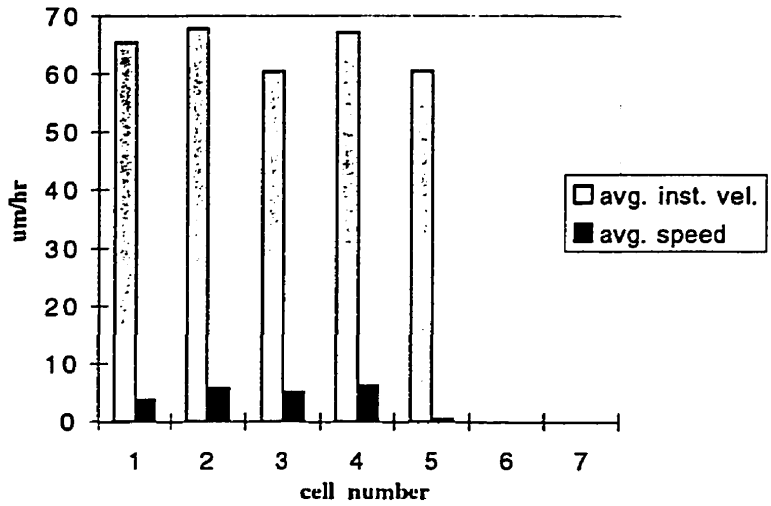


Figure 13: Comparison of average instantaneous velocity and average speed of a normal gingival tissue.

Figures 11-13 compared average instantaneous velocity and speed for each individual cell that was tracked within a malignant, benign, or normal tissue sample. The average speed shown, took into account the frequency distribution of stops and goes of a locomoting cell.

For all three samples represented, the instantaneous velocity and speed for each cell were averaged over a period of 6 hours. The average instantaneous velocity for the malignant sample ranged from 80 - 180 μm which was higher than for the benign (65 - 118 μm) and normal (60 - 78 μm) tissue sample. In addition, the velocities of the benign and malignant single cells had a wider range of velocities than normal cells. This was generally the case for the other specimens studied.

It was also noted that the average speed was closest to the average velocity values for the malignant locomoting cells, and it differed the most with the normal cells. This means that the malignant cells locomoted most frequently (that is, stopped the least amount of time); and the normal cells stopped the most amount of time.

4.2.1.2 Cell Population Data

a) Malignant

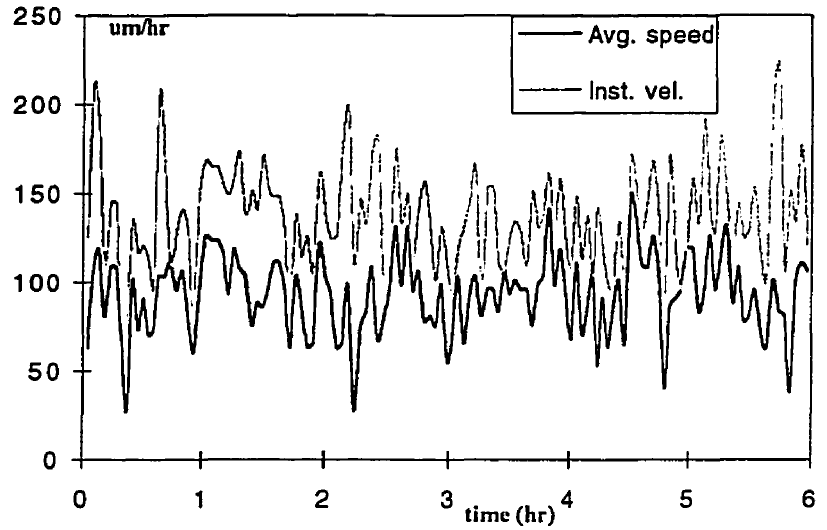


Figure 14: Average instantaneous velocity and speed at a given point in time for a squamous cell carcinoma cell population. Individual cell data were pooled over a period of 6 hours.

b) Benign

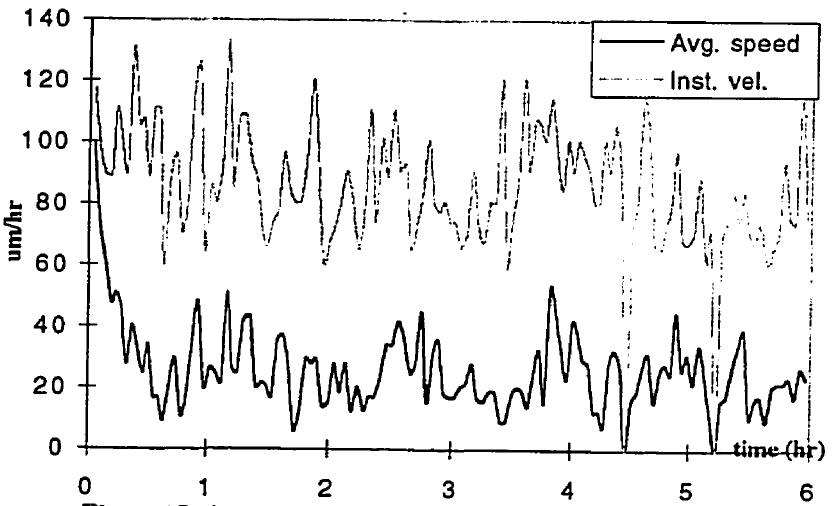


Figure 15: Average instantaneous velocity and speed of a benign colloid nodular thyroidectomy cell population at a given point in time. Individual cell data were pooled over a period of 6 hours.

c) Normal

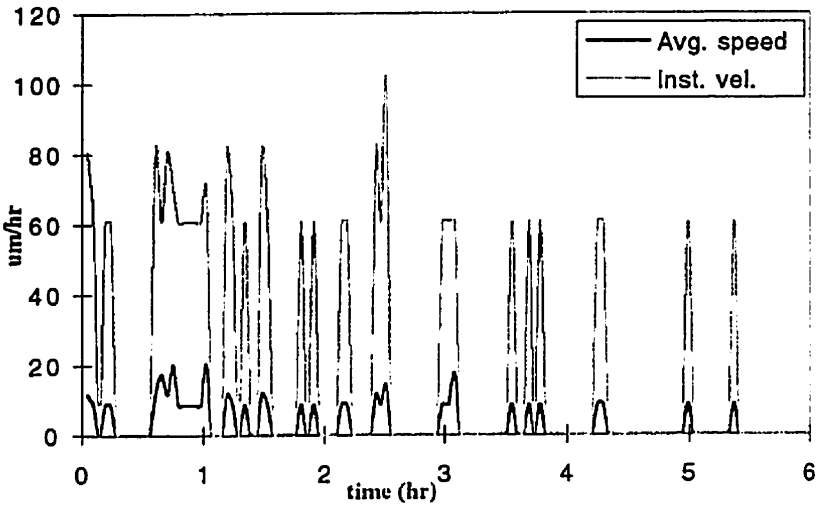


Figure 16: Average velocity and speed for a normal gingival tissue cell population at a given point in time. Individual cell data were pooled over 6 hours. Tracked normal cells included monocytic & epithelial-like cells since no tumor SLC behavior was observed .

Population data in Figures 14 -16 consisted of averaging the locomotory parameters - velocity and speed, in this case- of the pooled cells that were tracked at a given point in time. The total time of observation for all three samples was 6 hours. As with individual cell data (section 4.2.1), note average speed takes into account the frequency distribution of stops and goes.

The average velocity and speed for each of the cell populations remained relatively constant throughout the 6 hours of time-lapse recording and did not show any significant decline. The average velocity of the squamous cell carcinoma population (approximately 136 μm/hr) was higher than that of the benign or normal samples (app. 85 μm/hr and 10 μm/hr respectively).

The average speed for the malignant cell population was higher than that of the benign cells. Normal cells had the lowest average speed. Speed did not necessarily correlate with velocity, it depended on the frequency distribution of "stops" and "goes". For example, there was a higher frequency of "stops" within the benign cell population than with the malignant population; this can be seen in the difference between the average velocity and speed in Figures 14 and 15. Cell population behavior of normal cells (Figure 16) generally showed a much lower rate of locomotion and general activity.

4.2.2 3-D Representation of Individual Cell Data

Three cell locomotory parameters were used in a 3-D representation of individual cell data. These were: average velocity of a cell, total distance migrated during the period of observation, and percent of the time it was migrating. A representative sample is shown for a malignant, benign, and normal cell population.

a) Malignant

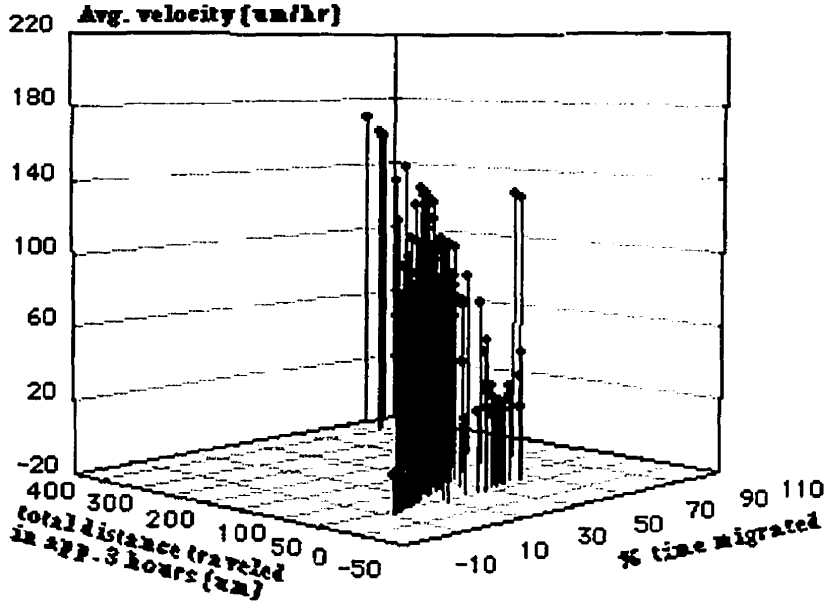


Figure 17: Functional subpopulations within a squamous cell carcinoma sample. This specific malignant sample suggests at least two distinct trends where a subset of cells (to the right) has a much lower velocity than the group of cells with the much higher velocity (to the left). Even if the latter migrated more frequently, the total distance was not as much as the subpopulation of cells with the higher velocity; this is because of the large difference in velocity which makes up for the difference. There are also several cells locomoting at high velocity and percent of migration.

b) Benign

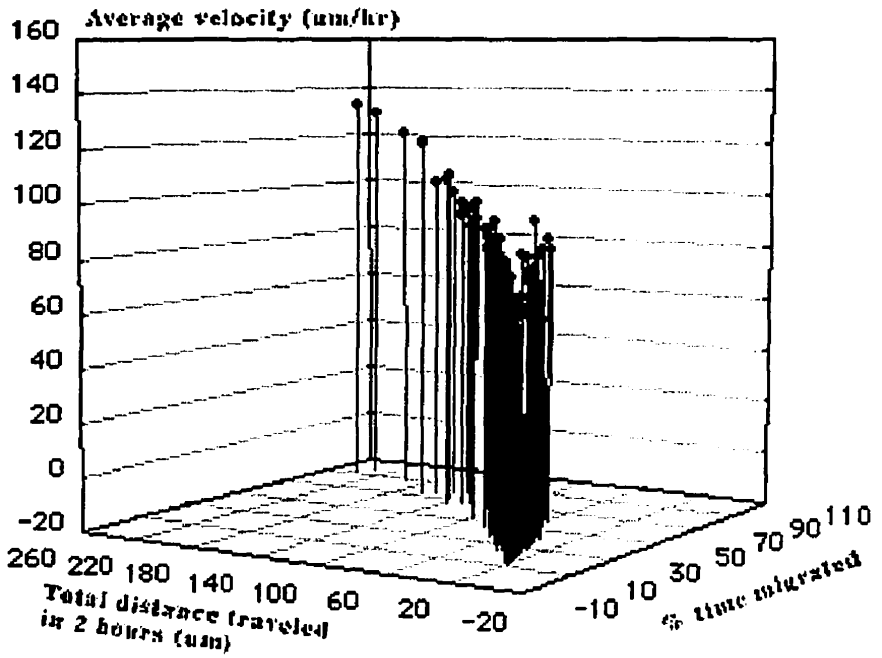


Figure 18: Functional subpopulations within a colloid nodular thyroidectomy sample. This benign sample suggests at least two subpopulations of locomoting cells. The first subset (to the right) showed a lower rate of increase in percent time migrated than the adjacent subset to the left. Since the velocities were comparable for both these subpopulations, the cells that migrated more frequently traveled further.

c) Normal

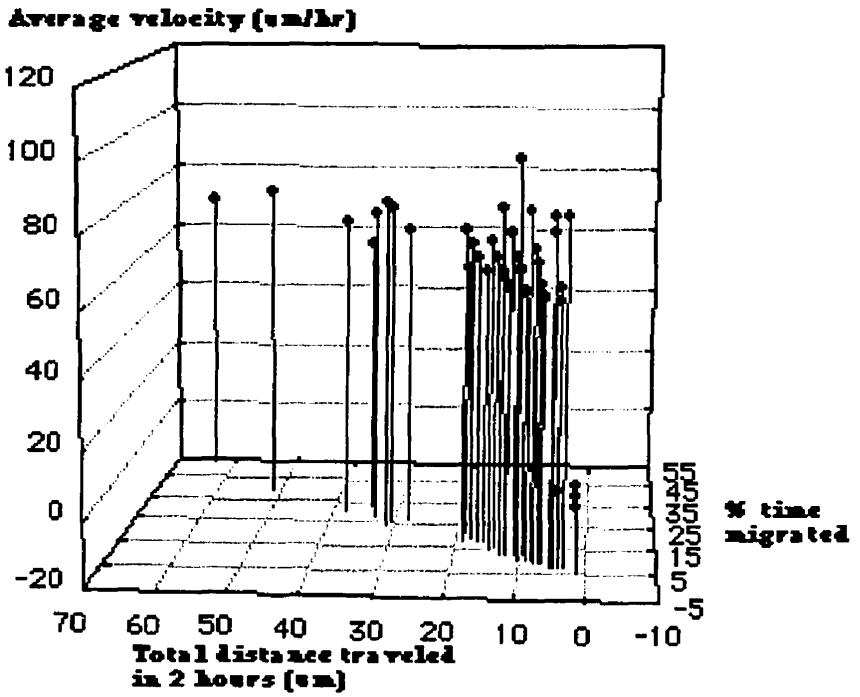


Figure 19: Functional subpopulations within a normal gingival sample. Normal cell locomotory parameters were plotted during 2 hour intervals. The cells fall within a relatively narrow range of parameters and show less variation than the majority of malignant or benign samples. The criteria for tracking normal cells were different from those of tumor cells; normal cells tracked consisted of monocytic or epithelial-like cells since no single locomoting tumor cell behavior was observed.

The graphs plotted in Figures 17 - 19 illustrated locomotory behavior of cell populations in a 3-dimensional format. First there was a " V-formation" of cells plotted from the malignant and benign plots (Figures 17 and 18, respectively). This could be indicative of cell subpopulations. With the malignant sample, there was a wider range of velocity differences than with the benign sample. Also, when compared to the benign 3-D plot, the malignant sample had a greater population of cells with a higher velocity and rate of migration. On the other hand, normal cells showed a much smaller range of variation of parameters when compared to either the benign or malignant plots. For example, velocity

was quite similar for the normal cells, and the percent of time the cells migrated ranged from 10-55%. The latter is compared with a percent migration rate of 0-90% for malignant and benign cells.

Upon further observation, the cell plots seemed to follow a characteristic "narrow corridor" - curved pattern. This phenomenon is explained in Table 3. For example, if a cell had a high velocity yet stopped frequently, its migrated distance would not necessarily have been high- and vice versa. The aforementioned would have then resulted in a characteristic pattern.

	↑ Average velocity of locomoting cell	↓ Average velocity of locomoting cell
↓ Percent Time Migrated	↓ distance traveled	↓↓ distance traveled
↑ Percent Time Migrated	↑↑ distance traveled	↑ distance traveled

Table 3: Correlation of average instantaneous velocity with percent time migrated of a single locomoting cell. This table explains the resultant cell distance traveled within a given time period. Distance migrated depended upon the combination of the two variables of velocity and migration rate of single cells. This table also explains why the plotted cells fell into a narrow corridor of values with a characteristic curved shape.

4.3 Differences Between Malignant, Benign, and Normal Locomotory Parameters

Locomotor parameters were compared in order to illustrate the existence of different locomotory behavior between malignant, benign, and normal samples

4.3.1 Population Components

Cell population data from malignant, benign, and normal were pooled to visually represent differences in average instantaneous velocity and average rate of locomotion of a cell population at a given point in time.

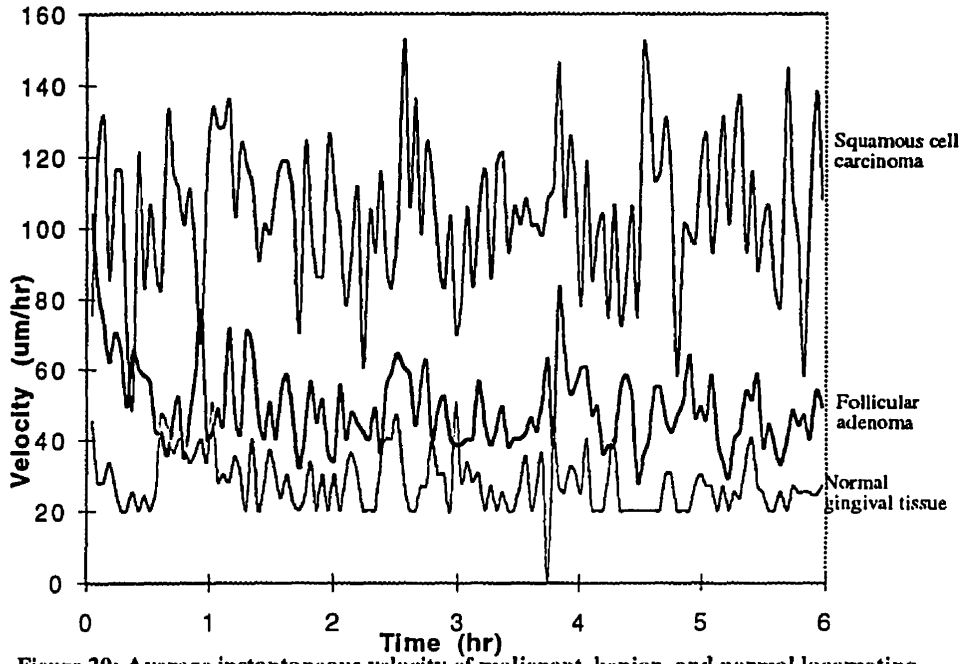


Figure 20: Average instantaneous velocity of malignant, benign, and normal locomoting single cells pooled at a given point in time. Velocity for pooled data from single cells within a population and plotted over a 6 hour observation period.

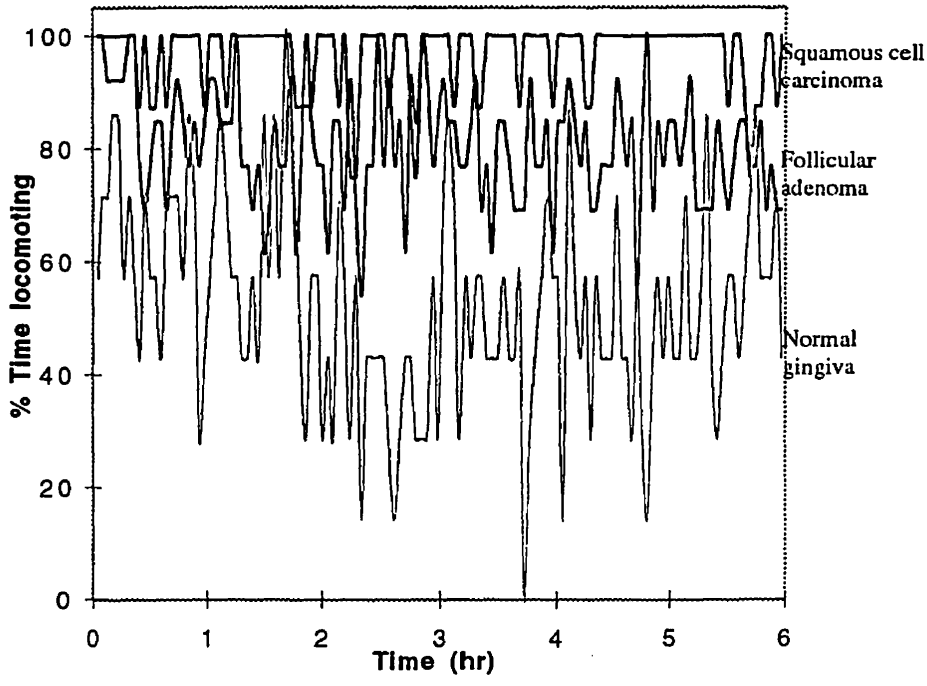


Figure 21: Average % time locomoting of malignant, benign, and normal locomoting single cells pooled at a given point in time. The amount of time that individual single cells within a population locomoted at a given point in time was pooled and plotted over a 6 hour observation period.

4.3.2 Individual Cell Components

Individual cell data from malignant, benign, and normal populations were pooled to visually represent differences in average velocity, rate of migration, and total distance traveled.

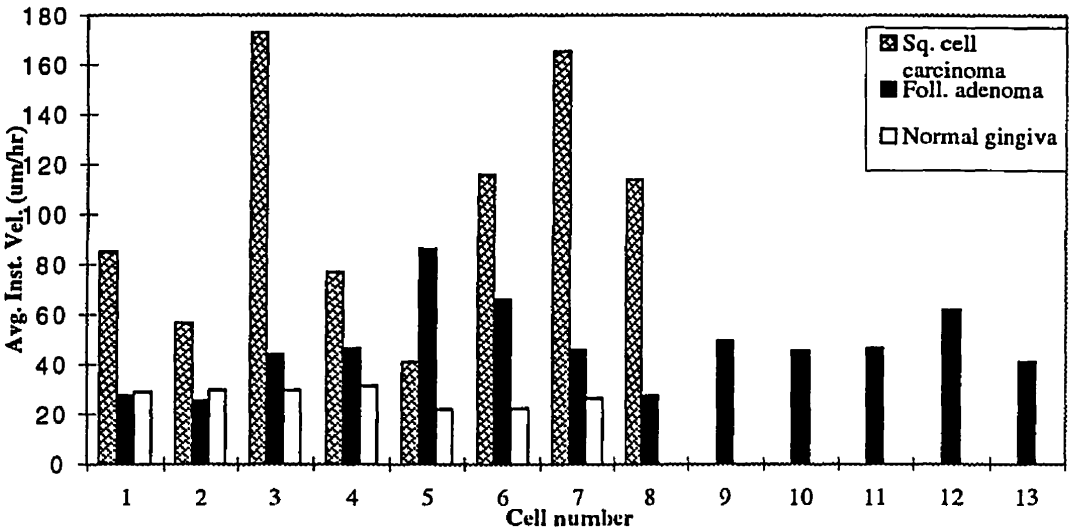


Figure 22: Average instantaneous velocity for individual cells within malignant, benign, or normal cell population. The instantaneous velocity over a period of 6 hours was averaged for each cell . A squamous cell carcinoma, follicular adenoma, and nonnal gingiva were used. The normal cells tracked were monocytic or epithelial-like since no SLC behavior was observed

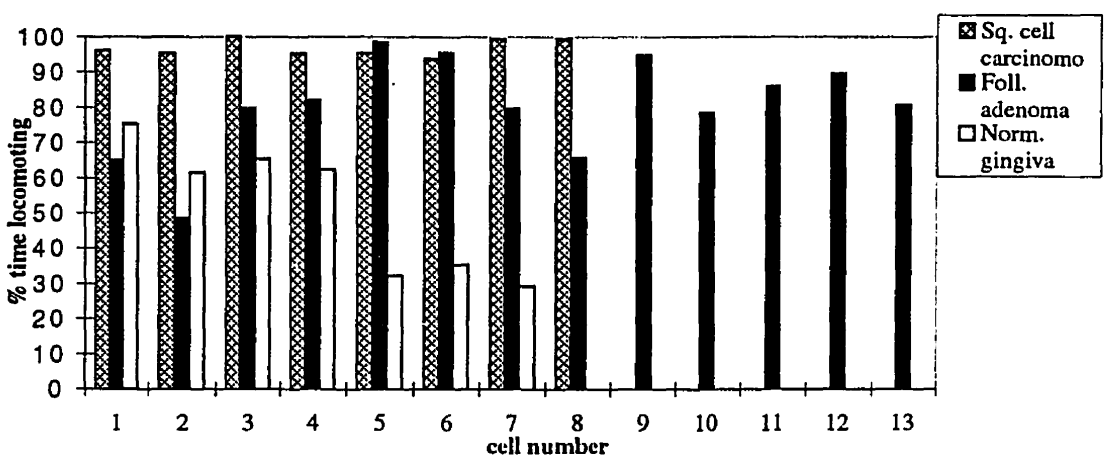


Figure 23: Average percent of time migrated by single cells within a malignant, benign, or normal cell population. The % time migrated over a period of 6 hours was averaged for each cell.

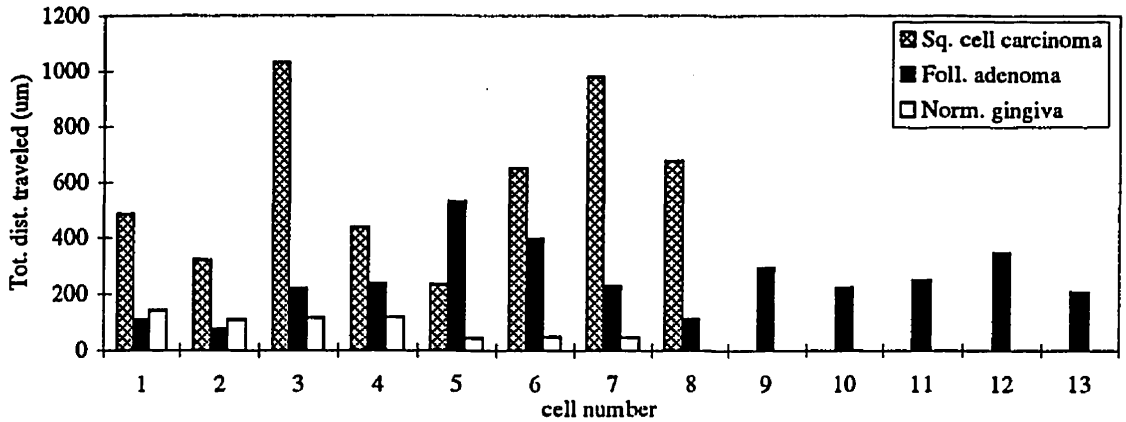


Figure 24: Total distance of traveled of individual cells within malignant, benign, or normal cell populations over a 6 hour period. The cumulative distance traveled was calculated for each cell within a squamous cell carcinoma, follicular adenoma or normal gingival tissue.

When malignant cell populations were compared to benign or normal populations, there generally was an obvious difference with respect to locomotory parameters. This was seen in Figures 20-24. For example, average instantaneous velocity, percent time migrated, and distance traveled of individual cells as well population at a given point in time were generally higher for malignant samples than for benign cells. Normal cell populations exhibited the lowest migratory activity.

4.3.3 General Analysis of Pooled Data

The following Table 4 shows a simple representation of the sum of *all* the analyzed (not shown) locomotory data of malignant, benign, and normal cells pooled and compared.

	MALIGNANT	BENIGN	NORMAL
AVG. VELOCITY (um/hr)	86.59 ± 26.8	74.33 ± 12.8	59.54 ± 5.1
% TIME MIGRATING	31.75 ± 20.3	15.76 ± 12.7	4.89 ± 4.2
AVG. DIST. TRAVELED /HR.	30.28 ± 39.9	12.06 ± 11.2	3.37 ± 3.1

Table 4: Locomotor parameters of pooled malignant, benign, and normal locomoting single cells. These values represent all the cells studied ± SD (standard deviation)

Analysis of single cell locomotory behavior showed differences in given measured characteristics. For example, malignant samples showed in general an average velocity of 86.59 μm/hr with locomotion occurring 31.75% of the time. Whereas pooled benign cells had an average velocity of 74.33 μm/hr and showed a tendency to stop more frequently (15.76%). Normal cells had a much lower average velocity of 3.37 μm/hr and locomoted 4.88 % of the time. One can therefore say that quantification of human single locomoting cells appearing from tissue cultured within a 3-D collagen gel matrix show differences between malignant, benign, and normal specimens.

Chapter 5

Discussion

The main aspect of cell motility examined in this thesis focused on quantification of single tumor cell locomotory behavior in a 3-D collagen matrix. Cellular behavior was recorded using time-lapse videomicroscopy and subsequently digitized through computer-assisted tracking. Further analysis of cell trajectory provided quantifiable data. Hence, the first objective of this thesis was met.

The second objective of this thesis was also met since different approaches of measurement were used in order to represent various facets of cell locomotion. By obtaining quantifiable locomotory parameters, differences amongst and between the samples were evaluated. Individual cell and cell population analyses were possible through quantifiable locomotory parameters such as velocity and percent time migrated, and frequency of distribution of "stops" and "goes". The 3-D population graphs provided a visual representation of cell subpopulations within a given sample; if only histology or end-point methods had been used, cell subpopulation behavior could not have been elucidated.

In this study, it has been shown that cells within a collagen type I matrix can be used to induce cellular locomotory behavioral responses that can be observed and measured. It was also shown that the tumor specimens could be maintained for future cultures for up to one year in frozen suspension. This is an advantage since the ability to go back and re-examine a patient's tumor lends itself quite readily to research and can serve as

a useful adjunct to conventional static techniques. Culturing cells within a 3-D collagen lattice was an improvement since it was a controllable in vitro system which likely provided a better approximation of the tissue environment [24,48,64]. Furthermore cellular behavior previously not seen on 2-D plastic or liquid culture systems was manifested with this collagen system. Not only that, but cell tracking and data analysis of single locomoting cells showed characteristic differences in locomotory parameters such as velocity, percent time migrated, and directional migration [34].

Even if the cell trajectory was within a 3-D collagen matrix, it was analyzed from a 2-D mathematical perspective since the z-axis was previously found not to contribute significantly in a cell's locomotory pattern under the experimental conditions in this thesis [1,6,55]. Also, due to the complexity of measurement of the z-axis in the particular experimental set-up employed, only the x-y coordinates were used. Perhaps future and more complex 3-D studies of cell locomotion should include all the planes.

Analysis of tumor cell locomotory behavior in a 3-D collagen matrix has previously revealed the existence of locomotory subpopulations within heterogeneous cell populations such as lymphocytes [55] and fibroblasts [13]. Trajectory analysis methods of tumor specimens could prove sensitive enough to measure cell behavioral parameters such as the existence of functional subpopulations, aggressiveness with respect to pathogenicity, invasion, and metastasis. This is in contrast to end-point determination methods where the resultant behavior of cells may not be sufficient to represent heterogeneity of cell populations as well as cell-cell and cell-matrix interactions [55]. It is possible that locomotory parameters other than the ones used in this thesis exist; these might even reveal different subpopulations within a heterogeneous tissue sample. However the main point here is that subpopulations do exist as demonstrated by the 3-D plots and various representations of locomotory behavior analysis which can be detected with the methodology discussed in this thesis [25]. For example, observation of the 3-D plots of a squamous cell carcinoma revealed the existence of subpopulations with high velocity and rate of migration, and vice versa. In contrast, benign samples generally showed less heterogeneous behavior and normal cells showed more or less uniform values. These results emphasize the heterogeneity of tumors and how cell trajectory analysis could reveal individual differences in cell locomotory profiles.

Furthermore, as was seen in the results of this thesis, cell trajectory analyses provided huge amounts of data that could be quantified and assessed in a myriad of ways. The importance of cell locomotion in metastasis and invasion was also illustrated by observed and measured behavior in 3-D collagen gels. Observation of tumor cell locomotion in the 3-D collagen lattice provided a wealth of information ranging from

pseudopodial extension to ruffling behavior to measurement of migration rates. These are just a few examples of how the use of a dynamic pathology approach could eventually help explain the role of tumor cell locomotion in invasion and metastasis of cancer. As was previously mentioned, the dissemination of cancer is usually an important factor in assessment of patient survival. Likewise, the 3-D cell population plots provided visual representation of the possible existence of functional subpopulations of locomotory behavior; this might not have otherwise been seen with simple 2-D analysis of one parameter at a time. This would signal the need for future research which should correlate the findings of the dynamic pathology approach of quantification of cell locomotory behavior with survival studies of the patient with the potential for use of tailor-made anti-neoplastic drug treatment protocol for the patient (as opposed to broad-spectrum "hit-or-miss" treatments). These are long term studies which require at least five years due to the remission time of cancer.

Due to the complexity and number of variables involved, more sophisticated and sensitive techniques (which are beyond the scope and time of this thesis) such as time series and cluster analysis or even exponential smoothing should be used to further describe cell population behavior and locomotory heterogeneity of tumor cell populations including single tumor cells and cell clusters [13,25]. These methods would allow simplification of data interpretation and determination of mathematical models most suitable for quantification of cell locomotory behavior within a 3-D environment.

A further application of 3-D matrices may be development of specific and more complex extracellular matrices for a given study. These matrices may include: interstitial components (such as fibronectin, laminin, and various glycosaminoglycans), growth factors, or a combination of tissue types (such as neoplastic tissue and basement membrane). Tumor cell behavior within 3-D in vivo culture systems could be used to understand anti-neoplastic drug and radiation resistance. Another future application of in vitro 3-D gel systems may include drug testing of anti-neoplastic [57] and antibiotic drugs as well as development of custom-made treatment protocols for the individual patient. This can prove to be less harsh since only a small tissue sample is sufficient to perform a battery of tests within the 3-D culture system rather than on the patient him/herself. Furthermore, this system can easily be used by the clinician, and is cost-effective since the equipment is readily available and is reasonably priced.

Using this approach, malignant tumors studied showed differences in velocity, percent time migration and total distance traveled; that is, they showed more locomotory aggressiveness than benign tumors and normal tissue. These locomotory parameters could be useful indicators of malignant aggressiveness of an individual patient's tumor and as

such constitute a "dynamic pathology" to supplement current "static" pathological methods The findings also reveal heterogeneity in individual cell locomotory behavior which could be of vital importance in selecting anti-neoplastic drug treatment protocols. The methodology described in this thesis and in the selection of anti-neoplastic drugs with the best chance of eliminating cell phenotypes expressed. Further research and analysis using 3-D cultures is required to further understand the role and implications of tumor cell locomotion- both single cells and clusters. Other than that, the appearance of certain characteristic features such as locomotion and other observable phenomena might in themselves be pathological and prognostic indicators of invasive and metastatic potential in vivo. For example, the existence of a cell subpopulation exhibiting a high velocity and percent time migration could indicate an aggressive malignant phenotype. In addition, the study of locomoting tumor cell clusters may help elaborate tumor invasion and metastasis mechanisms.

In conclusion, as opposed to end-point methods, trajectory analysis of cell locomotion within a 3-D collagen gel matrix should prove to be a more sensitive indicator of tumor cell locomotory behavior and therefore aggressiveness.

Bibliography

[1] Friedl, P., Noble, P.B., Walton, P., Laird, D.W., Chauvin,P.J., Tabah, R.J, Black, M., and Zanker, K.S. Migration of coordinated clusters in mesenchymal and epithelial cancer explants in vitro. Cancer Res. 55: 4557-4560; 1995.

[2] Harris, A. Cell surface movements related to cell locomotion. Locomotion of Tissue Cells. Ciba Foundation Symposium 14. Associated Scientific Publishers, Amsterdam, 1973.

[3] Middleton, C.A., and Sharp, J.A. Cell Locomotion In Vitro: Techniques and Observations. Croom Helm Applied Biology Series. P. Baron ed. London and Canberra, 1984.

[4] Lester, I., Korner, G., Ishai-Michaeli, R., Bashkin, P., Bar-Shavit, R., and Fuks, Z. Extracellular matrix resident growths factors and enzymes: possible involvement in tumor metastasis and angiogenesis. Can Met. Rev. 9: 203-226; 1990.

[5] Sträuli, P., and G. Haemmerli. Cancer cell locomotion: its occurrence during invasion. Invest. Exp. and Clinical Implications. Oxford Univ. Press. 1984.

[6] Friedl,P., Noble, P.B., and Zanker, K.S. T lymphocyte locomotion in three-dimensional collagen matrix: expression and function of cell adhesion molecules. J. Immunol.154: 4973-4986; 1995.

[7] Koutsilieris, M., Sourla, A., Pelletier, G., and Doillon, C.J. Three-dimensional type I collagen gel system for the study of osteoblastic metastases produced by metastatic prostate cancer. J. of Bone and Mineral Res. 9: 1823-1832; 1994.

[8] Applegate, K., Balch, C., and N. Pellis. *In vitro* migration of lymphocytes through collagen matrix: arrested locomotion in tumor-infiltrating lymphocytes. Can. Res. 50: 7153-7158; 1990.

[9] Kumar, V., Cotran, R., and Robbins, S. Basic Pathology 5th ed W.B. Saunders Company Harcourt Brace Ivanovich, Inc. Philadelphia. J. Mitchell ed., 1992.

[10] Simon, N., Noël, A., and J.M. Foidart. Evaluation of in vitro reconstituted basement membrane assay to assess the invasiveness of tumor cells. Invasion Met. 12: 156-167; 1992.

[11] Haramaki, M. Morphological and biological changes of a hepatocellular carcinoma cell line in a three-dimensional matrix of collagen. Acta Path. Japonica, 43: 490-499; 1993.

[12] Caplan, C. The extracellular matrix is instructive. BioEssays 5: 129-132; 1986.

[13] Noble, P.B., and Shields, E.D. Time-based changes in fibroblast three-dimensional locomotory characteristics and phenotypes. Expl. Cell Biol. 57: 238-245; 1989.

[14] Vlodavsky, I., Korner, G., Ishai-Michaeli, R., Bashkin, P., Bar-Shavit, R., and Fuks, Z. Extracellular matrix- resident growth factors and enzymes: possible involvement in tumor metastasis and angiogenesis. Can. Met. Rev. 9: 203-226; 1990.

[15] Toda, S., Yonemitsu, N., Hikichi, Y., Sugihara, H., and Koike, N. Differentiation of human thyroid follicle cells from normal subjects and Basedow's disease in three-dimensional collagen gel cultures. Path Res. Pract. 188: 874-882; 1992.

[16] Fong, C.J., Sutowski, D., Kozlowski, J., and C. Lee. Utilization of the Boyden chamber to further characterize in vitro migration and invasion of benign and malignant human prostatic epithelial cells. Invasion Met. 12: 264-274; 1992.

[17] Brown, B., Barnes, L., Mazariegos, J. Prognostic factors in mobile tongue and floor of the mouth carcinoma.Cancer 64:1195-1202; 1989.

[18] The Merck Manual of Diagnosis and Therapy. Fifteenth edition. Published by Merck Sharp & Dohme research laboratories; division of Merck & Co., Inc., Rahway, N.J., 1987.

[19] Friedl, P., Noble, P.B., Walton, P., Chauvin,P.J., Tabah, R.J, Black, M.,and Zanker, K.S. Invasion and migration of cooordinated cell clusters in rhabdomyosarcoma and other head and neck cancers. Cancer. 1995.

[20] Schor., S.L., Schor, A.M., Allen,T.D., and Winn, B. The interaction of melanoma cells with fibroblasts and endothelial cells in three-dimensional macromolecular matrices: a model for tumor cell invasion. Int. J. Can. 36:93-102 ; 1985.

[21] Schor., S.L., Allen, T.D., and Winn, B. Lymphocyte migration into three-dimensional collagen matrices: A quantitative study. J. Cell Biol. 96:1089-1096; 1983.

[22] National Cancer Institute of Canada: Canadian Cancer Statistics 1996. National Cancer Institute, Statistics Canada, Provincial Cancer Registries and Health Canada.

[23] Fenogolio-Preisner, C., Noffsinger, A., Miller, M.A., Davis, B., and Heffelfinger, S. "Imaging" the cell through its markers: insights into cell growth, cell death and cancer. Annals of Royal College of Physicians & Surgeons 28: 341-347; 1995.

[24] Schor., S.L., Schor, A.M., Winn, B., and Rushton, G. The use of three-dimensional collagen gels for the study of tumour cell invasion in vitro: experimental parameters influencing cell migration into the collagen matrix. Cancer (Lond). 29: 57-62; 1982.

[25] Shields, E.D., and Noble, P.B. Methodology for detection of heterogeneity of locomotory phenotypes in three-dimensional gels. Exp. Cell Biol. 55, 250-256; 1987.

[26] Liotta, L. Tumor invasion and metastases- role of the extracellular matrix: Rhoads memorial award lecture. Can. Res. 46: 1-7; 1986.

[27] Partin, A.W., Schoeniger, J.S., Mohler, J.L., and Coffey, D.S. Fourier analysis of cell motility: Correlation of motility with metastatic potential. Proc. Natl. Acad. Sci. USA 86: 1254-1258; 1989.

[28] Zetter, B. Cell motility in angiogenesis and tumor metastasis. Can. Investigation 8: 669-671; 1990.

[29] Mareel, M. Invasion in vitro: methods of analysis. Can. Met. Rev. 2: 201-218; 1983.

[30] Hart, I.R. Mechanism of tumor cell invasion. Can. Biol. Rev. (eds JJ Marchalonis, MG Hanna, IJ Filler) Vol. 2, pp 29-58, Marcel Dekker, New York, 1981.

[31] Robertson, N., Starkey, J., Hamner, S., and Meadows, G. Tumor cell invasion of three-dimensional matrices of defined composition: evidence for a specific role for heparan sulfate in rodent cell lines. Can. Res. 49:1816-1823; 1989.

[32] Pauli, B., Schwartz, D., Thonar, E., and Kvettner, K. Tumor invasion and host ECM. Can. Met. Rev. 2: 129-152; 1983.

[33] Liotta, L., Klenerman, J., and Saidel, G. Quantitative relationships of intravascular tumor cells, tumor vessels, and pulmonary metastases following tumor implantation. Can. Res. 34: 997-1004; 1994.

[34] Muradali, S. "Invasion and migration of clusters in oral carcinomas within Three-dimensional Collagen Matrices: the effects of ß1 and ß1α2 integrin function." Master's thesis, Department of Dental Sciences, McGill University, Montreal, July 1996.

[35] Liotta, L. , Rao, C., and S. Barsky. Tumour invasion and the extra-cellular matrix. Lab. Invest. 49 : 636-647; 1983.

[36] Luparello, C., Sheterline, P., Pucci-Minafra, I., and Minafra, S. A comparison of spreading and motility behavior of 8701-BC breast carcinoma cells on type I, I-trimer and type V collagen substrata. Evidence for a permissive effect of type-I trimer collagen on cell locomotion. J. Cell Sci. 100: 179-185; 1991.

[37] Wood, S. Pathogenesis of metastasis formation observed in vitro in the rabbit ear chamber. Arch. Pathol. 66: 550-568; 1958.

[38] Albini, A., Kleinman, H., Martin, G., Aaronson, S., Kozlowski, J., and R. McEwan. A rapid *in vitro* assay for quantitating the invasive potential of tumour cells. Can. Res 47: 3239-3245; 1987.

[39] Ratner, S. Lymphocyte migration through extracellular matrix. Inv. Met. 12: 82-100; 1992.

[40] Schor, S.L. Cell proliferation and migration on collagen substrata in vivo. J. Cell. Sci. 41: 159-175; 1980.

[41] Klominek, J., Robért, K-H., and K-G Sundqvist. Chemotaxis and haptotaxis human malignant mesothelioma cells: effects of fibronectin, laminin, type IV collagen, and an autocrine motility factor-like substance. Can. Res. 53: 4376-4382; 1993.

[42] Haston, W.S., Shields, J.M., and Wilkinson, P.C. Lymphocyte locomotion and attachment on two-dimensional and three-dimensional matrices. J. Cell Biol. 92: 747-752; 1982.

[43] Elsdale, T., and Bard, J. Collagen substrata for studies on cell behavior. J. Cell Biol. 54: 626; 1972.

[44] Boyden , S. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes. J. Exp. Med. 115: 453-466; 1962

[45] Merzack, A, Koocheckpour, S., and G. Pilkington. CD44 mediates human glioma cell adhesion and invasion *in vitro*. Can. Res. 54: 3988-3992; 1994.

[46] Brown, L., Lanir, N., McDonagh, J., Tognazzi, K., Dvorak, A., and Dvorak, H. Fibroblast migration in fibrin gel matrices. Amer. J. Pathol. 142: 273:283; 1993.

[47] Strauli, P. A concept of tumor invasion. P. Strauli, A.J. Barret, A. Baichi (ed.), Proteinases and Tumor Invasion, pp. 1-13, Raven Press, New York; 1980.

[48] Schor., S.L., Schor, A.M., Winn, B., Rushton, G., and Smith, L. Adult, foetal and transformed fibroblasts display different migratory phenotypes on collagen gels: Evidence for isoformic transformation during foetal development. J. Cell Sci. 73: 221-234; 1985.

[49] Sato, H., and Suzuki, M. Experimental studies on metastasis formation with special reference to the mechanism of cancer cell lodgement in the microcirculation. Exc. Med. int. Congr. Set., Atherogenesis-II (269): 168-176; 1972.

[50] Masi,L., Franchi, A., Santucci, M., Danielli, D., Arganini, L., Giannone, V., Formigli, L., Benvenuti,S., Tanini, A., Beghè, F., Mian, M., and Brandi, M.L. Adhesion, growth, and matrix production by osteoblasts on collagen substrata. Calcified Tissue Int. 51: 202-212; 1992.

[51] Iwasaka, K., Rogers, L., Barnett, G., Estes, M., and B. Barna. Effect of recombinant tumor necrosis factor-alpha on three-dimensional growth, morphology, and invasiveness of human glioblastoma cells *in vitro*. J. Neurosurg. 78: 953-958; 1993.

[52] Grill, B.C., and Z. Cully. Signal perception of fibroblasts for directional migration to platelet-derived growth factor in Boyden-type chamber. J. Cell Physiol. 143:172-177; 1990.

[53] Nabi,I., Watanabe, H., and A. Ras. Autocrine motility factor and its receptor: role in cell locomotion and metastasis. Can. & Met. Rev. 11: 5- 20; 1992.

[54] Schor., S.L., Schor.,A.M., Durning, P., and Rushton, G. Skin fibroblasts obtained from cancer patients display foetal-like migratory behavior on collagen gels. J. Cell Sci. 73: 221-234; 1985.

[55] Friedl, P., Noble, P.B., and Zanker, K.S. Lymphocyte locomotion in three-dimensional collagen gels: comparison of three quantitative methods for analyzing cell trajectories. J. Immunol. Methods 165: 157-165; 1993.

[56] Leighton, J. The growth patterns of some transplantable animal tumors in sponge matrix tissue culture. J. Natl. Can. Inst. Vol. 15: 275-293; 1954.

[57] Hoffman, R. The three-dimensional question: can clinically relevant tumor drug resistance be measured *in vitro*? Can. and Met. Rev. 13: 169-173; 1994.

[58] Allen, T.D., Schor., S.L., and Schor, A.M. An ultra-structural review of collagen gels: A model system for cell matrix, cell basement membrane, and cell- cell interaction. Scan. Elect. Micros. 1: 375-390; 1984.

[59] Gutman, H., Risin, D., Katz, B.P., and Pellis, N.R. Locomotion through three-dimensional type I rat tail collagen. A modified mini assay. J. Immunol. Meth. 157: 175-180; 1993.

[60] Strohmaier, A., and Spring, H. Three-dimensional analysis of the substrate-dependent invasive behavior of a human lung tumor cell line with confocal laser scanning microscope. Histochem. Cell Biol. 105: 179-185; 1996.

[61] Schor., S. The effects of EDTA and trypsin on the serum requirement for cell attachment to collagen. J. Cell Sci. 40: 271 ; 1979.

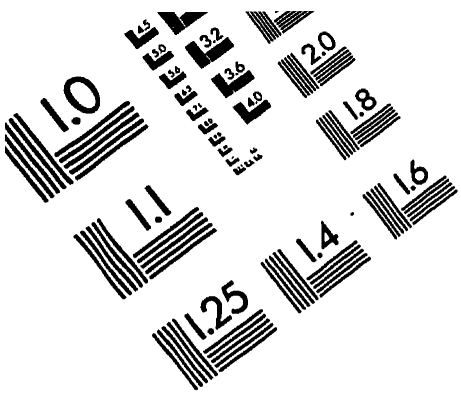
[62] Ratner, S., and Heppner, G. T-cell locomotion in the tumor microenvironment. I. A collagen matrix assay. J. Immunol. 135, 2220; 1985.

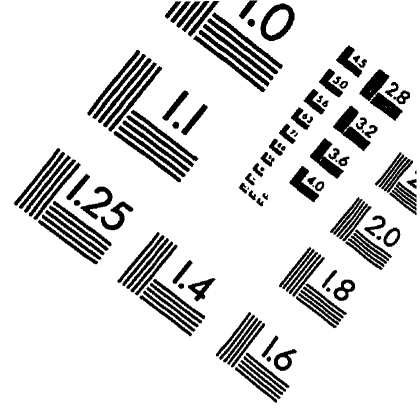
[63] Mareel, M., De Baetselier, P., and F. Van Roy. Mechanisms of invasion and metastasis. CRC Press Inc., 1991.

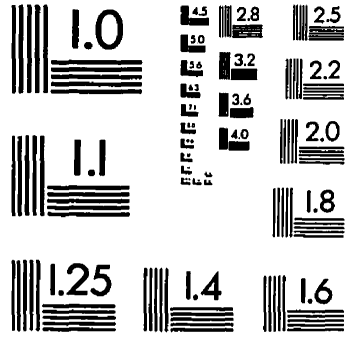
[64] Noble, P.B., Shields, E.D., and Walton, P. Changes in three-dimensional locomotory characteristics of 212 cells upon expression of the EJ-ras gene. Invasion and Metastasis 13: 113-118; 1993.

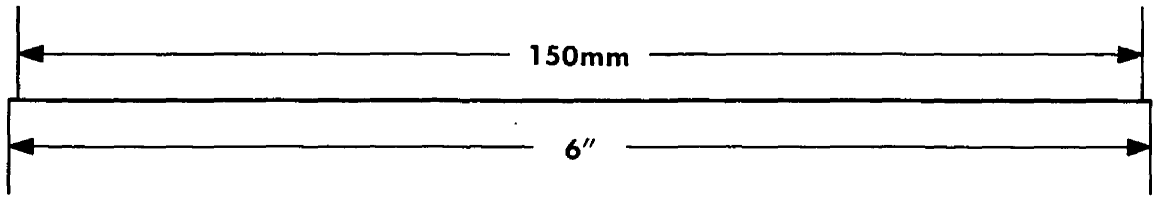
[65] Noble, P.B., and Levine, M.D. Computer-assisted analyses of cell locomotory characteristics and phenotypes. Exp. Cell Biol. 57, 238; 1989.

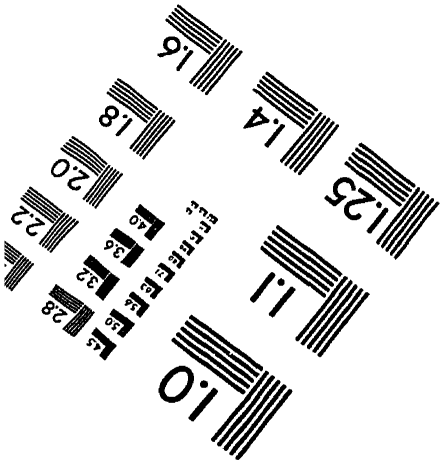
[66] Noble, P.B. The extracellular matrix and cell migration:locomotory characteristics of Mos-11 cells within a three-dimensional hydrated collagen lattice. J.Cell Sci. 87: 241-248; 1987.

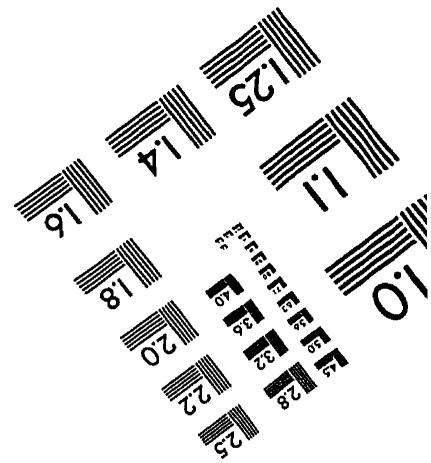












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