Image analysis of Transwell assays in the assessment of invasion by malignant cell lines

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ABSTRACT

This study aims to determine if layering of extracellular matrix (ECM) can achieve a physiological basement membrane thickness of 8 μm and to assess the use of paraffin wax-embedded Transwell plates coupled with digital image analysis as a means of determining invasion by malignant cell lines. Layers of Matrigel, a sarcoma-derived ECM was built to a concentration of 7.4 μg/mm^2 in the upper chamber of a Transwell plate invasion assay. Two cell lines from extrahepatic bile duct adenocarcinoma were tested in serum-free growth medium. Conditioned medium was added to the lower chamber to act as a chemoattractant. Following attachment, cells were incubated for 48 h and the Matrigel-coated insert cut from its holder and fixed in 10% unbuffered formalin saline. Each insert was bisected and processed to paraffin wax. Serial levels were stained by haematoxylin and eosin. A Kontron image analysis system was used to measure the mean thickness of Matrigel for each cell line and the degree of invasion was assessed by measuring the depth to which cells had degraded the Matrigel. A mean thickness of 8 μm was achieved using 5.0 μg/mm^2 for the OCUCh-LM1 cell line and 7.4 μg/mm^2 for the SKChA-1 cell line. No significant difference was seen in the ability of either cell line to degrade Matrigel. Immunocytochemistry for laminin and cytokeratin helped to identify ECM components and cells, respectively. In conclusion, digital image analysis of paraffin wax-embedded inserts can be used to determine the invasive capacity of various cell lines; immunocytochemistry may help to identify ECM components and cells; and the assay used to assess different cell lines and their ability to degrade Matrigel.

KEY WORDS: Extracellular matrix.
Image processing, computer-assisted.
Immunohistochemistry.
Neoplasm, invasiveness.

Introduction

In order for a metastatic colony to become established, malignant cells must invade a secondary site and proliferate within the tissue. Metastasis is a multistep process involving interactions between tumour cell, host cell, and cell matrix.[1-3] Interactions with the extracellular matrix (ECM), especially the basement membrane, that characterise invasion include the ability of the tumour cell to become attached, to proteolyse the matrix components, and then migrate through the matrix defect.[4] In order to initiate the metastatic process, carcinoma cells must penetrate the epithelial basement membrane and invade the interstitial stroma. However, to cross the basement membrane the tumour cells must have the appropriate integrins and type IV collagenase on their surface to adhere to ECM components (e.g. laminin) and help digest type IV collagen, respectively. Loss of the epithelial basement membrane is a hallmark of invasion.

A Boyden chamber[5] and its modifications[6,7] have been used successfully as an *in vitro* model of invasion since introduction of the chamber in 1962. A variety of *in vitro* systems have been developed to assess the invasiveness of tumour cells, and several of these assays use tissues that contain basement membranes, such as bladder wall,[8] amnion[9] and lens capsule.[10] In addition, a pressed disc composed of lyophilised collagen IV and laminin has also been used to assay for the invasiveness of tumour cells.[11]

Albini *et al*.[6] reconstituted a basement membrane matrix onto a filter in a Boyden chamber to assess the ability of various malignant and non-malignant cells to penetrate the coated filter, and discovered that only the malignant cell lines crossed the matrix. They varied the amount of basement membrane Matrigel and discovered that invasion took longer when the amount of Matrigel was increased.[6] The cells that penetrated the basement membrane were isolated and studied, and they appeared to be a more invasive subpopulation of the parent line when the assay was repeated.

Matrigel is a basement membrane matrix derived from Engeibreth Holm Swarm (EHS) tumour, and consists of type IV collagen, laminin, and certain other elements such as plasminogen activator.[12]

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Most *in vitro* systems for measuring invasion are based on an invasion chamber consisting of two compartments that are separated by a polycarbonate filter.[7] These assays have major disadvantages and pitfalls:[13] for example, the need for cells to be able to adhere to and pass through the rigid filter with fixed pores; the relative thinness of the filter; the random distribution of the pores, which 'cover' less than 30% of the filter surface, leading to uneven chances to invade; and the accuracy and evenness of the Matrigel coating are rarely controlled. Also, the quantitation of the assay is usually carried out manually, lacking a clear standardised procedure, and this may lead to contradictory and variable results.[7]

Schonermark *et al.*[7] designed a chamber and used a scanning electron microscope (SEM) to control the coating of the membrane, and images were obtained by confocal laser scanning microscopy (CLSM). This method and the degree of invasion by malignant cell lines attempted to eliminate the subjectivity associated with most other techniques. The assay enabled measurement of the distance travelled by invading cells, and the possibility of distinguishing cell migration from invasion.

The invasion chamber was a modification of the classical chemotaxis chamber as described by Boyden,[5] and carried a nitrocellulose filter that separated the lower and upper compartments, and the filter was coated with Matrigel. To ensure reproducible and effective coating, either a spin coater or successive manual coating was used. Tests of this system showed that an even matrix coat that covered all filter pores could be achieved only with Matrigel concentrations greater than 2.5 μg/mm^2. Also, in order to produce a basement membrane-like structure with a physiological thickness of approximately 8 μm (5.29 μg/mm^2), a greater amount of Matrigel was required than had been reported previously.

In our department we have an interest in bile duct cancer and developing cell line models,[14,15] and in this study we use cell lines derived from bile duct carcinomas to assess the ability of the layering technique to achieve a physiological 8 μm ECM thickness and determine whether or not image analysis can be used to assess cellular degradation of the basement membrane.

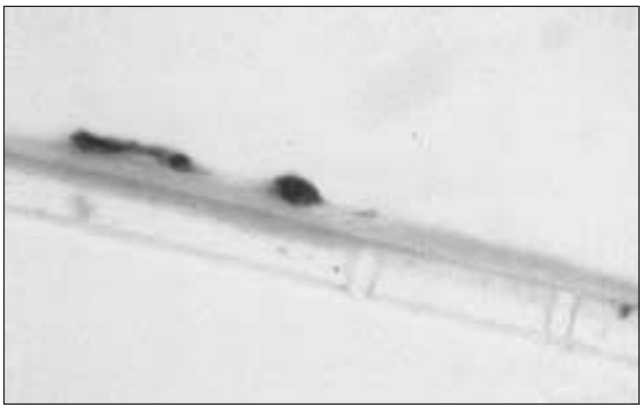


Fig. I. 600μL layering of Matrigel achieving a physiological ECM thickness of 8 μm (7.4 μg/mm^2).

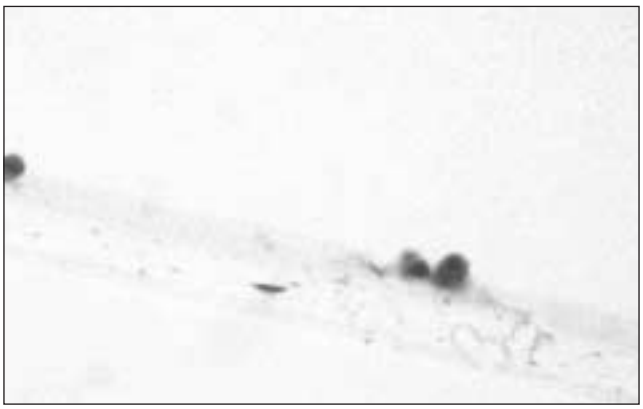


Fig. 2. OCUCh-LM1 cell line clumping together therefore affecting the overall thickness of Matrigel.

Materials and methods

In vitro *invasion assay*

Cell culture: Two cell lines derived from extrahepatic bile duct adenocarcinomas (SKChA-l and OCUCh-LM1) were used.[14,15] Cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, L-glutamine and 0.5% phenol red. No antibiotics were used in the experiments. Cells were detached from their flasks using a two-minute wash in 5 mL versene (Sigma, Poole, Dorset, UK.) at 37°C, followed by incubation in 0.5 mL 0.2% trypsin (Sigma) at 37°C. The cells were washed and resuspended in 10 mL serum-free medium. Prior to each assay, the cell number and viability was assessed by a dye exclusion method,[16] and the cell population confirmed as being >95% viable.

Transwell assays: Transwell units (Falcon) were coated with 200 μL, 400 μL or 600 μL Matrigel (Becton-Dickinson). These represented 280 μg (2.5 μg/mm^2), 560 μg (5.0 μg/mm^2) and 840 μg (7.4 μg/mm^2) respectively. Each layer of 200 μL was polymerised at 37°C for one hour before the addition of a further 200 μL. The Matrigel was dried overnight in the cell culture hood prior to hydration with 50 μL serum-free medium and then the addition of 0.25 x 10^5 cells. Following dilution of cells to l x10^5 viable cells per mL, 0.5 mL of each cell line was added to the upper chamber of the Transwell unit. Medium removed from confluent cell monolayers and filtered through a 0.2 μm Millipore filter was added to the lower chamber. Cells in the Transwell chambers were incubated for 48 hours at 37°C in 5% CO_2. Excess medium was removed, the membrane was cut from the upper chamber of each unit and then fixed in 10% unbuffered formalin saline (UBFS). The membranes were processed to paraffin wax, embedded on edge, bisected and labelled A and B, and then sectioned. Each was stained with haematoxylin and eosin (HE). Experiments were performed in triplicate.

Immunocytochemistry

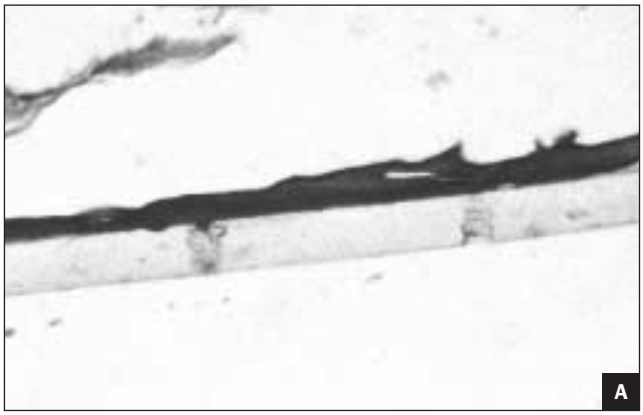
Sections cut from each block were pretreated in trypsin (ICN Flow) for eight minutes at 37°C, incubated for 30 minutes with antibody to cytokeratin (CAM 5.2, 1 in 10 dilution, Becton, Dickinson) and laminin (Sigma, 1 in 20 dilution), and the reaction detected using a standard peroxidase streptavidin-biotin method (Dako).

Image analysis

A Kontron image analysis system was used to measure the thickness of the Matrigel and to determine the amount of degradation produced by each cell line. Briefly, each slide was viewed at x400 magnification and an image transferred to a monitor using a Sony digital CCD TV camera. The digital image was measured using an interactive mouse cursor, and each field measured 140 μm^2. Measurements (50) were taken at four representative levels for each Matrigel concentration used and with both cell lines.

In order to determine the amount of Matrigel degradation, the distance from invading cell nuclei to the bottom of the Matrigel layer was measured and a mean distance for each set of results was taken. Only those levels that showed the

Fig. 3. Immunocytochemical localisation of ECM components and cell line.
(a) ECM components staining positive for laminin. (b) Cells staining positive for Cam 5.2 (arrow).



Matrigel µL	Concentration µg/mm²	Mean thickness µm (99% CL)
200	2.5	4.06 (3.61 – 4.52)
400	5	5.15 (4.52 – 5.79)
600	7.4	9.97 (8.92 – 11.02)

Table I. Effect of Matrigel concentration and SKChA-1 cells on mean Transwell membrane thickness.
CL: confidence limits

Matrigel µL	Concentration µg/mm²	Mean thickness µm (99% CL)
200	2.5	5.58 (5.08 – 6.07)
400	5	12.23 (10.36 – 14.1)
600	7.4	10.1 (8.11 – 12.09)

Table 2. Effect of Matrigel concentration and OCUCh-LMI cells on mean Transwell membrane thickness.
CL: confidence limits

Cell line	Mean thickness µm (99% CL)
SKChA-I	20.92 (17.23 – 24.61
OCUCh-LMI	26.47 (22.09 – 30.86)

Table 3. Mean invasion distance achieved by SKChA-1 and OCUCh-LMI cell lines when seeded on 600 µL Matrigel.
CL: confidence limits

support filter pores in cross sections were assessed. This was used as a means of ensuring a total distance of cell invasion.

Results

A total of 600 µL Matrigel applied in 200 µL layers was needed to achieve a minimum physiological ECM thickness of 8 µm (7.4 µg/mm²) (Figure 1).

Addition of cell lines affected the overall thickness of the Matrigel (Figure 2), with a mean Matrigel thickness of 9.97 µm (7.4 µg/mm²) at 600 µL Matrigel. (Table l) following the addition of SKChA-l cells, and a mean thickness of 10.1 µm at 600 µL Matrigel (Table 2) following addition of OCUCh-LM1 cells. However, there was no significant difference in how far each cell line invaded the Matrigel (Table 3).

Immunocytochemistry for laminin and CAM 5.2 demonstrated the Matrigel layer and cells within it (Figure 3).

Discussion

Basement membranes are thin (40-l20 nm), flexible layers of specialised ECM that underlie all epithelial cell sheets and tubes, comprising a meshwork of type IV collagen, specific glycoproteins – such as laminin and entactin – and heparin sulphate proteoglycans.[17-19] Integrins appear to be the major receptors by which cells attach to extracellular matrices[20] and migration occurs once this has happened.[21]

Localised degradation of matrix components is needed to enable cells to migrate through the basement membrane, and such components (collagen, laminin) are degraded by extracellular proteolytic enzymes that are secreted locally.[22,23] To initiate metastasis, cancer cells must first penetrate the epithelial basement membrane and then invade the interstitial stroma.[24,25]

A Transwell unit is a simple, rapid method to investigate the invasiveness of tumour cells through the basement membrane. This was found to be a successful *in vitro* assay, as the cell lines penetrated through to the nutrients. However, Schonemark[7] discovered problems with Albini's Transwell inserts, in particular the thinness of the filter corresponds to the approximate size of the single cell, and the unevenness of the Matrigel coating.

In the present study, using an Albini-type assay, we attempted to create a basement membrane-like structure of approximately 8 µm by layering the Matrigel. However, we found that Matrigel thickness increased with concentration, and that addition of the respective cell lines also influenced overall thickness (SKChA-1: mean thickness 9.97µm; OCUCh-LM1: mean thickness 1O.1 µm).

In this study, both were slightly higher than the 8 μm reported by Schonermark; however, these were achieved with a Matrigel concentration of 7.4 μg/mm^2, which was greater than the 5.3 μg/mm^2 used by Schonermark. In addition, we employed layering techniques rather than a spin coater as used by Schonermark.

There was no significant difference in the amount of Matrigel degraded by each cell line; both seemed equally invasive. Immunocytochemistry proved useful in highlighting ECM components such as laminin, and for detecting the invading tumour cells.

Conclusions

A physiological thickness of basement membrane material can be achieved using a layering technique in an Albini-type Transwell assay system. Matrigel thickness can be assessed in HE stained paraffin-wax sections using image analysis, and immunocytochemistry can highlight the ECM and tumour cells using antibodies to laminin and cytokeratin, respectively.

In most assays, cell morphology is not considered; however, the method described here has the advantage that visualisation of the model system can aid understanding of the mechanisms involved in malignant cell invasion.

In addition to identifying the molecules involved, immunocytochemistry can be used in inhibition experiments to investigate the roles of different integrins in the invasion process. Also, image analysis can be useful in the determination of malignant cell invasion.

The extrapolation of this system to fresh tumour tissue remains to be studied. □

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