RESEARCH COMMUNICATION

Distinctive Features of Advancing Breast Cancer Cells and Interactions with Surrounding Stroma Observed Under the Scanning Electron Microscope

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Abstract

Breast cancer cells undergo transformation when they spread into surrounding tissues. Studies have shown that cancer cells undergo surface alterations and interact with the surrounding microenvironment during the invasion process. The aim of the present study was to analyse these cancer cell surface alterations and interactions of cancer cells and stroma. Twenty 1-methyl-1-nitrosourea-induced breast cancer samples taken from five rats were fixed in McDowell-Trump fixative and then washed in 0.1 M phosphate buffer. The samples were then treated with osmium tetroxide before being washed in distilled water and subsequently dehydrated through graded ethanols. The dehydrated samples were immersed in hexamethyldisilazane (HMDS), then following removal of excess HMDS, the samples were air dried at room temperature in a dessicator. The dried samples were mounted onto specimen stubs and coated with gold coater before being viewed under a scanning electron microscope. We detected the presence of membrane ruffles on the surface of cancer cells and the formation of unique surface membrane protrusions to enhance movement and adhesion to the surrounding stroma during the process of invasion. Advancing cancer cells demonstrated formation of lamellipodia and invadopodia. The stroma at the advancing edge was desmoplastic with many collagen fibres laid down near the cancer cells. Our data suggest that all of these abnormalities could act as hallmarks of invasiveness for breast cancer.

Keywords: Breast cancer - cellular protrusions - desmoplastic stroma - scanning electron microscope - stroma

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Introduction

Cells interact with different groups of macromolecules in the surrounding microenvironment that form the extracellular matrix (ECM). The ECM regulates cells' advancement, organisation, function and signals from adjacent cells (Nikkhah et al., 2009, Schindler et al., 2005). Cancer occurs when a normal cell fails to function properly, leading to abnormal cell growth that subsequently interrupts the organisation of tissue (Kaul-Ghanekar et al., 2009). Atypically, a structurally dense form of the ECM, termed the basement membrane, makes contact with the basal surfaces of cells that form tissue, such as epithelial and endothelial cells (Schindler et al., 2005). Cancer cell penetration through the barrier-acting basement membrane and the ECM environment is an important part of malignant cancer cell progression and metastatic dissemination (Martin et al, 2001, Wolf and Friedl, 2009, Sugiura and Berditchevski, 1999). Cancer invasion is frequently facilitated by the proteolytic processing of ECM components. Wolf and Friedl reported that the cellular regions mediating proteolysis depend upon the physical structure, density and dimensionality of the ECM-cell surface communication (Wolf and Friedl, 2009). Furthermore, Provenzano et al. implied that the cancer cell infringement across the basement membrane and collagenous stroma before spreading into neighbouring ECM environments sets a pathway for further migration, resulting in metastatic growth in distant tissues (Provenzano et al., 2006).

One of the distinctive properties of cancer cells is the presence of membrane ruffles on their surface. This was highlighted by Kaul-Ghanekar et al. who used the tumour suppressor protein SMAR1 (Scaffold/Matrix Associated Region binding protein 1) as a phenotypic differentiation marker between cancerous and non-cancerous cells. They demonstrated that untreated tumour cells exhibit a rough surface, whereas tumour cells treated with SMAR1-P44 peptide exhibit a smoother surface profile. They suggested that the rough surface of cancer cells may be a consequence of overall reorganisation of cellular architecture as well as rearrangement of dynamic structures involved in cell motility and division (Kaul-Ghanekar et al., 2009).

Cancer cells, facilitated by proteolytic activity, can migrate to form a metastatic growth in distant tissues. The proteolytic activity is normally focused on

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plasma membrane protrusions, such as lamellipodia and invadopodia, which are formed at the leading edge of migrating cells. These protrusions mediate specific interruption of the ECM and rebuild the tissue scaffold (Wolf and Friedl, 2009, Sugiura and Berditchevski, 1999 and Kelly et al., 1998).

Genetic alterations of epithelial cells cause tumour formation; however, the epithelial-stromal connection also plays an important role in tumour invasion (Provenzano et al., 2008). Connective tissues have an abundance of fibrillar collagen type I, elastin and other ECM elements present in and around most organs, such as skin, breast or gut. Cancer cells need to penetrate the surrounding ECM before reaching vessels for haematogenic or lymphogenic dissemination (Wolf and Friedl, 2009). During the invasion process, the amount of fibrillar collagen deposition significantly increases. Based on this finding, Provenzano et al. hypothesised that increased collagen density promotes tumourigenesis and migration (Provenzano et al., 2008).

The present study focused on obtaining three-dimensional images of breast cancer tissue to generate information regarding the invasion properties of advancing cancer cells. Cell surface alterations, the presence of cytoplasmic protrusions and the formation of desmoplasia were examined in detail.

Materials and Methods

Twenty breast tumour samples (tumours induced with 1-methyl-1-nitrosourea) were randomly taken from five Sprague Dawley rats (Animal House Unit of the University Science Malaysia (USM)). Ethical clearance for using experimental animals was obtained from the USM's Animal Ethics Committee and the rats were handled according to standard laboratory practice. The samples used in this study were derived from samples generated for a previous study (Jaafar et al., 2009). The samples were fixed in McDowell-Trump fixative prepared in 0.1 M phosphate buffer (pH 7.2) at 4oC for a minimum of 24 hours. After fixation, the samples were washed three times in 0.1 M phosphate buffer (10 minutes for each wash). The samples were then treated with osmium tetroxide for two hours, followed by three washes in distilled water (10 minutes for each wash). A series of graded alcohol was used to dehydrate the samples: 50% ethanol for 15 minutes; 75% ethanol for 15 minutes; 95% ethanol for 30 minutes; and absolute alcohol for one hour. The dehydrated samples were then immersed for 10 minutes in 2 ml of hexamethyldisilazane (HMDS; Sigma Chemical, St Louis, USA). Following removal of excess HMDS, the samples were air dried at room temperature in a dessicator before being mounted onto scanning electron stubs with double-sided sticky tape. Finally, the samples were coated with gold coater (Fisons Instruments, VG Microtech, Sussex, UK) then viewed under a Leo Supra 50 VP Field Emission Scanning Electron Microscope (SEM) equipped with an Oxford INCA 400 energy dispersive x-ray microanalysis system (Carl Zeiss SMT, Oberkochen, Germany and Oxford Instruments Analytical, Buckinghamshire, UK). During the processing of the samples, the tissues were not allowed to dry at any stage until HMDS immersion.

Results

Scanning electron microscopy analysis was carried out to investigate the surface interactions between tumour cells and the stroma in invasive breast carcinoma. We analysed twenty invasive breast carcinoma samples using standard scanning electron microscopy procedures incorporating the HMDS technique (Dysktra, 1992). The HMDS technique was chosen as it does not contribute to artefacts or structural damage to tissues whilst preserving the ultrastructural surface details (Araujo et al., 2003).

Ultrastructural analysis revealed distinctive features of advancing breast cancer and its relationship with the stroma environment. Membrane ruffles were found to exhibit 'cabbage-like' structures due to the cancer cell outer membrane peeling off. Furthermore, lysed cells were detected near the cancer cells, indicating the occurrence of apoptosis (Figure 1.1). Figure 1.2 demonstrates the presence of membrane ruffles on cancer cells at a higher magnification. To enhance the survival rate of cancer cells, displacement of the prominent leader cancer cell advance followed by other cancer cells looking shrunken was observed (Figure 1.3). Apoptosis was indicated by membrane blebs scattered on the cancer cell surface, with the presence of lamellipodia contributing to the invasive properties of cancer cells by engulfing normal cells (Figure 1.4). We detected a large amount of thin hair-like structures protruding from the cancer cell body and cross-linking with collagen fibres in the surrounding environment (Figures 1.5 and 1.6). Accumulations of collagen fibrils surrounding cancer cells with membrane ruffles on their surface were observed (Figure 1.7). It is thought that these abnormalities have a direct influence on the invasiveness of cancer cells, possibly by the cell body being held in position by fibrils. Figure 1.8 illustrates the hair-like protrusions emerging from a cell body and readily attaching themselves to adjacent cells. Finally, Figure 1.9 demonstrates an abundance of collagen with the cancer cells, which are flat in nature, gliding onto the matrix.

Discussion

A major role played by tissue microenvironments is to maintain normal cell behaviour. Thus, any changes induced by oncogenes or other external influences which cause rearrangement of the cytoskeleton will transform the properties of cells (Brinkley et al., 1980; Russo et al, 1991; Provenzano et al, 2006). Cytoskeleton rearrangement leads to disorganisation of the membrane of cancer cells. This is a result of changes to internal biopolymeric protein, an important component of the cell cytoskeleton, which subsequently alter the surface of cancer cells (Brinkley et al., 1980; Rajah et al., 2001; Kaul-Ghanekar et al., 2009).

Our SEM images demonstrate the presence of membrane ruffles on the surface of breast cancer cells. Several authors have speculated on the nature and role of membrane ruffles. Johnston et al. and Stoica et al. proposed that membrane ruffles are specialised plasma membrane

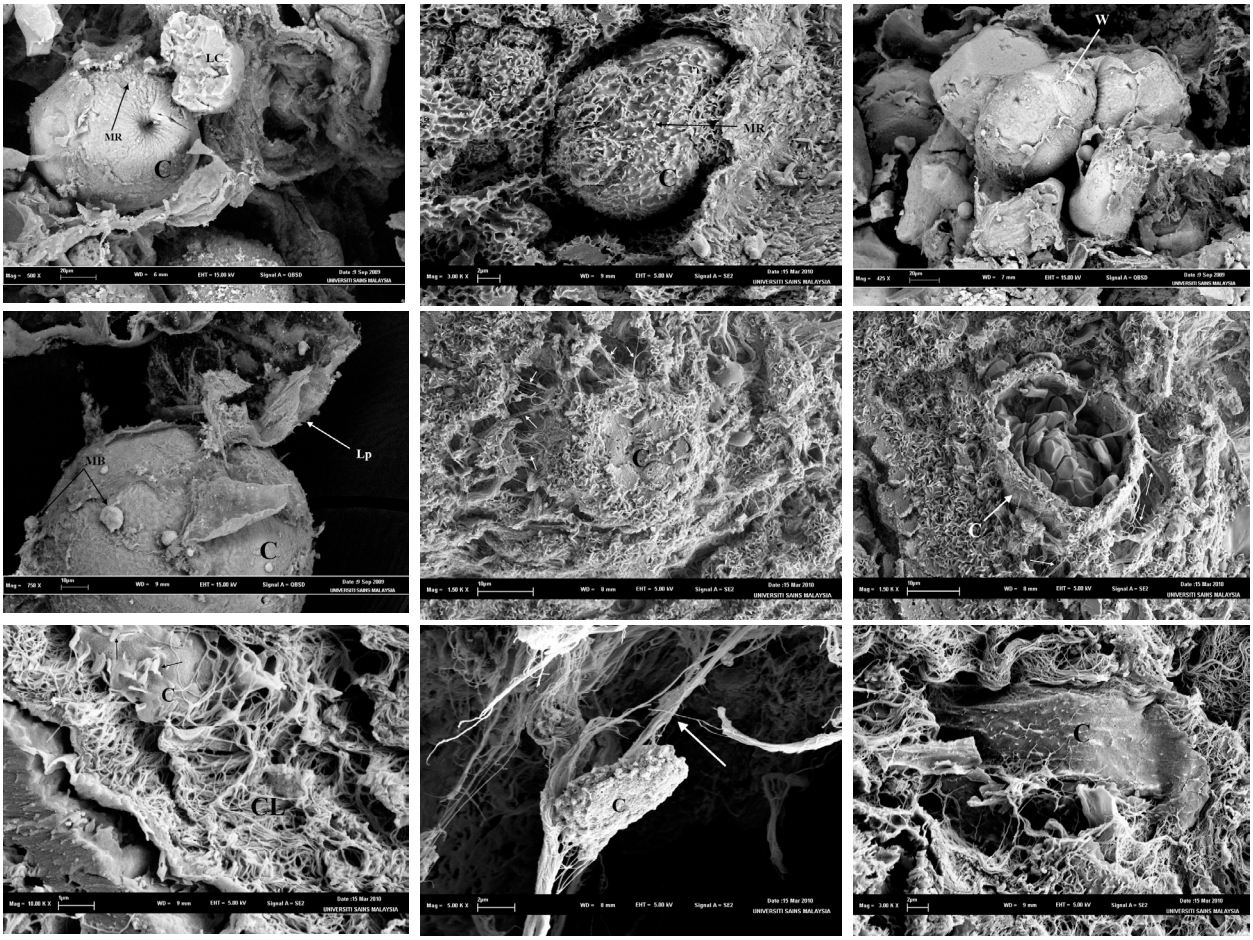

Figure 1. Scanning Electron Microscope Features. a) The presence of membrane ruffles (MR) on a cancer cell (C): A lysed cell (LC) is shown nearby. Magnification 500×, b) Breast cancer cell (C) exhibiting membrane ruffles (MR). Magnification 3000×, c) Displacement of the prominent leader cancer cell (W) advance followed by other cancer cells looking shrunken enhances the survival rate of cancer cells. Magnification 425×, d) The presence of membrane blebs (MB) on the surface of a cancer cell (C). Flat protrusions, lamellipodia (Lp), are also visible. Magnification 758×, e) Collagen fibres in the surrounding matrix cross-link (arrows) with protrusions from the cancer cell body (C). Magnification 1500×, f) A string of protrusions from the body of a cancer cell (C) cross-links (arrows) with collagen fibres from the surrounding matrix. Magnification 1500×, g) Accumulations of collagen fibrils (CL) surrounding cancer cells (C) with membrane ruffles (arrows) on their surface. Magnification 10000×, h) Collagen fibrils and protrusions cross-link (arrow) to anchor the cancer cell (C). Magnification 5000×, i) Cancer cells (C) begin their migration by gliding onto the matrix. Magnification 3000×

ultrastructures which contain fine actin filaments and depend on actin polymerisation. Furthermore, they suggested that membrane ruffles have a role in the growth, development and motility of cancer cells, and so are important in determining the metastatic potential of cells (Johnston et al., 1995; Yan et al., 2001; Stoica et al., 2003). Rajah et al. investigated the role of membrane ruffling in cell behaviour using a breast cancer cell line. Their findings led them to conclude that the presence of membrane ruffles on the surface of cancer cells facilitates ligand-receptor binding processes by acting as a platform for adhesion foci and signal transducing events that lead to the activation or suppression of genes. Furthermore, they proposed that membrane ruffling is one of the cancer cell's properties. In their study, they showed that conditioned media from NIH 3T3 fibroblasts (3T3-CM) used as a chemoattractant in matrigel invasion chambers increases the invasiveness of breast cancer cells. Additionally, they found that MCF-7 breast cancer cells treated with 3T3-CM exhibit an increased rate of membrane ruffling of the plasma membrane and concluded that 3T3-CM increases the invasiveness of breast cancer in direct correlation with increased membrane ruffling (Rajah et al., 2001). Our SEM images reveal that the presence of membrane ruffles on the surface of breast cancer cells gives them a rougher appearance than normal breast cells. A similar finding has previously been reported by Kaul-Ghanekar et al. who found that control tumour samples (mice injected with melanoma cells) show surface roughness, whereas treated tumour samples (mice injected with melanoma cells followed by the tumour suppressor protein SMAR1-P44) exhibit a smoother surface (Kaul-Ghanekar, 2009).

Our SEM images show membrane blebs on the cancer cell surface and lysed cells near the cancer cells. Membrane blebs and lysed cells are characteristics indicative of apoptosis. Apoptosis or programmed cell death is a cell suicide mechanism where cells actively pursue a course towards death upon receiving certain stimuli (Kataoka and Tsuruo, 1996; Xanthopoulos et al., 2005). Since all the tissues examined in this study were not treated with any anti-cancer drugs, we can assume that the apoptosis is naturally occurring, even among the

cancer cells. This finding is supported by previous works showing that cells not treated with the anti-cancer drug anastrozole exhibit oncogene-induced membrane blebs and lysis (Rizk and El-Rakhawy, 1981; Kataoka and Tsuruo, 1996; Xanthopoulos et al., 2005).

As part of the enhancement of the survival rate of cancer cells, displacement of the prominent leader cancer cell advance followed by other cancer cells looking shrunken was observed here. Previous reports have indicated that this event occurs naturally among cancer cells during cell motility when they undergo shape adaptations and contact relationship changes. Transformed cancer cells displace each other irrespective of the presence of cell-cell contacts in order to enhance their ability to survive and become more resistant to apoptosis incurred by anti-cancer drugs (Luparello et al., 1991; Kataoka and Tsuruo, 1996). In order to increase our knowledge of this phenomenon of apoptosis of untreated breast cancer cells, further studies are required.

Cancer cell invasion is a dynamic process that involves alterations in cell shape and adhesion, with actin cytoskeleton changes a key regulator. It is well established that oncogene-induced protrusion formation is linked to alterations in cortical actin in a variety of cells (Rizk and El-Rakhawy, 1981; Russo et al., 1991; Jing et al., 2009; Yamada et al., 2009; Abbineni et al., 2010). Our finding of the presence of membrane ruffles can be related to cellular protrusion changes. Membrane ruffles arise from lamellipodia that are extended (McHardy et al., 2005), unattached and retracted over the surface of the cancer cell (Bailly et al., 1998). Several authors have highlighted the importance of cellular protrusions during cancer cell invasion (Bailly et al., 1998). In 1986, Sapino et al. reported that the cytoskeletal structure of MCF-7 breast cancer cells cultured in oestrogen-supplemented medium displays F-actin-rich cytoplasmic protrusions sprouting from the whole peripheral rim (Sapino et al., 1986). Yamada et al. demonstrated the importance of cytoplasmic protrusions using dynasore (a potential anti-cancer drug), a dynamin inhibitor which potently destabilises F-actin in vitro and in vivo. It was found to inhibit the formation of protrusions and to suppress cancer invasion and metastasis, indicating that cytoplasmic protrusions are an important tool for cancer cell invasion (Yamada et al., 2009). Using clotrimazole (an anti-fungal azole derivative) to induce membrane protrusion dysfunction, Meira et al. observed the absence of membrane alterations to adjacent cells, thus inactivating the invasion process (Meira et al., 2004). Studying the role of cellular protrusions using reconstituted matrices, Kramer et al. found that tumour cell invasion begins with the formation of unique projections that are thought to maximise the cell membrane surface area (Kramer et al., 1986). The cytoskeleton plays an important role in cell motility, especially actin filaments which control breast cancer invasion by reorganising into three-dimensional networks to form lamellipodia and invadopodia (Jiang et al., 2009). Invading cancer cells continue to alter at the leading and retracting edges, finally leading to the formation of actin-rich plasma membrane protrusions, such as lamellipodia, to facilitate invasion and migration (Jing et al., 2009 and Yamada et al., 2009). In relation to this process, we were interested in determining the three-dimensional shape, role and mode of activity of lamellipodia. Our SEM images reveal the presence of lamellipodia as broad and sheet-like protrusions that are extended from the cell cortex and adhere to the matrix surface. This finding is consistent with previous works. According to Jiang et al., lamellipodia are sheet-like and broad membrane protrusions at the invasive margin of cancer cells during movement (Jiang et al., 2009). Lamellipodia are extended from the cell cortex and localised to the border of adherent cells as well as near the matrix surface, thus initiating programmed movement which involves translocation of the cell body in the forward direction (McHardy et al., 2005, Bailly et al., 1998 and Ada-Nguema et al., 2006). Lamellipodia play a crucial role in cancer cell invasion, as demonstrated by Wang et al. investigating the anti-invasive properties of troglitazone on the cell spreading of MDA-MB-231 and T47D breast cancer cells. They found that troglitazone inhibits lamellipodia formation by downregulating actin polymerisation at these structures in both cell lines (Wang et al., 2008). Kramer et al. proposed that the leading edge of invading cancer cells projects lamellipodia which spearhead the direction of cell movement. Furthermore, lamellipodia represent active sites of penetration into matrices, and may provide an efficient mechanism for the local enzymatic hydrolysis of matrix macromolecules (Kramer et al., 1986).

Our SEM images reveal another type of cellular protrusion – invadopodia – which are thin, protrude perpendicularly from the cancer cell body and cross-link with the ECM. This finding is consistent with previous reports describing invadopodia as thin protrusions that are perpendicular to the cancer cell body and that cross-link into the three-dimensional network of the ECM (Wolf and Fried, 2009 and Jiang et al., 2009). An important role for invadopodia in enhancing human breast cancer invasion, facilitated by matrix metalloproteinases (MMP), has been documented by Kelly et al. (Kelly et al., 1998). Using human breast cancer cell lines and collagen type I gels, they demonstrated that invadopodia enhance the invasion potential of breast cancer by degrading ECM proteins such as collagen type I. Furthermore, when they reduced the proteolytic activity of invadopodia by administration of the MMP inhibitor batimastat, invasion was found to decrease.

In order to metastasise, tumour cells need to invade across the surrounding ECM where the majority of stroma is fibrillar collagen, particularly collagen type I (Provenzano et al., 2006 and Kusafuka et al., 2008). Collagens are the main structural proteins responsible for the structural integrity of vertebrates and many other multicellular organisms (Luparello et al., 1991). The expression of collagen type I is spatially and temporally regulated during mammary ductal formation. Our SEM images demonstrate the accumulation of collagen fibres around cancer cells, as previously reported by Provenzano et al. Their study illustrated that the disruption of stromal-epithelial communications leads to tumourigenesis and an excessive amount of collagen surrounding the invasive cancer cells, termed desmoplasia (Provenzano et al.,

2006). An increased amount of collagen deposition is another important feature for the location of cancer cells. Following their invasion into the ECM, the next step for cancer cells is to achieve prominent contact with collagen fibrils (Provenzano et al., 2006 and Luparello et al., 1991). In relation to this step, our SEM images demonstrate the cross-linking of collagen fibrils and cellular protrusions, thereby anchoring the breast cancer cell. This finding is in line with the concept proposed by Huang et al. who stated that cells in culture have the ability to pull up matrix fibrils so that they attach strongly to the ECM (Huang et al., 1999). This concept is consistent with the role of collagen type I described by Provenzano et al. and Luparello et al. who indicated that collagen type I is important in the control of the movement of invasive cancer cells as well as in containing and anchoring single cells (Provenzano et al., 2006 and Luparello et al., 1991).

Another role of collagen is to assist the movement of cancer cells during invasion. Our SEM images support this collagen role in that they show breast cancer cells beginning their migration by gliding onto the matrix. This finding is in agreement with the SEM analysis of breast cancer cells performed by Benbow et al. They observed that cancer cells undergo three steps of adhesion, invasion and migration. The process begins with attachments between cancer cells, followed by the formation of protrusions and finally movement along the matrix. The cells possess the ability to tunnel into the matrix and migrate away from their entry point, thus indicating that stromal-tumour cell interaction can facilitate invasion and metastasis (Benbow et al., 1999)

In conclusion, the present study reveals that cytoskeleton changes and cell surface alterations are vital in detecting the mode of action of the interaction of invasive breast cancer with the surrounding microenvironment. SEM analysis provides a new dimension to the detection of cell surface abnormalities. Our data suggest that the presence of cellular protrusions and desmoplasia could act as benchmarks for further diagnosis regarding stages of breast cancer. Further research should be focused on the role of invasion-assisting proteases, such as MMP-2 and MMP-9, which amass at the edges of cellular protrusions, particularly lamellipodia.

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