A novel breast cancer cell line initially established from pleural effusion: Evolution towards a more aggressive phenotype

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Received September 11, 2006; Accepted October 30, 2006

Abstract. Many human breast cancer cell lines have been in culture for several years, serving as model systems for studying aspects of breast cancer biology. Molecular alterations might occur in these cells during cultivation, and it remains unknown to which extent findings in these cell lines can be related to human disease. Hereby, we describe the establishment of a breast cancer cell line, MW1, from malignant pleural effusion. We compare expression patterns of several molecular markers in breast biopsy tissue, in cultivated tumor cells derived from pleural effusion reflecting the metastatic state, and in late passages of a lineage derived from the pleural culture. Our data show that expression of estrogen and progesterone receptors was lost in the cultivated tumor cells derived from pleural effusion as shown by immunohistochemical staining. Cytokeratin expression patterns remained luminal. During cultivation, the growth rate of MW1 cells increased dramatically and the morphology underwent alterations. As shown by Western blotting, E-cadherin expression remained unchanged whereas P-cadherin expression had increased after 4 years of cultivation of the cell line. Integrin ß4 expression was low in early passages of the pleural effusion whereas the cell line exhibited high expression levels of ß4. HGF receptor (c-Met), EGF receptor, VEGF and VEGF receptor-2 (KDR) expression was detectable by semiquantitative RT-PCR and remained unchanged during cultivation. In contrast, VEGF receptor-1 (flt-1) expression showed lower expression after 4 years of cultivation. The cell line migrated towards HGF, but not towards VEGF. This study provides exemplary insight into the molecular metamorphosis tumor cells undergo *in vivo* or *in vitro* on their way from the primary tumor via an equivalent of the metastatic state and during the development of a clonal cell line.

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Abbreviations: ATCC, American Type Culture Collection; CK, cytokeratin; EGF, epidermal growth factor; ER, estrogen receptor; FGF, fibroblast growth factor; FISH, fluorescence *in situ* hybridization; HGF, hepatocyte growth factor; HIER, heat-induced epitope retrieval; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PR, progesterone receptor; VEGF, vascular endothelial growth factor; RT-PCR, reverse transcriptase polymerase chain reaction

Key words: breast cancer, cytokeratin, cadherin, HGF, c-Met, EGFR, integrin α6ß4, VEGF, flt-1, KDR

Introduction

Worldwide, breast cancer is the most common cancer and the leading cause of cancer death among women. Despite improvements in breast cancer treatment and a decline in the mortality rate in developed countries, the death rate from breast cancer is still around 38 deaths per 100000 women (1). Prognostic and predictive gene signatures have been identified by microarray technology and point into the direction of individualization of breast cancer management to further improve breast cancer treatment. Molecular fingerprinting is performed on the primary tumor tissue and later on might be applied for treatment of metastatic patients as well, in spite of the fact that molecular alterations might occur during the process of metastasis. Molecular pathology of breast cancer is mostly studied in breast cancer cell lines. To date, a large number of breast cancer cell lines have been established and used worldwide. Most of the established breast cancer cell lines have been in culture for many years and it remains questionable to which extent data derived from these models are relevant for human disease. In this study, we describe the

development of a breast cancer cell line from pleural effusion and compare the cell line with cultivated tumor cells derived from pleural effusion and with the breast cancer tissue from the patient's primary disease. This longitudinal analysis gives insight into the quantitative or qualitative changes which might occur on the way to metastatic disease *in vivo* and how further selective pressure might alter molecular features during the process of cultivation. We have developed a breast cancer culture from pleural effusion of a patient and used early passages [6-9] of the culture as equivalent of the metastatic state. We compared these early passages with the primary tumor of the breast biopsy and with late passages [47-65] of a clone derived from the pleural culture.

In our analysis, we focused on a selection of cytokeratins, glycoproteins and growth factors that have been shown to play a role in tumor cell invasion and metastasis and are considered to be prognostic markers for poor clinical outcome.

One reason for the heterogeneity of breast cancers might be their origin from different cell populations within the normal breast. Breast cancer subtypes have been classified inter alia according to their cytokeratin expression pattern. Expression of CK 5 is considered to identify 'basal like' tumors (2) and is associated with a distinct molecular gene expression pattern characterized by ER-, HER2-negativity and EGF receptor expression (3). Expression of the luminal marker CK 7 has been reported to occur predominantly in breast cancer (4). We therefore used CK 5 as a marker for basal cells and CK 7 as a marker for luminal cells.

Cadherins are cell-cell adhesion glycoproteins that are critical for tissue stratification (5). Functional losses of cadherins on the one hand and overexpression on the other have been implicated in carcinogenesis (6). Subfamilies of cadherins show different specific tissue distribution patterns. E-cadherin is expressed in all epithelial cells whereas the expression of P-cadherin in breast tissue is restricted to myo-epithelial cells (7). In ~30% of breast carcinomas P-cadherin expression is upregulated and it has been reported to correlate with poor clinical outcome (8,9). Therefore, we characterized the cadherin expression patterns in early and late passages of MW1 cell line applying Western blotting.

Integrins are glycoproteins that form heterodimers between α and ß subunits and serve as receptors for extracellular matrix proteins. Integrin α6ß4 is a receptor for some laminin isoforms and has been shown to play a role in tumor cell invasion and metastasis (10-13). α6ß4 expression has been reported to correlate with reduced patient survival in breast cancer (14,15). Since the ß4 subunit associates exclusively with α6, we compared ß4 protein expression in early and late MW1 passages.

Hepatocyte growth factor (HGF) plays a role in proliferation, dissociation, migration, and invasion of tumor cells and is a potent angiogenic factor (16). HGF binds to its specific receptor, c-Met. Expression levels of c-Met have been correlated with tumor progression and poor clinical outcome in breast cancer (17,18).

The epidermal growth factor receptor (EGFR) belongs to the type I receptor tyrosine kinases, the ErbB receptors. Apart from EGFR (ErbB1/HER1) the family includes ErbB2 (HER2/neu), ErbB3 (HER3), and ErbB4 (HER4) (19). In many human tumors including breast carcinoma, EGFR is frequently overexpressed (20) and has been correlated with poor clinical outcome in a subset of breast carcinoma patients (21). Monoclonal antibodies directed at EGFR such as gefitinib (ZD1839/Iressa) have proved to inhibit breast cancer growth *in vitro* and *in vivo* (22) and are currently under clinical investigation.

Vascular endothelial growth factor (VEGF) expression is crucial for tumor angiogenesis and has been correlated with poor prognosis of breast cancer (23). VEGF as well as its receptors VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR) and neuropilin (NP-1/NP-2) are expressed in breast carcinoma cells (24,25). VEGF acts in a paracrine fashion on endothelial cells and displays autocrine activity on carcinoma cells (26,27). Up to now, it is unclear which role the different VEGF receptors play within the autocrine signaling loop. Therefore, we studied the expression patterns of VEGF and VEGF receptors in early and late passages of MW1 cell line.

Materials and methods

Clinical history. The breast cancer cell line described herein, designated MW1, was developed from the malignant pleural effusion of a 39-year-old patient suffering from metastatic breast cancer. The patient was diagnosed with breast cancer in 1998 at the age of 36. She had a grade 2 invasive carcinoma with excessive lymphangiosis carcinomatosa (pT1c multifocal, pN2, M0). The patient underwent breast conservative treatment and axillary dissection. Metastases were found in 8 out of 20 lymph nodes examined. The lymph node metastasis reached up to 1 cm in diameter. The patient received 6 cycles of standard dose epirubicin (90 mg/m^2) and cyclophosphamide (600 mg/m^2) i.v. every third week. Post-operative radiation therapy (50 Gy) was applied to the ipsi-lateral regional lymph nodes and the breast between cycles 3 and 4 of chemotherapy (sandwich irradiation). Subsequently, the patient received adjuvant tamoxifen treatment (20 mg per day). In September 2000 the patient presented with bone metastasis and in 2001 lymphangiosis carcinomatosa of the lung was diagnosed. The patient received taxol monotherapy as treatment for distant metastatic disease. Initially, a partial response was achieved but by the end of 2001 the patient suffered from dyspnoea due to pleural effusions. Palliative punction of the pleural cavity were clinically necessary and some of the drained pleural fluid was used for cell culture. Subsequent palliative procedures including pleurodesis did not improve the patient's condition and, unfortunately, she died 3 months later.

Establishment of a primary breast cancer culture. Prior to cultivation of malignant cells, approval therefore was obtained from the patient and the local ethics committee. Fresh pleural effusion was collected, centrifuged and treated as described previously (27). The resulting cell line, designated MW1, was grown continuously upon medium changes thrice weekly in CRML medium (Gibco) containing 10% fetal bovine serum, 5 μg/ml insulin, penicillin and streptomycin. Confluent cultures were trypsinized for 5 min at 37˚C and split into new cultures at ratios of 1:5. The cells were cloned by seeding single cells into 96-well plates and one of the resulting colonies (clone 3) was used for further cultivation and experimental procedures.

Clone 3 of MW1 had by December 2005 undergone 65 passages. Cryopreservation was done in 90% culture medium/ 10% DMSO. To compare the cell line MW1 with established cell lines, T47d, MCF-10A and MDA-MB-231 were grown under conditions recommended for each of the individual cell lines by ATCC. The cell lines PAEC/KDR (28) and LIM 1863 (29) were grown as described previously.

Growth rate assay. MW1 cells in passage 9 (p9) and cloned cells in passage 65 (p65) were plated in 24-well plates at a concentration of 1000 cells/well. Duplicate wells were counted daily for 2 weeks. Values were calculated from the log phase of the growth curves: Doubling time, tD; MW1 p9, tD = ln2/0.0087/h = 79.7 h; MW1 clone 3 p65, tD = ln2/ 0.0179/ h = 38.7 h.

Genetic and immunohistochemical characterization. Karyotyping by multicolour fluorescence *in situ* hybridization (mFISH) has been described elsewhere (30,31).

Tumor specimens from the primary surgery and paraffin-embedded cell pellets of early and late passages of the pleural cell culture and cell line MW1, respectively were immunohistochemically studied. Immunoperoxidase staining was performed on 5-μm sections of formalin-fixed, paraffin-embedded tissue. Slides were first deparaffinized, and rehydrated. Antigen retrieval was carried out as follows: slides were either placed in 0.01 M citrate buffer at pH 6.0, or were placed in 0.001 M EDTA buffer at pH 8.0. Both sets were heated in a 770-W microwave oven for 14 min. After cooling and rinsing, slides were stained on the Dako Autostainer (Dako, Carpinteria, CA). Endogenous peroxidase activity was blocked by treating tissue sections with Dual Endogenous Enzyme Block (Dako Cytomation, Carpinteria, CA) for 10 min. The slides were then rinsed with Tris-buffer, and the sections were incubated with the primary antibody for 30 min. The sources, dilutions and pretreatment of the antibodies used were: ER (mouse monoclonal, Dako, 1:160, HIER: EDTA pH 8.0), PR (mouse monoclonal, Dako, 1:1280, HIER: EDTA pH 8.0), CK5/6 (mouse monoclonal, Dako, 1:50, HIER: EDTA pH 8.0), CK7 (mouse monoclonal, DAKO, 1:200, HIER: citrate pH 6.0).

Following a buffer rinse, a goat anti-mouse and goat anti-rabbit-labeled polymer conjugated with horseradish peroxidase [Envision + Dual Link HRP (Dako)] was applied for 30 min. After rinsing, the slides were treated with DAB for 10 min (Dako) to visualize the end-product, rinsed again, and toned with DAB Enhancer (Dako) for 2 min. Finally, the sections were counterstained with hematoxylin, dehydrated, cleared and mounted with permanent media. For HER-2 immunoassaying, sections were stained with the anti HER-2/neu antibody available from the Dako HercepTest kit (DakoCytomation Carpinteria, CA) on a Dako Autostainer following manufacturer's protocol including pretreatment.

Positivity of the primary antibody was defined as strong brown staining. For estrogen and progesterone receptor, only nuclear staining was considered positive. In the case of CK5/6 and CK7, membrane and cytoplasmic staining was considered positive. HER-2 immunostaining was evaluated using the HerCep Test scoring scheme, in which tumor cell membrane staining was graded as 0, 1+, 2+ and 3+ based on the intensity of immunostaining.

Semiquantitative reverse transcription-PCR. We isolated mRNA using the One-step RNeasy kit (Qiagen) according to the recommended protocol. The indicated primers and 2 μg of RNA were added to the RT-PCR reactions. Reverse transcription was performed at 50˚C for 30 min, followed by 15 min of 95˚C for inactivation. The resulting cDNA was then subjected to 35 cycles of amplification, followed by final extension for 10 min at 72˚C. The primers and conditions for VEGFR-1 (flt-1) and VEGF were described elsewhere (29). For the detection of other transcripts of interest, the following primers and cycling profiles were used: EGFR: sense, 5'-TCT CAG CAA CAT GTC GAT GG-3'; antisense, 5'-TCG CAC TTC TTA CAC TTG CG-3'; 94˚C for 20 sec, 61˚C for 30 sec, and 72˚C for 45 sec, HGF receptor c-Met: sense, 5'-ATA ATG AAG GCC CCC GCT GTG CTT-3'; antisense, 5'-ATT CAT CAC GGC GCG CTT CAC A-3'; 94˚C for 40 sec, 58˚C for 60 sec, 72˚C for 90 sec, VEGFR-2 (KDR, flk): sense, 5'-ACG CTG ACA TGT ACG GTC TAT-3'; antisense, 5'-GCC AAG CTT GTA CCA TGT GAG-3'; 95˚C for 45 sec, 55˚C for 45 sec, 72˚C for 1 min. Normalization with GAPDH was performed for each sample.

Protein expression. Proteins were extracted from cells in RIPA buffer containing EDTA and EGTA (Boston BioProducts) supplemented with protease inhibitor cocktail tablets (Roche Diagnostics). Lysates were centrifuged at 13000 rpm for 15 min at 4˚C to remove cellular debris, and the concentration of total cellular protein was determined using Bradford assay. Equal amounts of total cellular protein were treated with SDS-sample buffer and run on polyacrylamide gels, followed by transfer to nitrocellulose membranes (Bio-Rad). Membranes were probed with antibodies against ß4 integrin (505) (32), E-cadherin (H-108, Santa Cruz), P-cadherin (6A9, Santa Cruz), estrogen receptor α (D-12, Santa Cruz), or ß-actin (A-2066, Sigma). Incubation with the primary antibody was followed by probing with a horseradish peroxidase-linked secondary antibody (Mouse immunoglobulins/HRP, Rabbit immunoglobulins/ HRP, Pierce). Enhanced chemiluminescence (Pierce) was detected using X-ray film (HyBlot Cl, Denville).

Migration assay. Migration of breast carcinoma cells was quantified using a modified 48-well Boyden chamber (Nucleopore, Corning Costar Corp.) and polycarbonate membranes with a pore diameter of 5 μm. Cells were seeded in a concentration of $5x10^5$ cells/ml in CRML and allowed to migrate for 3 h at 37˚C with 5% CO_2. The filter membrane with adherent cells was fixed in 99% ethanol for 10 min and stained using Giemsa dye. Thereafter, cells on the upper side of the filter membrane were scraped off and the migrated cells were counted. A total of 15 high power fields from 3 different wells (5 each) was evaluated. Cell migration was stimulated with either HGF (0-100 ng/ml) (Sigma) or VEGF-A165 (0-100 ng/ml) (Reliatech).

Results

The MW1 cell line. We have established a new breast cancer cell line, MW1, from metastatic pleural fluid. The cells have been grown in culture for more than 4 years and grow as an adherent monolayer. In culture, the cells have an appearance of

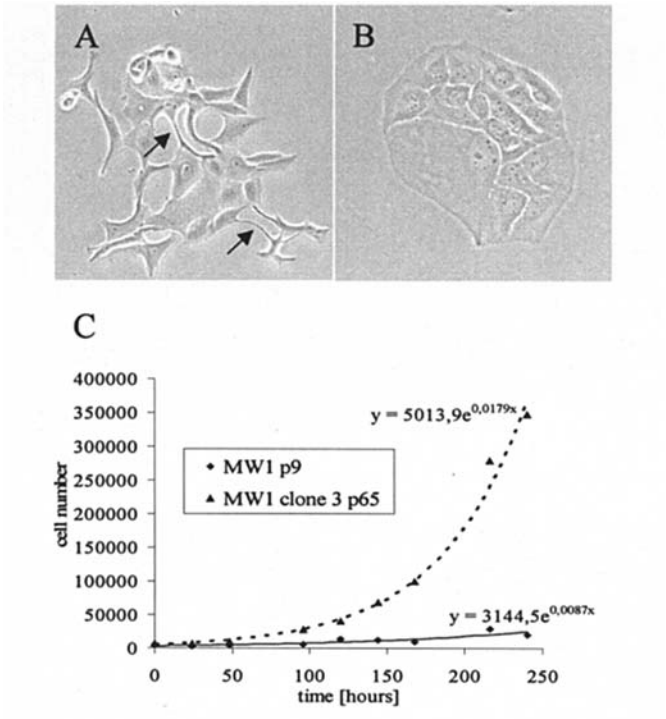

Figure 1. Morphology and growth characteristics of MW1 cell line. A, Early passage (p8) of the primary breast cancer culture MW1 shows epithelial monolayers and typical cellular apophyses that disappeared in later passages (p65). B, Growth curve. MW1 passage 9 and MW1 clone 3 passage 65 were grown and counted daily. Doubling times were calculated from the exponential phase of the growth curves.

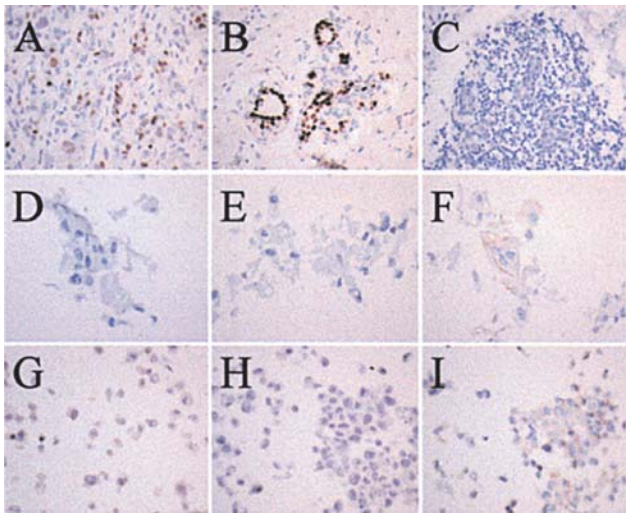

Figure 2. Immunohistochemical characterization of MW1. Immunohistochemical staining of the patients primary tumor (A-C), passage 6 of the primary breast cancer culture MW1 (D-F) and passage 57 of clone 3 of the breast cancer cell line MW1 (G-I). Estrogen receptor (A, D and G), progesterone receptor (B, E and H) and HER2 (C, F and I) expression were analyzed.

medium-sized epithelioid cells with variable nuclear size. In December 2005, the cells had undergone 65 passages, showed continuous growth and had the capacity of recovering from cryopreservation. For comparative purposes, early passages of the pleural culture (passage 3-9) were cryopreserved, thawed 3 years later and compared with passages 49-65 of clone 3. Morphologically, the cells of early passages of the pleural effusion exhibited the same cobblestone formation as late passages but were characterized by long cellular apophysises that were lost during continuous growth (Fig. 1A, MW1 p8; B, MW1 clone3 p65). During cultivation doubling times shortened dramatically from 79.7 h in passage 8 to 38, 7 h in passage 65 (Fig. 1C). The epithelial origin of MW1 was confirmed by pan-cytokeratin staining (27). Multiple colour FISH analysis was performed in early passages of the cultured tumor cells derived from pleural fluid, in order to confirm malignant origin of the cell line. Cells (82) were cytogenetically analyzed and 15 additional carcinoma cells underwent multiple colour FISH analysis. Multiple structural and numeric aberrations were found. Almost all chromosomes showed aberrations: 53,X,i(Xq), t(1p;5), +1, t(3q;6), t(3q;17), +3, t(5q;1), +del(5q), t(7p;2),

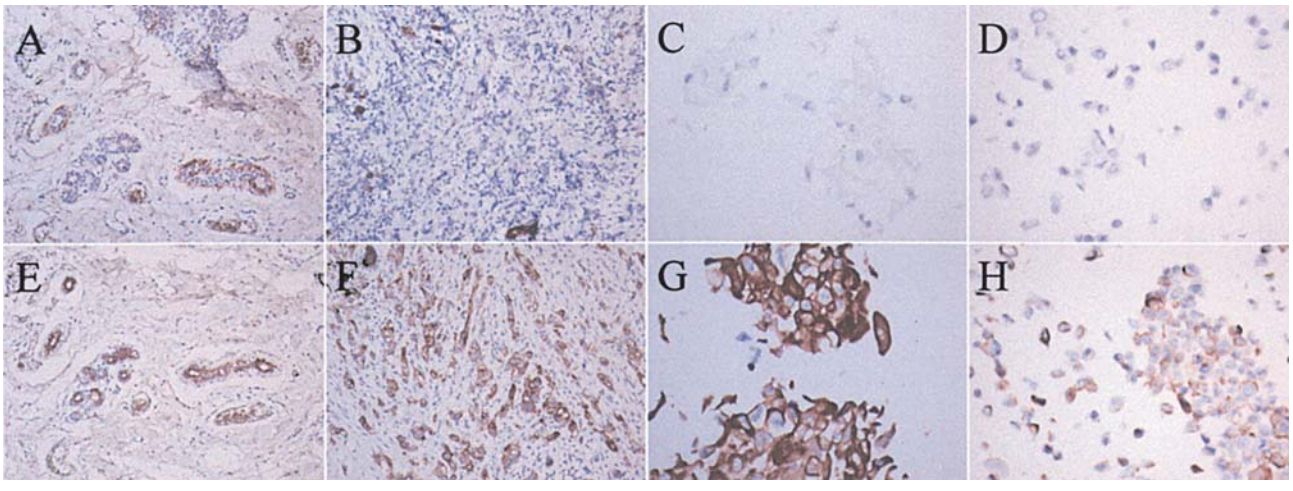

Figure 3. Cytokeratin expression pattern. Immunohistochemical staining for cytokeratins 5/6 (A-D) and cytokeratin 7 (E-H) is shown. Areas of normal breast tissue of the same patient (A and E) are compared to the primary breast cancer tissue (B and F), passage 6 of breast cancer culture MW1 (C and G) as well as passage 57 of clone 3 of MW1 cell line (D and H).

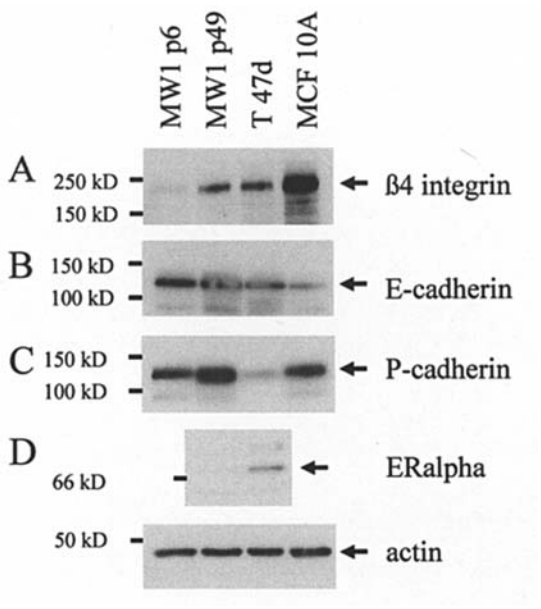

Figure 4. Expression of α6ß4 integrin, cadherins and estrogen receptor α. Passage 6 of breast cancer culture MW1, passage 49 of clone 3 of MW1 cell line, breast cancer cell lines T47d and immortalized breast cell line MCF-10A were lysed, immunoprecipitated and blotted against ß4 integrin (A), E-cadherin (B), P-cadherin (C) and ERα (D). Protein loading was controlled by blotting for actin on the same membrane.

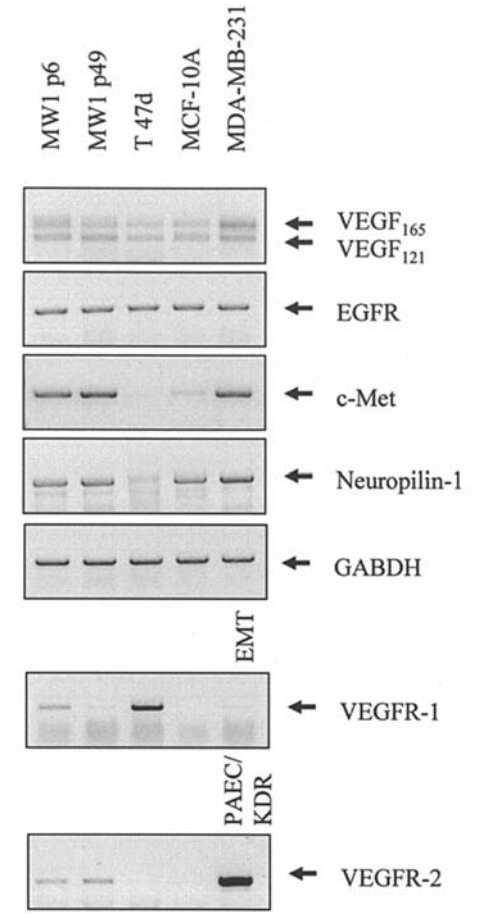

Figure 5. Expression of VEGF, EGFR, c-Met, Neuropilin-1, VEGFR-1 and VEGFR-2. Passage 6 of MW1, passage 49 of clone 3, breast cancer cell lines T47d, MCF-10A and MDA-MB-231 were examined for expression of VEGF, EGFR, c-Met, Neuropilin-1, VEGFR-1 and VEGFR-2 by reverse transcription PCR. GABDH was examined as control. For VEGFR-1 mRNA of colon carcinoma cell line LIM 1863 after induction of epithelial-mesenchymal transition (EMT; 29) was used as positive control. For VEGFR-2 mRNA of a porcine aortic endothelial cell line overexpressing VEGFR-2 (PAEC/KDR; 28) served as positive control.

+7, t(8p;12), t(8p;22), i(9q), +del(10q), +t(13q;14), -13, i(14q), -14, i(15q), +del(16q), -17, t(18q;22), +del(18q), t(22q;16), t(22q;18).

The genetic changes we identified include chromosomal translocations leading to structural rearrangements in genes and numeric changes causing imbalance in gene dosage. There is growing evidence that mutations in genes controlling chromosome segregation during mitosis causes chromosome instability and leads to aneuploidy in cancers as we see it in the MW1 cell culture (33).

We have established a clonal breast cancer cell line derived from pleural effusion that can be grown as a monolayer under cell culture conditions and serves as *in vitro* model system for breast cancer.

Immunohistochemical characterization. Immunohistochemical staining of the patient's primary breast tumor revealed positivity for estrogen and progesterone receptors (Fig. 2A and B) but no staining for HER2 (Fig. 2C). Comparison with early passages of tumor cells derived from pleural effusion and with late passages of the clonal cell line showed that the estrogen and progesterone receptor expression was lost in the pleural effusion (Fig. 2D, E, G and H). HER2 was negative in the primary tumor and showed low expression level in the metastatic state and in the cell line. Estrogen receptor negativity of MW1 cell line was confirmed by Western blotting (Fig. 4D).

Subtyping the primary tumor with antibodies specific to cytokeratins of basal origin (CK 5/6) and to luminal origin (CK 7) showed a predominantly luminal origin of the carcinoma cells (Fig. 3B and F), although single cells within the carcinoma cell structure showed positvity for CK 5/6. Early passages of MW1 (Fig. 3C and G) as well as late passages of clone 3 (Fig. 3D and H) stained positive for CK 7, exclusively.

Expression levels of ß4 integrin, E- and P-cadherin. The expression levels of ß4 integrin, E- and P-cadherin were compared between early and late passages of MW1 as well as the established breast cancer cell line T47d and the immortalized breast cell line MCF-10A by Western blotting. Early passages of MW1 showed low ß4 expression whereas late passages of clone 3 exhibited high expression levels (Fig. 4A). E-cadherin expression remained unchanged in early and late MW1 passages (Fig. 4B) whereas P-cadherin expression increased in late passages (Fig. 4C).

Expression of EGFR, HGFR (c-Met), VEGF, VEGFR-1, VEGFR-2 and neuropilin-1. We analyzed mRNA expression of EGFR, HGFR (c-Met), VEGF, VEGFR-1, VEGFR-2 and neuropilin-1 in passage 6 of MW1, passage 49 of clone 3, and in the established breast cancer cell lines T47d, MCF-10A, MDA-MB-231. Fig. 5 shows similar VEGF and EGFR mRNA transcription levels for all cell lines tested. Neuropilin-1 levels are likewise essentially similar; with only the cell line T47d exhibiting lower levels of neuropilin-1 transcription. The expression level of VEGFR-1 was high in passage 6 of MW1, but low in passage 49. As positive control we used mRNA from a colon carcinoma cell line after stimulation of

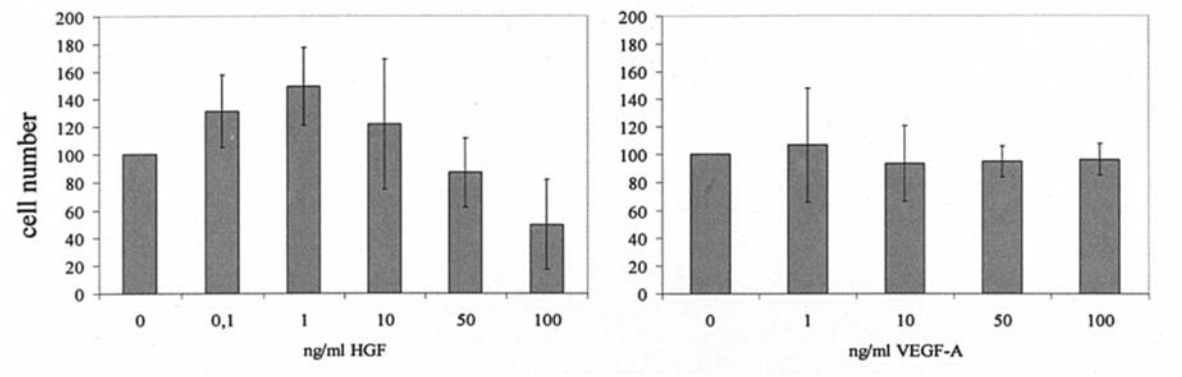

Figure 6. HGF and VEGF-A stimulated chemotaxis of MW1 breast cancer cells. MW1 cells were stimulated with different concentrations of either HGF of VEGF-A using a modified Boyden chamber. A total of 15 high power fields were counted for each sample. Baseline control value was set to 100%.

epithelial-mesenchymal transition (EMT, 29). VEGFR-2 and c-Met were detectable in both MW1 early and late passages and the levels of expression detected showed no alterations after years in culture. As positive control for VEGFR-2, mRNA from a porcine aortic endothelial cell line over-expressing VEGFR-2 was used (PAEC/KDR, 28).

Tumor cell migration. HGF and VEGF-A induced MW1 tumor cell migration was assessed using a modified Boyden chamber assay. The migration of MW1 cells could be stimulated to 149% with HGF as compared to the 100% baseline control value. The maximal stimulatory effect was reached at 1 ng/ml HGF (Fig. 6). An increase of HGF concentration beyond 50 ng/ml appeared to inhibit tumor cell migration. VEGF-A stimulation showed no effect on MW1 tumor cell migration (Fig. 6).

Discussion

We herein describe the establishment and characterization of a breast cancer cell line, MW1, derived from pleural effusions of a patient suffering from metastatic breast cancer. To our knowledge, this is the first longitudinal analysis of a cell line over more than 4 years, applying molecular methods to compare the primary tumor of a patient to her pleural effusion and to different passages of a monoclonal cell line that we established. The cell line displays stable growth characteristics under standard culture conditions as recommended by ATCC for breast cancer cell cultures. We ensured that the cells originated from the breast carcinoma by staining for several cytokeratins and by fluorescence *in situ* hybridization. Genetic changes in numerous chromosomal regions have been described to be involved in the development and progression of breast cancer (34). Some of the changes observed in cell line MW1 involve known loci such as 8p12 (FGFR-1) others do not appear to be associated with established genes. Changes in loci not yet described might be of assistance in the search for critical genes involved in breast cancer development and progression.

A significant number of human breast carcinoma cell lines are available as tumor model systems. Most of the cell lines have been in culture for many years, e.g., the cell line T47d, which was first described in 1979 (35). There are some reports of short-term breast cancer cultures (25) or new breast cancer cell lines (34,36,37). Long-term cultivation of breast cancer cells is difficult and only in a very small percentage of approaches results in a stable cell line (27,38). We assume, that growing tumor cells under culture conditions encourages genetic alterations and selects for cells with rapid doubling times. One challenge described before by Ethier *et al* (38) is the initially very long doubling time of 70-100 h that the cells display. This is consistent with the biology of human breast carcinoma in patients. We have confirmed these observations by comparing the growth rate of an early passage of the cell culture to that of a late passage of the cloned cell line MW1. The doubling time reduced to half from 79.7 to 38.7 h. In addition to accelerated growth, the cells also changed their appearance in the culture plates. In particular, characteristic cellular apophyses observed in early passages were lost in late passages. The molecular changes underlying the changes in growth characteristics of the MW1 cell line are as yet unclear.

We used a selection of molecular markers to gain insight into the extent to which the changes occur during cultivation. In addition to changes resulting from cultivation we were also interested in molecular changes occurring *in vivo* on the way from the primary tumor to the metastatic state reflected by early passages of the pleural culture. In this regard, we were limited by the fact that only paraffin-embedded tissue of the primary tumor was available. Since very early passages [1-5] of a primary culture are inevitably mixed cultures of mesothelial and tumor cells, we had to cultivate the cells for 4-6 months in order to obtain pure tumor cell cultures, as confirmed by staining for cytokeratin 19 (38, staining not shown). Therefore, some changes might have already taken place within the first months of cultivation. Nevertheless, early passages of breast cancer cells derived from pleural effusions closely resemble the metastatic state of breast carcinoma progression.

The primary tumor stained positive for estrogen and progesterone receptor whereas the cultivated metastatic cells as well as the cell line showed no expression of either estrogen or progesterone receptor. This might be due to the antihormonal treatment the patient received in the adjuvant setting. In contrast, HER2/neu expression was not detectable in the primary tumor whereas in the cultivated cells and the cell line, low levels of expression were observed. Both the loss of estrogen and progesterone receptor expression as well

as gain of HER2/neu expression reflect changes towards a phenotype described as being clinically more aggressive.

As regards cytoceratin expression, the primary tumor expressed predominantly CK 7, as reported for most primary breast cancer tissues (4). Since the luminal and basal staining patterns may show variations between different individuals (39), we stained a section of normal breast tissue of the same patient to ensure that the markers we used could differentiate between luminal and basal cytokeratin expression patterns (Fig. 3A and E). The cytokeratin expression patterns remained luminal in the metastatic state and during cultivation. The staining patterns became more uniform and single cells positive for CK 5/6 as observed in the primary tumor could not be detected in the cultivated cells. We compared E- and P-cadherin expression between passage 6 of the breast cancer culture and passage 49 of the cell line MW1. E-cadherin expression remained unchanged whereas P-cadherin expression increased. This is consistent with the observation that P-cadherin is upregulated in ~30% of breast carcinomas. The same is true for ß4 integrin. In summary, our data suggest that the tumor cells developed a more aggressive phenotype on their way to metastatic disease as well as during cultivation. Hormone receptor expression was lost, weak HER2/neu expression developed, P-cadherin and ß4 integrin expression were upregulated. On the way to metastatic disease, this may be due to a selective pressure caused by antihormonal and cytostatic treatment. Apparently, the cell culture conditions also induced selection of the more aggressive phenotype, characterized not only by faster growth but also by harbouring molecular features that have previously been found to correlate with poor clinical outcome. This selection might be due to the fact that only certain cells in a mixed population of the primary tumor are capable of approaching metastatic state and of surviving culture conditions. Alternatively, the selective pressure might enable cells carrying spontaneous mutations towards more aggressive behaviour to survive more efficiently than the original tumor cells.

We were also interested in receptor tyrosine kinase expression patterns and in autocrine signaling loops involving these receptors. VEGF isoforms are known to be expressed by tumor cells and to correlate with poor clinical outcome (23). We found similar mRNA levels of VEGF-A_{165} and VEGF-A_{121} isoforms in early and late passages as well as in all control cell lines tested. In previous studies, we found higher levels of VEGF-A_{165} in the supernatant of MW1 as compared to established control cell lines applying ELISA (27). This might reflect differences either at the level of protein versus mRNA expression or at the level of VEGF secretion into the culture medium. EGF receptor is known to be overexpressed in breast carcinoma and showed a uniform expression pattern at the mRNA level in the cell lines we tested (Fig. 5). More variations were seen for HGF receptor c-Met. HGF is known to be involved in autocrine and paracrine signaling in tumor cells (40), and c-Met expression is considered to be an independent prognostic factor in breast cancer (41). Our data show that both early and late passages of MW1 as well as the established cell line MDA-MB 231 express c-Met whereas the breast cancer cell line T47d and the immortalized breast epithelial cell line MCF-10A display no or merely mRNA levels of c-Met. VEGF produced by tumor cells acts in a paracrine fashion on endothelial cells but also induces autocrine signaling in the tumor itself (26,27,42). The autocrine signaling loop enhances survival and invasion of breast carcinoma cells (26). It is as yet not known which role each of the VEGF receptors VEGFR-1 (flt-1), VEGFR-2 (KDR) and Neuropilin-1 play in the autocrine VEGF signaling loop. We found expression of all of the VEGF receptors in MW1 early and late passages, with VEGFR-1 mRNA levels being lower in late passages of the MW1 cell line. The control cell line T47d expressed VEGFR-1 whereas MCF-10A expressed Neuropilin-1 only. It is unclear why VEGF receptor expression patterns vary between cell lines and what the functional impact thereof might be.

To investigate whether HGF and VEGF signaling have an influence on migration, we studied chemotaxis of MW1 cells towards HGF and VEGF-A. We observed increased migration towards HGF, but no influence on migration could be detected by stimulation with VEGF-A. One possible explanation could be that autocrine VEGF signaling is already saturated in the cells that we have shown to produce VEGF themselves (27, Fig. 5) and that addition of external VEGF has no further effects on signaling.

We established a new cell line that is of special interest for studying the autocrine VEGF signaling loop since VEGF as well as its receptors VEGFR1, VEGFR2 and neuropilin-1 are expressed. We analyzed several molecular markers and if these markers are representative for the patients either primary disease or metastatic state as starting point for further investigations.

Here, we provide an exemplary overview of the molecular changes that may occur on the way from a primary breast cancer lesion via metastatic pleural effusion to the establishment of a stable cell line. This longitudinal analysis might be helpful in relating results gained in breast cancer cell lines to human disease.

Acknowledgements

We thank Arthur Mercurio for his generous help and for the opportunity to cooperate with his laboratory. We also thank Kurt Weigand, Jennifer Ring, Richard Bates, Dongoo Bae, Keith Merdek and Pia Hantel for valuable discussions and technical help as well as Marianne Madlener for critical review of the manuscript. This study was supported in part by the Medical Faculty of the University of Ulm and in part by the NIH grant CA89209.

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