Breast Cancer Cells Isolated by Chemotaxis from Primary Tumors Show Increased Survival and Resistance to Chemotherapy

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Abstract

In this study, we have collected a migratory population of carcinoma cells by chemotaxis to epidermal growth factor-containing microneedles held in the primary tumor. The collected cells were subjected to microarray analysis for differential gene expression. The results show that anti-apoptotic genes are up-regulated and pro-apoptotic genes are down-regulated coordinately in the migratory subpopulation. Induction of apoptosis by doxorubicin, cisplatin, and etoposide in these cells demonstrates that they exhibit a lower drug-induced apoptotic index and lower cell death compared with carcinoma cells of the whole tumor. Our study indicates, for the first time, the capability of using a rat alograft model for evaluating the apoptotic status of a migratory subpopulation of tumor cells and the ability to study their resistance to chemotherapeutic agents directly. In addition, these results indicate that tumor cells that are chemotactic and migratory in response to epidermal growth factor in the primary tumor have a survival advantage over stationary tumor cells.

Introduction

Alterations in gene expression along with protein activation by cancer cells lead to transformation, proliferation, invasion, intravasation, dissemination in blood or lymphatic vessels, and eventually growth of distant metastases. For a tumor cell to become metastatic, it must be able to survive in the circulation and respond appropriately to new environments. This includes being able to migrate both within and beyond the primary tumor, in and out of blood and lymph vessels, and to use growth factors available at the site of metastasis for attachment and growth (1).

We have studied the motility-associated behavior of metastatic and nonmetastatic mammary tumor cell lines by intravital imaging within primary tumors (2–4). These studies have shown that the metastatic cells migrate to blood vessels and intravasate in a series of steps that involve active cell motility and may involve chemotaxis (3–5). A novel observation resulting from intravital imaging of these tumors is that they exhibit a lower drug-induced apoptotic index and lower cell death compared with carcinoma cells of the whole tumor. Our study indicates, for the first time, the capability of using a rat alograft model for evaluating the apoptotic status of a migratory subpopulation of tumor cells and the ability to study their resistance to chemotherapeutic agents directly (6).

Materials and Methods

Needle Collection and Fluorescence-Activated Cell Sorting of Primary Tumor Cells. We used MTLn3-derived mammary tumors in rats (2) and the microneedle collection method described previously (4, 6) to study the gene expression pattern of invasive subpopulation of carcinoma cells within live primary tumors. In brief, the invasive cells were collected from MTLn3 tumor using microneedles containing epidermal growth factor. Macrophages were removed from this population by using MACS CD11b Microbeads (Miltenyi Biotec, Auburn, CA) as described previously (6). The residual carcinoma cells were lysed for RNA extraction. To isolate the general population of carcinoma cells from primary tumor, a small piece of tumor was minced and filtered twice through a nylon filter to obtain a single cell suspension. Fluorescence-activated cell sorting (FACS) was performed on the resulting single cell suspensions based on their green fluorescent protein (GFP) expression in tumor cells using a FACSVantage cell sorter (Becton Dickinson, San Jose, CA). GFP-positive tumor cells were collected and lysed directly for RNA extraction. All of the procedures were done on ice or 4°C.

Ribonucleic Acid Extraction and Amplification. RNA extraction was performed using the RNeasy kit (Qiagen, Valencia, CA) per the manufacturer’s protocol and eluted with 30 μL of RNase-free water. The total RNA was reverse-transcribed and amplified directly using the SMART PCR cDNA synthesis kit (Clontech, Palo Alto, CA) as described previously (6).

Use of Pooled Reference Ribonucleic Acid as Control. An equal quantity of reference RNA (pooled RNA from rat liver, spleen, brain, and kidney, 4:2:1:1; Ambion, Austin, TX) was used as a control in all our microarray experiments, which allowed us to use one of the channels as a hybridization control for all of the spots on the microarray. The use of pooled reference RNA from the same species as the MTLn3 cells allowed the same interspecies cross hybridization as the background, allowing us to use mouse cDNA microarrays for our experiments. The pooled reference RNA covers a very broad range of gene expression and is routinely used as a control in cDNA microarray studies (7).

Probe Labeling and Microarray Hybridization. After amplification, cDNAs were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and eluted with Tris-EDTA buffer 10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA buffer. Labeling was performed using Label IT (Mirus Corporation, Madison, WI) following the manufacturer’s instructions. In brief, labeling reactions were prepared by mixing 10× Muris Labeling Buffer A, purified cDNA, and Cy5 (or Cy3) dye. After incubating the reaction mix at 37°C for 1 hour, the two resulting probes were purified by passing through gel filtration columns. The purified probes were then combined and concentrated using Microcon columns. The concentrated cDNA probes were denatured at 94°C and hybridized to an arrayed slide overnight at 50°C. Details of slide washing and image collection were described in previous studies (3, 6).

Quality Control and Data Analysis for Microarrays. The scanned images were analyzed using the software GenePix (Axon Instruments, Foster City, CA), and an absolute intensity value was obtained for both the channels. The entire raw data set was filtered to accommodate a requirement of at least two good quality measurements for each triplicate experiment. Values from only the good quality measurements (in which the signal strength was more than twice the SD of the background plus the background) were considered for
additional analysis. Two types of normalization were performed routinely in tandem on all of the experiments using the GeneSpring software package (Silicon Genetics, Redwood City, CA). First, intensity-based normalization was performed to take into consideration the overall signal strength of both channels and to normalize the signal strength between all of the different chips, reducing the chance of chip-to-chip variability. Second, a reference channel–based normalization was performed, which takes into consideration the reference channel (which in this case is pooled reference RNA) and normalizes the values in all of the spots. This reduces the chance of spot to spot variability. The final data were a result of both of these types of normalization.

**Significance Analysis of Microarrays.** To determine the significance of up-regulated and down-regulated genes, we performed significance analysis using the software Significance Analysis of Microarrays (SAM; ref. 8). In brief, after normalizing the data as mentioned above, the data were log transformed to Log2 and subjected to SAM analysis. The algorithm performs a significance analysis by comparing the relative variance of the replicates between the samples. The results were determined at 5% false discovery rate.

**Real-Time Polymerase Chain Reaction Confirmation.** To verify the data obtained from microarrays, quantitative real-time PCR analysis of selected overexpressed and underexpressed genes was performed by using the ABI 7900 (Applied Biosystems, Foster City, CA) with sequence-specific primer pairs for all genes tested (see Supplementary Table 2 for primer sequences, 7900 (Applied Biosystems, Foster City, CA) with sequence-specific primer pairs for all genes tested (see Supplementary Table 2 for primer sequences, and annealing temperature) as described previously (3). SYBR Green was used for real-time monitoring of amplification. Results were evaluated with the ABI Prism SDS 2.0 software. All of the genes tested for regulation were compared with at least two housekeeping genes (β-actin and glyceraldehyde-3-phosphate dehydrogenase).

**Cell Culture and Apoptosis Assay.** The cells extruded from the needles and tumor cells FACS sorted were cultured in Dulbecco’s modified Eagle’s medium 20% fetal calf serum along with streptomycin and penicillin for 16 hours. Subsequently, the cells were challenged with doxorubicin (17 μM/L), cisplatin (50 μM/L), or etoposide (50 μM/L) for 1 hour; washed; and allowed to recover for 24 hours. The cells were then subjected to an apoptosis assay kit containing annexin V Cy5 for staining the apoptotic cells and propidium iodide for staining the dead cells (BD Biosciences, San Jose, CA). After staining the cells using the manufacturer’s protocol, the cells were observed under a fluorescent microscope in the green, red, and high red channel for GFP, propidium iodide, and Cy5, respectively. The total number of GFP cells counted was compared with the number of propidium iodide-positive and annexin V-Cy5-positive cells.

**Results and Discussion**

GFP-labeled tumor cells were injected into rat mammary fat pads, and primary tumors were allowed to grow for 2 to 2.5 weeks. To provide insight into the pattern of gene expression associated with chemotactic and migratory carcinoma cells in vivo, we compared the gene expression profile of a subpopulation of tumor cells collected from the primary tumor by chemotaxis into a microneedle, called the invasive cells, with that of the general population of GFP-expressing tumor cells sorted from the whole primary tumor by FACS (Fig. 1). Differential gene expression analysis comparing the invasive and general populations of tumor cells was performed using SAM analysis at 5% false discovery rate level, revealing 679 genes that were differentially expressed significantly relative to all genes on the array (Supplementary Table 1). These results were the same regardless of whether the group of cells was FACS sorted from tumors that were subjected to needle collection, indicating that the needle collection procedure did not affect the gene expression pattern.

The genes that are previously known to be associated with the epidermal growth factor response (28 genes) were removed from this population. As shown in Fig. 1, genes with known functions whose regulation was changed in the chemotactic and migratory population of cells in the primary tumor were divided into six different functional categories based on the definitions provided by the gene-ontology consortium.1 It was evident that among the functional categories mentioned here, the largest change was in the genes associated with the cell cycle, indicating a big change in the cell proliferation pattern of migratory cells. A detailed scrutiny of these cells showed that the genes associated with increasing cell proliferation were down-regulated and those genes associated with a reduction in cell proliferation were up-regulated.

Another category of genes found to be significantly regulated in the chemotactic and migratory population of cells in the primary tumor is that of cell motility. These genes have been explained in detail in an accompanying paper. Because there are five steps of the motility cycle that are coordinated to assure efficient cell motility, the up-regulation of genes for major effectors in the pathways of each step predicts that the invasive cells will have a heightened migratory activity compared with carcinoma cells of the general tumor population, and this is consistent with the high velocities of migration seen in tumors (5).

**Regulation of Pro- and Anti-apoptotic Genes along with Mechanical Stability Genes.** Of particular relevance to survival, stress- and apoptosis-associated genes showed large changes in regulation (Fig. 2). The up-regulation of the heat shock proteins indicates a survival phenotype (9) This is particularly interesting here because the MTLn3 cells used to generate the primary tumors in this study have been shown to overexpress heat shock proteins compared with nonmetastatic cell lines (MTC) derived from the same tumor (10). This indicates that in the chemotactic and migratory population of cells in the primary tumor, there is additional up-regulation of the heat shock gene expression over that in the MTLn3 cells used to generate the primary tumor.

A potential explanation for mechanical stability and survival advantage observed in invasive cells (9, 11) is the large relative overexpression of cytokeratins 19 and 13 by 8- and 3-fold, respectively, by carcinoma cells and the suppression of apoptosis gene expression in metastatic tumors when compared with nonmetastatic tumors (3). Keratins form the largest subfamily of intermediate filament proteins that play critical roles in the mechanical stability of epithelial cells subjected to shear forces (12). In addition, it was found that carcinoma cells in metastatic tumors and in culture express laminins, cadherins, and apoptosis suppressor genes at high levels, all of which might contribute to survival during invasivation and in the circulation (3). In contrast, carcinoma cells in nonmetastatic tumors and in culture express genes involved in programmed cell death at higher levels. The combination of these factors may contribute to the

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1 Internet address: http://www.geneontology.org/.
in vivo
genes were up-regulated in a cell line (MTLn3), which causes metastasis. This is consistent with a previous study where the apoptosis suppressor genes were unregulated or down-regulated. A significant number of the anti-apoptotic genes were up-regulated, whereas the pro-apoptotic genes were unregulated or down-regulated.

Increased numbers of viable carcinoma cells in the circulation of metastatic tumors and to fragmentation during intravasation and cell death seen in nonmetastatic tumors (4, 11).

In addition, the anti-apoptotic and pro-apoptotic genes are inversely regulated in the chemotactic and migratory population of cells in the primary tumor (Fig. 2A). The ratio of expression of each gene in the invasive cells, when compared with the general population, indicates that a significant number of the anti-apoptotic genes were up-regulated, whereas the pro-apoptotic genes were unregulated or down-regulated. This is consistent with a previous study where the apoptosis suppressor genes were up-regulated in a cell line (MTLn3), which causes metastasis in vivo when compared with another cell line from the same lineage (MTC), which does not (3). In this study, we show a similar difference between the invasive and general populations of the primary tumor even though the tumor is derived from the same parental cells (MTLn3). This is important because it means that the microenvironment that induces the chemotactic and migratory behavior of tumor cells induces the survival expression pattern in cells with a previously genetic background.

We have verified the array results using real-time PCR for selected genes by quantitative real-time PCR. Real-time PCR was performed by using the ABI 7700 and SYBR Green PCR Core Reagents system (Applied Biosystems) along with sequence-specific primer pairs for all genes tested. Results were evaluated with the ABI Prism SDS 2.0 software. Comparison of expression analyses in needle-collected invasive tumor cells gives similar results for cDNA microarrays and quantitative real-time PCR.

Drug Resistance in Invasive Cells Measured by Apoptosis Assay. The finding that the anti-apoptotic genes are up-regulated in the invasive cells prompted us to study the functional importance of this finding and whether these cells indeed have a survival advantage over the resident population. The invasive cells were challenged with the three most commonly used anticancer drugs: doxorubicin, cisplatin, and etoposide. Previous studies have shown that these drugs induce apoptosis in the MTLn3 cells (13). We performed these studies on the invasive and general populations of cells from MTLn3-derived tumors. After treatment with the drugs, the cells were allowed to recover for 24 hours. Subsequently, the apoptotic index and cell viability were measured as described in Materials and Methods. The results are shown in Fig. 3 and demonstrate that as a percentage of all of the carcinoma cells, the invasive cells are able to tolerate all three drugs better than the general population of tumor cells. The process of FACS by itself did not cause any change in the apoptotic index of the tumor cells (data not shown).

Most of the anticancer drugs like doxorubicin, cisplatin, and etoposide are designed against the proliferative cells (14), making them cytotoxic. Recently, there is an increasing effort to make cytostatic drugs, which prevent the proliferation and invasion as opposed to killing the cells. There has been a demand in the field to have a method to isolate these invasive cells and look for the effect of cytostatic drugs specifically on invasive cells. We believe that in our studies, we have demonstrated a method that makes possible this analysis on migratory cells of the primary tumor.

Coordinate Regulation of Survival Genes in the Invasive Cells. Previous studies have shown that the anti-apoptotic pathways are overexpressed in the metastatic cell lines (15), and these cells have a survival advantage via Stat3-dependent overexpression of BCL-2. In our study, we find a number of anti-apoptotic genes to be up-regulated. These genes belong to all three pathways that mediate apop-

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nis, rendering a survival advantage to the cells. On one hand, up-regulation of the defender against death 1 (DADI) gene indicates that the extrinsic pathway is blocked in these invasive cells. On the other hand, there are signs of down-regulation of the intrinsic pathway as well by the overexpression of ornithine decarboxylase 1 (ODC1). Up-regulation of the expression of apoptosis inhibitor 1, 4, and 5 (Api1, Api4, and Api5) genes indicates an involvement of the convergence pathway as well. Finally, there is the robust overexpression of the genes like immediate early response gene 3 (IE3), which is a multipathway regulator involving the nuclear factor κB family of transcription factors (16). Simultaneously, a number of the pro-apoptotic were down-regulated; significantly, a key regulator of the intrinsic pathway, APAF-1, was down-regulated in the invasive cells. Fig. 4 summarizes these findings and indicates the extent of change that is observed in the transcriptome of invasive cells.

In this study, we have attempted to investigate the pathways leading to metastasis, which provides this survival advantage to these cells. In previous studies, investigators have used cell lines derived from an established secondary tumor (15). We, on the other hand, have performed a dynamic assessment of the process of metastasis and have captured the cells before the entry into the blood.

In our studies, we have identified pathways that get regulated in the invasive cells, which are not proliferative (Fig. 1). The majority of the genes indicated in the functional category of “cell cycle” are genes that cause a reduction in cell proliferation and prevent the progression of the cell cycle. Recent studies have shown that the overexpression of BclII in MTLn3 cells causes the cells to become resistant to doxorubicin (13) as observed by a reduction in drug-induced DNA fragmentation. Previous studies using cell lines derived from metastatic and resident cells from human breast adenocarcinoma have shown that the metastatic cell lines are more resistant to anticancer drug treatment compared with the cell lines from the primary tumor (15). However, it remains unknown at which stage of cancer progression (i.e., transformation, proliferation, invasion, intravasation, or dissemination of metastases) the selection of the cells that have a survival advantage occurs. In this study, we show for the first time that this selection of cells with a survival advantage probably takes place at the very initial stage of invasion, as is evident by the overexpression of anti-apoptotic genes and resistance to anticancer drugs by the invasive cells. The gene expression pattern observed here is associated with an invasive signature unique to these cells. Hence, we have identified an expression pattern of survival genes that offer a survival advantage to nonproliferating invasive cells. Studies are under way to find out what initiates and maintains this selection. We are attempting to understand whether the microenvironment that induces invasion causes this dramatic change in the survival expression pattern.

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References

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