Progression and Enhancement of Metastatic Potential after Exposure of Tumor Cells to Chemotherapeutic Agents


Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, Minnesota 55455

Abstract

Data presented in this report indicate short-term in vitro treatment of nonmetastatic MCF-7 breast carcinoma cells with the chemotherapeutic agents Adriamycin and/or 5-fluoro-2'-deoxyuridine (5-FU), induced changes in the expressed phenotype. Cells treated sequentially with Adriamycin and/or 5-FU rapidly increases, in a dose-dependent manner, the release of the angiogenic cytokine, interleukin-8 (IL-8), which is released at consistently higher levels in metastatic cell lines. Cell populations surviving a single treatment with either one or both of these chemotherapeutic agents continue to stably release IL-8. Survivors of sequential treatment with Adriamycin and/or 5-FU (MCF-7 A/F) release the most IL-8 and express the greatest phenotypic variance from the parental, MCF-7 cells. Parental MCF-7 cells and MCF-7 A/F cells both form primary tumors when used in an orthotopic tumor model; however, the MCF-7 A/F tumors have a more rapid initial growth phase in situ and give rise to spontaneous lung metastases within 10 weeks. A cell line that is established from lung metastases releases more IL-8, has a higher cloning efficiency, and forms looser colonies in monolayer than do their parental cells. These experiments indicate the in vitro exposure of tumor cells to chemotherapeutic agents either selects more aggressive cells or enhances the metastatic potential of the surviving cells.

Introduction

Breast cancer is a devastating disease that continues, unabated, to rob women of their health and, all too often, their lives (1). Breast tumors that recur after such treatments as radiation or chemotherapy are characterized as the most detrimental. Because most breast carcinomas are considered to be responsive to chemotherapy, their initial response is often very good. However, in many patients a portion of treated tumor cells becomes less sensitive to chemotherapeutic compounds (2). Tumor cells that survive chemotherapy often express phenotypes distinctly different from those expressed by the primary tumor prior to chemotherapy. The levels and activity of many cellular factors are distinctly different in the survivors; for example, cells enduring chemotherapy often express higher levels of MDRs (3). Although the expression of MDRs can confer drug resistance and thereby increase the survival potential of tumor cells, there is not a direct association between MDRs and lymph node metastases, estrogen receptor status, tumor size, tumor grade, or tumor histology (3). Although MDR expression correlates with poor prognosis, it does not contribute to other cellular characteristics associated with a more aggressive phenotype, such as increased metastatic potential. Other factors thought to contribute to the growth and progression of tumor cells include the angiogenic cytokine, IL-8. Its angiogenic properties could enhance neovascularization at the tumor site allowing for increased growth and metastasis because of poorly formed basement membranes of tumor-associated blood vessels (4–7). Our previous studies have shown a correlation between the level of IL-8 released and the metastatic potential of breast tumor cell lines, suggesting that an increase in IL-8 level is indicative of a more metastatic phenotype (8). The mechanisms underlying the correlation between metastatic potential, coexpression of MDR, and the other cellular functions are unknown. Cells that coexpress these properties may be selected for from a preexisting minor population of tumor cells that are present prior to the initiation of chemotherapy; alternatively, these attributes may be induced by the chemotherapeutic agents. If chemotherapeutic agents can induce tumor cells to express factors that enhance their survival and increase their metastatic potential, the tumor cells are much more likely to survive chemotherapy, to progress, and to eventually kill the host. In this study, we developed a series of cell lines demonstrating that those cells surviving short-term in vitro treatment with chemotherapeutic agents have an enhanced ability to survive and progress to a metastatic phenotype in vivo. This approach may enable us to determine whether tumor cells are selected for survival because of expression of certain cellular factors or are induced to express these factors by the chemotherapeutic agents. Understanding the mechanisms by which tumor cells are able to circumvent elimination by chemotherapeutic agents and adopt a more aggressive behavior will hopefully lead to the development of more efficacious chemotherapeutic protocols to eradicate tumor cells.

Materials and Methods

Cell Lines and Culture Conditions. The MCF-7 cell line (American Type Culture Collection, Rockville, MD) and the chemotherapeutic selected cell lines were cultured with antibiotic-free DMEM (Mediatech, Herndon, VA) plus 10% FBS (Bio Whittaker, Walkersville, MD) in sterile tissue culture flasks and incubated at 37°C/6%CO₂. The cell lines were certified to be mycoplasma-free. Cells were subcultured by trypsinizing in 5 mg/ml of trypsin (Sigma, St. Louis, MO) and 0.5 mM of EDTA in HBSS without Ca²⁺ or Mg²⁺ in a laminar flow hood during their logarithmic phase of growth.

ELISA for Human IL-8. The breast carcinoma cell lines were seeded in six-well plates containing 2 ml of complete medium per well. At 80% confluency, the medium was aspirated and 2 ml of fresh complete medium were introduced to each well along with the varying concentrations of the chemotherapeutic agents, Adriamycin and/or 5-FU. After a 24-h treatment, 1.5 ml of medium were collected from each well, clarified of cells and cellular organelles, stored at −20°C, and the number of cells per well determined. The IL-8 ELISA was performed according to the manufacturer’s instructions (OptEIA Human IL-8 set; Pharmingen, San Diego, CA). This kit is specific for human IL-8. Neither NIH 3T3 cells nor tissue culture strains, developed from athymic mice tested with the IL-8 kit, released IL-8 cross-reacting products constitutively or when induced with IL-1β (a gift from Jim Cone of Otsuka Pharmaceutical Company, Rockville, MD) or TNF-α (Peprotech Inc., Rocky Hill, NJ).

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2 To whom requests for reprints should be addressed, at Department of Laboratory Medicine and Pathology, University of Minnesota, 420 Delaware Street SE, Minneapolis, MN 55455. Phone: (612) 624-6608; Fax: (612) 625-1121; E-mail: delar001@tc.umn.edu.

3 The abbreviations used are: MDR, multidrug resistant factor; IL, interleukin; FBS, fetal bovine serum; FUdR, 5-fluoro-2'-deoxyuridine; NF-xb, nuclear factor xB; ROS, reactive oxygen species; TNF-α, tumor necrosis factor α.
Selection of Cell Populations Using Chemotherapeutic Agents. MCF-7 cells were plated at a density of $0.75 \times 10^5$ cells per well in six-well tissue culture plates. After 24 h, the medium was changed, and a series of concentrations of FUdR or Adriamycin were added. Drug treatment was terminated after 72 h for the FUdR and after 5 days for the Adriamycin. Over the next two weeks, the medium was changed approximately every 4 days as the cells repopulated the culture. Cells surviving 10 $\mu$g/ml FUdR became the MCF-7-F cell line, and cells surviving 25 ng/ml Adriamycin became the MCF-7-A cell line. To produce the doubly selected cell line, MCF-7A cells were plated at a density of $10^5$ cells per well in six-well tissue culture plates. Twenty-four h later, varying concentrations of FUdR were added to the plates. The medium and FUdR was changed every 3 or 4 days for 2 weeks, after which the medium and FUdR was replaced with medium containing no selective pressure. MCF-7A cells that survived treatment with 20 $\mu$g/ml FUdR became the MCF-7-A/F cell line.

Animal Studies. Female, athymic nu/nu mice (4–6 weeks old) were purchased from the National Cancer Institute, Bethesda, MD to be used in an orthotopic model of spontaneous metastasis. One week after arrival of the mice, human breast carcinoma cells (2.5 $\times 10^5$ cells suspended in DMEM without serum) were injected directly into the second left mammary fat pad of each mouse through an incision just below the nipple. The cells were injected in a volume of 25 $\mu$l using a 0.3-ml syringe with a 29-gauge needle (Monoject; Sherwood Medical, St. Louis, MO). The mice were also implanted s.c. with 90-day release 17-estradiol pellets, which contain 0.72 mg of estrogen per pellet (Innovative Research of America, Sarasota, FL). After the tumors grew to a measurable size, usually 3 weeks, the tumor diameters were measured weekly using a Bel-Art metric, dial caliper, and the tumor volumes were determined using the following formula: $V = \frac{4}{3}\pi r^3$ where $r$, the radius, is the mean of three measurements. At the end of each trial, tissue from the primary tumor and lungs from each mouse was used for histological analysis and to establish cell cultures. The number of mice having metastatic lung tumors was determined by culturing sections of lung tissue in 1 mg/ml collagenase for 1–2 h and then transferring the cells and tissue sections to 10-cm tissue culture dishes with DMEM containing 10% FBS and 2X antibiotic/antimycotic (Life Technologies, Inc., Grand Island, NY). After culturing the cells, medium was collected and an IL-8 ELISA performed. Cultures containing MCF-7 cells produced IL-8 detectable by the IL-8 ELISA set, whereas cultures containing only murine cells did not produce detectable IL-8.

Results and Discussion

IL-8 Induction by Chemotherapeutic Agents. Our previous studies demonstrated that a strong correlation exists between IL-8 expression and the metastatic potential of breast carcinoma cells (8). In the course of establishing, during a separate study, a toxicity curve for MCF-7 cells treated with Hygromycin B (Calbiochem, La Jolla, CA) used to select transfectants containing vectors that express the Hygromycin B resistance marker, we observed that this treatment induced an increase in IL-8 release, as well as changes in cellular morphology (data not shown). Because it is possible that these effects were attributable to stress caused by the toxic nature of the compound, we tested chemotherapeutic agents on the assumption that their effect on the cells would be similar to the stresses created by the Hygromycin B used to titrate resistance markers. In initial experiments, dose curves were performed with MCF-7 cells under acute (24-h) exposure to Adriamycin or FUdR. This short-term exposure of the MCF-7 cells to the chemotherapeutic agent Adriamycin induced, in a dose-dependent manner, an increase in the release of IL-8. During the 24-h exposure to 400 ng/ml of Adriamycin, the level of IL-8 that was released increased by >3-fold the constitutive level of expression, observed in the presence of the solvent alone (Fig. 1A). The levels of IL-8 released by the MCF-7 cells treated with FUdR are similarly large to those seen during Adriamycin treatment (Fig. 1B). The IL-8 level released by the cultures treated with 1 $\mu$g/ml FUdR treatment was ~4 times that of the constitutive level seen in the solvent control. However, at 10 $\mu$g/ml FUdR, a decrease in the level of IL-8 released was observed. This decrease, seen at the highest level of FUdR treatment, is likely attributable to acute cellular toxicity from the FUdR. Also, as part of the toxic response to the higher concentration of FUdR, protein synthesis may have been inhibited early on in treatment, thereby inhibiting IL-8 synthesis.

During the 24-h treatment, the number of viable cells varied only slightly, and the treatment period was too short to account for these differences to have been caused solely by the selection and expansion of cells that produce more IL-8. We hypothesize that it is likely that these increases in IL-8 release were attributable mainly to induction of IL-8 by the chemotherapeutic agents as a protective mechanism. It is, therefore, possible that the increase in IL-8 release was one of the survival responses to oxidative stress that induced the activation of AP-1 and NF-κB. These two transcription factors are involved in IL-8 expression (9). During the metabolism of Adriamycin, ROS are generated (10). Oxidative stress is a pleiotropic modulator involved in gene expression directed by both physiological and environmental (such as xenobiotic) stimuli (11). Thus, the generation of ROS during the metabolism of Adriamycin could be a secondary messenger for growth factors and inflammatory cytokines, such as NF-κB, which, when activated, in turn stimulates a large number of genes, including IL-8 (12).

IL-8 Released by MCF-7 Cells and Cells Surviving Chemotherapeutic Treatment. The characterization of cells that have survived treatment with Adriamycin (MCF-7A), FUdR (MCF-7F), or cells that were subjected to sequential treatment with Adriamycin and FUdR (MCF-7 A/F) was performed to determine whether populations of
CONTRIBUTION OF CHEMOTHERAPY TO METASTATIC POTENTIAL

In Vivo Growth Characteristics of Cells Surviving the Combined Chemotherapeutic Treatment. To determine whether survivors of sequential chemotherapy treatment expressed altered *in vivo* growth properties and an increased metastatic potential, $2.5 \times 10^5$ cells from both MCF-7 and MCF-7 A/F were injected into the mammary fat pads of athymic nu/nu mice that had estrogen pellets implanted s.c. The rate of tumor growth was determined by measuring the tumors weekly using a caliper. Table 1 shows the differential growth rates between the primary tumors established from MCF-7 or MCF-7 A/F cells. By week 3, both groups of mice developed tumors that could be readily measured. The initial growth rate of the MCF-7 A/F tumors, as determined by the growth curve between weeks 3 and 7, was greater than that of the parental, MCF-7 tumors. The MCF-7 A/F tumors grew noticeably faster during this phase of their growth. After week 7, the MCF-7 A/F tumors still had a larger volume overall than the MCF-7 tumors; however, the rates of growth between the two groups appeared similar. One potential explanation is that there was an initial lag phase in the growth of the MCF-7 cells not seen in the MCF-7 A/F cells. We observed a similar growth pattern *in vitro*. The MCF-7 cells had an initial lag phase in their growth, whereas the MCF-7 A/F cells had little or no lag phase in their growth pattern. Known metastatic breast carcinoma cell lines, such as the MDA-MB-435, have similar growth patterns *in vitro* and *in vivo* (8), further supporting the theory that tumor cells surviving chemotherapy become more aggressive.

MCF-7 A/F cells not only developed tumors faster (Table 1), but they also attracted more superficial blood vessels to the inoculation site and grew as less-spherical, irregularly shaped tumors (data not shown). The tumors that developed from the MCF-7 cells were spherical with regular, smooth edges. After 10 weeks, the animals were euthanized, their lungs were removed, minced, and treated with collagenase, and the dissociated cells were established in tissue culture. Human breast tumor cells were obtained from the lungs of three of the five animals that received orthotopic implants of the MCF-7 A/F cells. No human tumor cells were obtained from cultures of animals in the MCF-7 group. Presence or absence of human cells was determined using an indirect immunoperoxidase stain for keratin. Cells from each of the cell lines were seeded in six-well tissue culture plates in DMEM containing 10% FBS. After 24 h, the medium was changed and either was left untreated or had 4 ng/ml TNF-α or 1 ng/ml IL-1β added to the wells. After 24 h of treatment, medium was collected from each well, clarified of cells and organelles, and stored at $-20^\circ C$. The number of cells per well was determined, and an IL-8 ELISA was performed.

The constitutive levels of IL-8 released into their conditioned media were elevated in all of the cell lines that survived treatment with the chemotherapeutic agents. The MCF-7 A/F was 6-fold more IL-8 than the control MCF-7 cultures, the MCF-7/F released 2.5-fold more, and the MCF-7 A/F released 11.8-fold more than the levels of the MCF-7 cultures (Fig. 2). The constitutive level of IL-8 released by the MCF-7 A/F is as high as the constitutive level of IL-8 released by the very metastatic MDA-MB-435 (8). We also tested the effects of IL-1β and TNF-α on these cells, because these cytokines have been shown to increase IL-8 release (8, 13). The levels of IL-8 that were induced by the inflammatory mediators IL-1β and TNF-α showed similar results. Cells that survived treatments with the chemotherapeutic agents released much more IL-8 in response to the inflammatory mediators than did the untreated MCF-7 control cells (Fig. 2). To determine whether the increase in IL-8 release induced by the chemotherapeutic agents was stable, the cells that survived treatment with the relatively high doses of chemotherapeutic agents were examined at low passage and at high passage (data not shown). The increased levels of IL-8 released by these cells have remained elevated for as long as we have carried these cells, which was greater than 20 passages in chemotherapeutic free media. This suggests that a relatively short treatment of nonmetastatic cells with chemotherapeutic compounds can produce stable changes in cellular phenotype, a property consistent with our hypothesis that cancer cells that survive chemotherapy may express an increased metastatic potential. Also, the continued elevated levels of IL-8 indicate that in addition to the changes in gene expression brought about by metabolism of chemotherapeutic agents, heritable genetic or epigenetic changes may have taken place. Genes that had previously been silent may be activated, and/or genes that had been active may be silenced (14, 15).

Table 1. Primary tumor growth time course

<table>
<thead>
<tr>
<th>Week</th>
<th>MCF-7</th>
<th>MCF-7 A/F</th>
</tr>
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<tbody>
<tr>
<td>3</td>
<td>57.64 ± 29.81</td>
<td>92.56 ± 30.47</td>
</tr>
<tr>
<td>4</td>
<td>41.98 ± 28.85</td>
<td>145.99 ± 78.22</td>
</tr>
<tr>
<td>5</td>
<td>55.52 ± 36.49</td>
<td>234.96 ± 80.67</td>
</tr>
<tr>
<td>6</td>
<td>109.38 ± 44.80</td>
<td>322.11 ± 158.62</td>
</tr>
<tr>
<td>7</td>
<td>142.76 ± 73.35</td>
<td>446.33 ± 176.36</td>
</tr>
<tr>
<td>8</td>
<td>270.12 ± 202.17</td>
<td>566.92 ± 241.04</td>
</tr>
<tr>
<td>9</td>
<td>470.85 ± 272.51</td>
<td>716.08 ± 270.50</td>
</tr>
<tr>
<td>10</td>
<td>602.86 ± 296.67</td>
<td>929.31 ± 370.94</td>
</tr>
</tbody>
</table>
determined by testing each culture for IL-8 release by ELISA. Mouse cells did not release any human IL-8 or other antigens detectable by the IL-8 ELISA set. Histological analysis (pap smear and H&E staining, data not shown) further confirmed that the cells obtained from the lungs were human carcinoma cells, showing that short-term treatment of nonmetastatic breast carcinoma cells with chemotherapeutic agents can induce a metastatic phenotype.

Changes in in vitro Growth Characteristics. Cultures from the three mice with lung metastases, Mouse 30, 32, and 33, were saved as metastatic cultures, assayed for quantifiable IL-8 levels, and compared with the MCF-7 and MCF-7 A/F cell lines used in the orthotopic model. All three metastatic cultures released more IL-8 than the MCF-7 A/F parental culture. The MCF-7 A/F released 1560 ± 160 pg/ml/24 h/10⁶ cells, whereas the M30L, M32L, and M33L released 4940 ± 290, 1670 ± 50, and 3760 ± 60 pg/ml/24 h/10⁶ cells, respectively. The elevated levels of IL-8 released by lung-derived metastatic cells remained stable for longer than 20 passages (data not shown), suggesting either that the cells releasing more IL-8 are more likely to metastasize to and grow in the lungs than the rest of the parental cell population or that, once the metastatic tumor cells take up residency in the lungs, their IL-8 release is enhanced by the neighboring mouse cells in a paracrine mechanism. In the later case, it would suggest that the paracrine mechanism induced stable genetic or epigenetic phenomena.

Morphological differences were observed in vitro between cells surviving chemotherapeutic treatment and untreated, control MCF-7 cells. The parental, untreated MCF-7 cells grew as cohesive, discrete epithelial-appearing colonies. Cells that survived chemotherapeutic treatment, when grown in monolayer, formed looser colonies with the cells at the borders tending to have more protruding filopodia (Fig. 3). We observed a progression from epithelial colony morphology with close cell-cell interactions to colonies having fewer, looser cell-cell interactions. The progression of cell morphology proceeded from the characteristic, epithelial features of the parental MCF-7 to the less invasive MCF-7/F, MCF-7A, and MCF-7 A/F to the M33L cells, which expressed the loosest colony morphology and the greatest numbers of filopodia. Although the M33L cells still formed colonies to a certain extent, it appeared that they were progressing toward a mesenchymal-like or fusiform morphology, which is commonly observed with metastatic breast carcinoma cells (8). An epithelial-to-fibroblastic transition has also been observed for other breast carcinoma cells resistant to either Adriamycin or vinblastine (16). Thompson et al. (17) found that breast cancer cell lines that have undergone the epitheliod-to-fibroblastic conversion express a more invasive phenotype, both in vitro and in vivo.

In addition to displaying a more transformed morphology in vitro, the M33L cells also showed increased colony formation when monolayer plating efficiency was tested. The parental MCF-7 cells had an overall plating efficiency of 33.2%, the MCF-7 A/F cells were higher at 52%, and the M33L cells had the highest plating efficiency of 73.2%. Plating efficiency trials were conducted in triplicate using 10% FBS. Increased colony formation is suggestive of a more transformed, more metastatic cell line.

The changes seen in the growth characteristics between the parental MCF-7 cells and the selected MCF-7 A/F cells, along with the in vivo data, show that short-term (acute) treatment with chemotherapeutic agents can induce a metastatic phenotype in previously nonmetastatic cells. The cells do not need to be selected as resistant to the chemotherapeutic agents. All of the observed changes reported here are attributable to a very short-term treatment process, being more homologous to the doses of chemotherapy cancer patients receive, which is cleared from the body in a few days. This may help to explain the ineffectiveness of chemotherapy in many cases. For example, if a portion of the patient’s tumor cells survive the initial doses, elevated doses of chemotherapeutic agents would eliminate these cells; however, the patient could not withstand the toxic effects of the chronic, high doses.

Survival mechanisms used by tumor cells that live through chemotherapy may be either activation of factors in a survival pathway, selection because of constitutive expression of protective factors, or a combination of both. Possible mechanisms for survival of the tumor cells include increases in levels of the multidrug resistance protein and P-glycoprotein (18, 19). These may certainly contribute to the survival of tumor cells but are unlikely to be the sole mechanism responsible for survival. Another factor that may be increased in the treated cells is activated NF-κB, a known inhibitor of apoptosis (20). Activated NF-κB could thereby contribute to the survival of the treated cells by inhibiting apoptosis. Decreases in level or activity of certain factors can also predispose cells toward an increased metastatic potential and ability to survive. ROS are known to down-regulate the activity of the p53 tumor suppressor protein by inhibiting its DNA binding ability (10).

Some of these factors, whose expression are changed because of treatment with chemotherapeutic agents, may also contribute to the transition from epithelial to mesenchymal phenotype. Relatively little is known about the factors that mediate the transition from normal breast epithelium to carcinoma in situ (21). It appears that increased levels of IL-8 release correlate with tumor progression from a nonmetastatic to a metastatic phenotype (5, 22–24). The increased levels of IL-8 released by the chemotherapeutically treated cells may be...
indicative of a series of cellular changes contributing to the transition of a carcinoma in situ to a metastatic carcinoma.

In summary, the results presented in this study suggest that recurrent breast tumors in patients treated with chemotherapy will be more aggressive than recurrent tumors in patients who have not undergone chemotherapy after surgery. If this is the case, it would argue for the need to determine the cellular processes that confer resistance to those tumors that use chemotherapy. Inhibiting such processes and the consequent survival advantage should yield more efficacious chemotherapy protocols. If markers for resistance to different classes of chemotherapeutic agents can be identified, a portion of surgically removed tumors could be screened to determine which chemotherapeutic agents would provide the most effective protocol to administer postoperatively.

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References

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