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12/17/95
The Role of Autocrine-Paracrine Cascades in Breast Tumor Metastasis

Joseph E. DeLarco, Ph.D.

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19990105 125

We had previously shown a very metastatic clone of NRK, RC-20, is non-invasive in an in vitro assay. These cells produce large quantities of an α-chemokine. It was proposed that the neutrophils the chemokine were attracting to the tumor could act as enablers in the metastatic process of these metastatic, non-invasive tumor cells. The aim of the project is to ascertain if metastatic breast tumor cell lines produce and release more α-chemokines than their non-metastatic counterparts. If this is so, these findings would be consistent with the hypothesis that tumor associated neutrophils can act as enablers in the metastatic process. Four human breast cell lines, two estrogen dependent, non-metastatic lines and two estrogen independent, metastatic lines were examined for their expression of IL-8. Conditioned media from these lines were analyzed for their IL-8 protein levels. The constitutive levels of IL-8 are not detectable in the non-metastatic lines, whereas both of the metastatic lines constitutively release measurable quantities of IL-8. In the presence of the inducing agents IL-1β or TNFα, cytokines often found in a tumor’s milieu, the non-metastatic lines produce low levels of IL-8, whereas the metastatic cells are very responsive and release high levels of IL-8.
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Joseph D. Fezza 8/11/98
Signature Date
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INTRODUCTION

Relevance: The standard treatment for breast cancer includes local surgery and/or radiation therapy with or without adjuvant chemo- or hormone therapy. Several significant problems continue to adversely affect the outcome of the current breast cancer therapy regiments, the worst being several years after the initial surgery many of the patients die of disseminated breast cancer. Currently much of the adjuvant chemotherapy targets the growth component of the remaining malignant cells after surgery and irradiation. This chemotherapy not only does not kill all of the remaining tumor cells, but it may also suppress normal populations of rapidly dividing cells, such as those in the bone marrow, the gastrointestinal tract, etc.

In this proposal, it is hypothesized that breast tumors produce chemokines that are the product of an autocrine-paracrine axis to recruit cells of the inflammatory system to the site of the tumor. The mediators released by the inflammatory cells at the tumor site supply the tumor cells, via a paracrine mechanism, with factors that assist the tumor cells in completing the steps required to establish an additional round of metastasis. Preliminary data show the existence of an autocrine-paracrine axis in tumor cells and that autocrine mediators are able to up-regulate the expression of chemokines in both the tumor cells producing the autocrine factors and in the normal parental cells. Inhibition of any of the steps in a tumor cell's autocrine-paracrine cascade should decrease the metastatic potential of the tumor cells and thus decrease the chances of disseminated breast cancer postoperatively. Such therapies as adjuvant to the radiation and/or chemotherapy treatments may increase the survival rate and decrease the chances of
dissemination of breast tumors postoperatively with minimal toxicity or discomfort.

The experiments proposed will test if there is a correlation between the metastatic potential of breast tumor lines and their production of chemokines via an autocrine-paracrine series. If the breast cells are producing autocrine factors enhancing the expression of the α-chemokine in the breast cells they will be identified. The experiments will also ascertain to what extent such autocrine-paracrine sequences can be interrupted by anti-inflammatory drugs. This series of experiments may validate a novel mechanism used by tumor cells to achieve or enhance the potential of their establishing a metastatic cascade. If the results from these experiments show a positive correlation between α-chemokine production by a tumor cell and its metastatic potential, this information may reveal new strategies for diagnosis and illuminate new targets for intervening in the growth and metastasis of breast tumors.

**Background:** The objective of this proposal is to determine if metastatic breast cancer cell lines are producing more α-chemokines than their non-metastatic counterparts. If this is so, the results would be consistent with the hypothesis that the ectopic production of α-chemokines by tumors can contribute to metastatic potential of these cells by recruiting new blood vessels and the tumor associated neutrophils. It is proposed that the tumor associated neutrophils can play a supportive role in the metastases of the tumor cells.

The α-chemokine family is composed of several peptides that are closely related with respect to both structure and function. They are potent chemoattractants for granulocytes -- in particular neutrophils. Historically, IL-8 and the rat equivalent, cytokine-induced neutrophil chemoattractant (CINC), were first purified on the basis of their neutrophil chemoattractant
properties (1,2). The α-chemokines are also inducers of new vessel formation, angiogenesis.

The chemokines’ roles in tumor pathophysiology have been studied for over a decade. The melanoma growth stimulating activity (MGSA) was first isolated from human melanoma cell lines in culture. MGSA was first described as an autocrine growth factor for melanoma cells (3). It was later demonstrated to be identical to GRO-α which was originally isolated as a cDNA from a tumor cell (4). The GROs -α, -β and -γ are α-chemokines and potent chemoattractants for neutrophils. Preliminary screening of eight human melanoma cell lines shows six to be producing significant levels of IL-8. The IL-8 was reported to be a growth factor for these cells, although it did not appear to be a potent mitogen for them (5). In an experimental metastasis model, it was established that the “metastatic” potential of several human melanoma cell clones correlates with their level of IL-8 expression (6). This correlation is consistent within a given series, i.e., A375 clones or the TXM series. In this model the tumors are injected intravenously and the metastatic foci in the lungs are counted. It has been demonstrated for an IL-8 secreting non-small cell lung carcinoma that the in vivo growth rate and the number of spontaneous lung metastases forming is dependent upon the level of IL-8 secreted. Passive immunization of SCID mice bearing this tumor, with a neutralizing anti IL-8 monoclonal antibody, depressed the rate of tumor growth by greater than 40% with an accompanying decline in lung metastases (7). However, this anti-IL8 antibody did not inhibit the in vitro growth of these cells. This demonstrates that an ectopically expressed α-chemokine, IL-8, can contribute to tumor growth and metastasis independent of an autocrine role. A further assessment of the role of IL-8 expression in tumor growth and metastasis was accomplished by transfecting IL-8 into a human melanoma line that is poorly tumorigenic and nonmetastatic in athymic nude mice. Transfected
clones producing IL-8 form tumors faster than the parental line or mock transfected clones; the growth rates of tumors produced by these clones correlate with their level of IL-8 expression. The IL-8 transfected clones are metastatic whereas the parental line is not (8). The data indicates IL-8 expression correlates with both the growth rate and with the rate of tumor metastasis.

BODY

The hypothesis put forth in this proposal was developed from our findings of the early 1990's. These observations indicated that there are cell lines that are highly metastatic, when tested in a spontaneous metastatic model in athymic nude mice, yet non-invasive, when tested in a Matrigel invasion assay. These results are in disagreement with the commonly held paradigm that the invasive potential of tumor cells directly correlates with their metastatic potential. Assuming a cell must invade the extracellular matrix in vivo before it can metastasize, one possible explanation for our results is that the host's cells are supplying a missing function to the non-invasive metastatic cells; such a function(s) could implement the invasive potential of these cells, and thereby enable them to complete the required steps of the metastatic cascade.

The non-invasive, metastatic cells we observed produce α-chemokines which have two basic activities; they are neutrophil chemoattractants and induce new vascular vessel formation i.e., they are angiogenic. It is obvious that the angiogenic properties of the α-chemokines may add to the metastatic potential of a tumor cell producing them. The release of the angiogenic factor by tumors will augment new tumor blood vessel formation, which will increase the supply of oxygen and nutrients to the tumor, thus enhancing the growth and progression of the tumor as
well as supplying a more proximal route for the tumor cells to leave the site of the primary tumor. It was theorized that chemoattractants released by these tumor cells may be serving in a paracrine function, i.e., to recruit cells from their "host's" inflammatory system. In the model proposed here, the ectopic release of chemokines by the tumor cells recruit tumor-associated inflammatory cells -- neutrophils -- that help the tumors to progress and eventually to metastasize. The neutrophils digest and remodel the extracellular matrix (ECM) between the sites from which they extravasate and the tumor with the help of their receptor systems and proteases. Once the neutrophils have arrived at the tumor site, their hydrolases can potentially hydrolyze some of the peptide bonds of the proteins making up the tumor “capsule” or the basement membrane surrounding the tumor. The growth factors released during the remodeling of the ECM, i.e., basic FGF, can potentially act as chemoattractants for both the tumor cells and the endothelial cells. The neutrophils will have digested parts of the ECM through which they have migrated, hence “loosening” the matrix proteins which may facilitate the tumor cell’s migration through the remodeled ECM, thus enhancing their chances of entering the circulation and migrating to distant sites where they can establish metastatic foci.

Based on the above model we hypothesized that the ectopic release of such activities might be a more general phenomena and proposed comparing the levels of α-chemokines released by metastatic breast cell lines with those of non-metastatic breast cell lines. The hypothesis predicts the levels of α-chemokines released by the metastatic cell lines is higher than the levels released by the non-metastatic cell lines.
The proposal had four objectives. They are: (1) To ascertain the degree of correlation between the metastatic potential of human breast lines and the amount of α-chemokine they release. (2) To purify and/or identify the major α-chemokine from the breast cancer cell line releasing the highest level of α-chemokine activity. (3) To determine if the major α-chemokine being expressed by one of these breast tumor cell lines is being enhanced by the ectopic release of autocrine factors produced by the tumor cell line. (4) To test if any of the classes of anti-inflammatory agents can inhibit the expression of any of the elements in the autocrine-paracrine cascade and thereby mitigate the expression of the α-chemokine by the metastatic tumor cells.

Upon examining the conditioned media from four human breast lines, two estrogen dependent, non-metastatic (MCF-7 and T47D) and two estrogen independent, metastatic lines (MDA-MB-231 and MDA-MB-435s), the non-metastatic lines constitutively produce little or no detectable IL-8, not detectable to 1.4 picograms per 1.25 × 10⁵ cells per 24 hours (Table 1.). However, both of the metastatic lines constitutively produce significant levels of IL-8, 280 to 1300 picograms per 1.25 × 10⁵ cells per 24 hours (Table 1.) Upon stimulation of these cell lines with the cytokines, IL-1β or TNF-α, the responses were generally dose dependent. These cytokines were selected as inducing agents since they are often found in tumor milieus. The IL-8 released into their media by the non-metastatic cells in response to these cytokines ranged from not detectable to 122 picograms per 1.25 × 10⁵ cells per 24 hours (Table 1 & Figure 1). The response of the metastatic cells to these cytokines was much greater than for the non-metastatic cells. The induced levels of IL-8 released into the conditioned media ranged from 1195 and
50,450 picograms per 1.25 X 10^5 cells per 24 hours (Figure 2.). Both metastatic lines appear to respond similarly to IL-1β but the MDA-MB-435s cells consistently have a much greater response to TNF-α than do the MDA-MB-231 cells. The differences in the IL-8 released between the non-metastatic and metastatic cells are so great that the data had to be graphed separately in order to see the levels of IL-8 released upon stimulation in the non-metastatic cells. The much larger quantities of IL-8 being released by the metastatic cells either constitutively or upon induction are consistent with the hypothesis of this proposal. These assays were repeated four times with similar results in all cases.

The RNA from these cells was examined using RT-PCR for the presence of IL-8 mRNA using 25 cycles of PCR none of the uninduced cells (controls) produced detectable levels of IL-8 mRNA as measured by visualizing a double stranded PCR product. The only RNAs that gave visible double stranded PCR product from IL-8 mRNA were those derived from the cytokine induced metastatic cells (Figure 3.).

Since the IL-8 is being quantified using two monoclonal antibodies that are specific for human IL-8, it can be said, with a fair amount of confidence, that the α-chemokine activity we are measuring in these ELISA assays is IL-8. To determine if IL-8 is the only major α-chemokine being released by these cells we will do an antibody deletion experiment using a neutralizing monoclonal antibody to human IL-8 and a neutrophil chemotaxis assay. If there is a substantial quantity of neutrophil chemoattractant activity remaining in the conditioned media after titrating the activity using the neutralizing anti-IL-8 antibody, the non-IL-8 activity will be
purified and identified. The purification of the activity will be done using reverse phase and ion exchange HPLC with fractions assayed by means of a neutrophil chemotaxis assay. The identification of this activity will be done, using either immunological means or by sequencing the purified peptide.

If the ectopic expression of IL-8 by tumor cells increases their metastatic potential, it appears the control of its expression might represent a point of intervention in the metastatic process. The activation of NFκB is known to enhance the expression of IL-8 and the IL-8 gene has a NFκB recognition sequence in its 5' sequence. There are several antioxidants that inhibit the activation of NFκB. These will be tested, along with several anti-inflammatory compounds, to determine if, at non-toxic doses, these compounds can modulate the expression of IL-8 in the metastatic breast carcinoma cell lines.

Our previous data on the rat line RC-20 showed that these cells were producing autocrine factors that up regulated their α-chemokine (CINC). This was shown using the sensitive parental line 49F and measuring the CINC released into the conditioned media. We have tried using the conditioned media from MDA-MB-231 cells and are unable to find an increase in the release of IL-8 by MCF-7 cells. This will be done again using MDA-MB-231 conditioned media and partially purified fractions of this media to see if they enhance the mRNA expression of IL-8 in the MDA-MB-231 cells. This may be a more sensitive assay since these cells are more responsive to the modulators of IL-8 used up until now. If there is evidence these cells are releasing an autocrine factor for the enhancement of IL-8 expression, we will attempt to purify
and characterize the factor. If such a factor exists, this information would give us a better understanding of the metastatic mechanisms involved in breast cancer as well as a potential target for prognosis and intervention.

CONCLUSIONS

The current results show that metastatic breast cancer cell lines are producing much more IL-8, an α-chemokine, than the non-metastatic breast cancer lines. There is a correlation between the cell's production of IL-8 and their reported metastatic potential. We have seen that there is also a differential response between the metastatic and non-metastatic cell lines with regards to the IL-8 inducing cytokines IL-1β and TNF-α. The metastatic cells display a strong response to these cytokines, whereas the non-metastatic cells have a minimal response toward these agents. This is consistent with the hypothesis that the ectopic production of α-chemokines by tumor cells increase their metastatic potential. The RNA of these cells was extracted, and the relative quantities of IL-8 mRNA was determined by RT-PCR. After 25 cycles of PCR, the expected amplification product is only detected in the cytokine induced metastatic cells. Under none of the conditions tested did the non-metastatic cells show signs of a PCR product from IL-8 mRNA nor is there product derived from the mRNA isolated from the uninduced (control) metastatic cells.

The four cell lines will be examined for the expression of other α-chemokine activities; if present, they will be identified and quantified; their levels will be compared with those found for IL-8. Further examination of the conditioned media from the metastatic cells will be made to
ascertain if they are producing an autocrine factor that may be instrumental in their IL-8 expression. Antioxidants and anti-inflammatory compounds will be examined to determine if they can lower the level of IL-8 expression in the metastatic cells.

REFERENCES:


APPENDICES:

(Following pages)

- Table 1: IL-8 Levels as Detected by ELISA
- Figure 1: Expression of IL-8 in Non-Metastatic Cell Lines
- Figure 2: Expression of IL-8 in Metastatic Cell Lines
- Figure 3: Digital image of a 2% agarose gel stained with ethidium bromide
### IL-8 Levels as Detected by ELISA

**Table A.**

<table>
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<tr>
<th>Cell Line</th>
<th>Constitutive Expression</th>
<th>IL-1 0.8 ng/ml</th>
<th>IL-1 0.4 ng/ml</th>
<th>IL-1 0.2 ng/ml</th>
<th>IL-1 0.1 ng/ml</th>
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<tbody>
<tr>
<td>MDA-MB-231</td>
<td>284 ±243 pg/ml</td>
<td>11042 ±153 pg/ml</td>
<td>5541 ±107 pg/ml</td>
<td>2060 ±246 pg/ml</td>
<td>1195 ±70.0 pg/ml</td>
</tr>
<tr>
<td>MCF-7</td>
<td>ND</td>
<td>121.2 ±3.89</td>
<td>60.5 ±7.02</td>
<td>34.6 ±12.8</td>
<td>12.2 ±21.1</td>
</tr>
<tr>
<td>MDA-MB-435s</td>
<td>1377 ±180 pg/ml</td>
<td>13232 ±106 pg/ml</td>
<td>8102 ±199</td>
<td>5679 ±77.1</td>
<td>3258 ±235</td>
</tr>
<tr>
<td>T47D</td>
<td>ND</td>
<td>22.2 ±5.84</td>
<td>2.1 ±6.74</td>
<td>ND</td>
<td>ND</td>
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</table>

**Table B.**

<table>
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<th>Cell Line</th>
<th>Constitutive Expression</th>
<th>TNF-a 4 ng/ml</th>
<th>TNF-a 2 ng/ml</th>
<th>TNF-a 1 ng/ml</th>
<th>TNF-a 0.5 ng/ml</th>
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<tbody>
<tr>
<td>MDA-MB-231</td>
<td>360 ±319 pg/ml</td>
<td>8637 ±195 pg/ml</td>
<td>6023 ±432 pg/ml</td>
<td>3057 ±398 pg/ml</td>
<td>2917 ±505 pg/ml</td>
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<tr>
<td>MCF-7</td>
<td>ND</td>
<td>188.1 ±6.76</td>
<td>96.4 ±6.76</td>
<td>47.6 ±8.94</td>
<td>24.2 ±6.76</td>
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<tr>
<td>MDA-MB-435s</td>
<td>557 ±43.9</td>
<td>50450 ±485</td>
<td>33983 ±1170</td>
<td>29850 ±676</td>
<td>17237 ±1351</td>
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<tr>
<td>T47D</td>
<td>1.4 ±3.4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2.35 ±11.7</td>
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ND = not detected

Table 1. These data are the levels of IL-8 (picograms/ml/1.25x10^5 cells) found in the conditioned media by a series of human breast carcinoma lines. MDA-MB-231 and MDA-MB-435s are estrogen independent metastatic lines whereas MCF-7 and T-47D are estrogen dependent non-metastatic lines. The cells were seeded at low density in Costar 6-well plates (3506) in DME containing 10% fetal calf sera. The media was changed after 24h and fresh media added. This media was either DME with 10% FCS which represents the constitutive levels of IL-8 production, or DME with 10%FCS and the stated amount of inducing agent (IL-1β or TNF-α).
Figure 1.

Expression of IL-8 in Non-Metastatic Cell Lines

[Graph showing IL-8 expression levels in different cell lines and treatments]
Figure 2.

Expression of IL-8 in Metastatic Cell Lines

[Graph showing the expression of IL-8 in MDA-MB-231 and MDA-MB-435s cell lines treated with different concentrations of TNF-α.]
Figure 3. Digital image of a 2% agarose gel stained with ethidium bromide. Total RNA was extracted from untreated cells (control) and cells treated with one hour with either 0.1 ng/ml of IL-1 beta or 10 ng/ml of TNF alpha. cDNA was produced by amplification of 1 microgram of RNA with oligo-dT primers. Twenty-five cycles of PCR was subsequently performed using primer sets amplifying either a 289 bp segment of the human IL-1 beta or a 643 bp segment of the human beta actin.

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<td>MCF-7 IL-1 Stimulated</td>
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<td></td>
<td>MB231 Control</td>
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<td></td>
<td>MB231 TNF Stimulated</td>
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<tr>
<td></td>
<td>MB231 IL-1 Stimulated</td>
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<td></td>
<td>T47D Control</td>
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<td></td>
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<td>T47D IL-1 Stimulated</td>
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<td></td>
<td>MB435s Control</td>
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<td></td>
<td>MB435s TNF Stimulated</td>
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<tr>
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<td>MB435s IL-1 Stimulated</td>
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