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<td>Breast cancers can not grow larger than several millimeters in diameter without the ingrowth of new blood vessels (angiogenesis). Recently, Interleukin 8 (IL-8) has been found to be an angiogenic factor. The general hypothesis for this project is that breast cancer cells are capable of producing angiogenic factors (AF) which support tumor growth and metastasis. In order to test our hypothesis, we are examining the expression of IL-8 antigen in human breast tissue using immunohistochemical methods, examining breast cancer cells grown in culture, and establishing an in vivo model in immunodeficient mice. In the first year of this grant, we have examined 29 breast cancers, 8 in situ cancers, and 11 normal breast tissues for IL-8 expression. In addition, blood vessel staining was done using an antibody to vWF. The results confirmed the expression of IL-8 by breast cancer cells. Currently, we are correlating IL-8 expression with angiogenesis. Additional specimens are being processed. We have begun testing the ability of breast cancer cells in vitro to express IL-8. Nine cell lines have been tested. We have found that in breast cancer cells, IL-1 is a potent inducer of IL-8 production. These results support our hypothesis that breast cancer cells produce AF and likely result in tumor growth and metastasis.</td>
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Introduction

Breast cancer is the most common malignancy in women in the United States. The overall survival for all patients with this diagnosis is approximately 70%. Factors associated with poor prognosis include presentation with advanced stage disease or invasion of neural and vascular structures. Recently, it has become clear that patients whose tumors demonstrate a high degree of angiogenesis have a shorter disease free survival, and that these patients are more likely to develop distant metastases [1]. It is also well accepted that tumor can not grow larger than several millimeters in diameter (approximately 10^6 cells) without ingrowth of additional blood vessels [2]. Angiogenesis has been shown to be critical for the growth and metastasis of human breast cancer [3]. Understanding the underlying mechanisms and factors that control angiogenesis will provide not only insights into the basic mechanisms of tumor growth and metastasis, but also will likely provide novel new therapeutic approaches to invasive breast cancer.

Although the relationship between neovascularization and poor outcome in breast cancer is well established, the mechanism of this relationship is not well understood. Undoubtedly, multiple angiogenic factors (AF) are involved in the recruitment and development of tumor angiogenesis [4]. The possible sources of these factors include the tumor cells themselves, tissue cells or infiltrating leukocytes. Previous studies have demonstrated that AF function by inducing vascular endothelial cell proliferation and/or migration. A variety of AF have been recently identified, and include Interleukin 8 (IL-8), Vascular Endothelial Cell Growth Factor (VEGF), and Fibroblast Growth Factor (FGF).

We and others have hypothesized that tumors behave as wounds that do not heal. Normal wounds heal as a result of infiltration of leukocytes and other cells releasing signaling factors which influence the local environment. As a result, a multitude of reactions occur which result in the promotion of wound healing. These reactions include production of fibrin, collagen and other repair factors, as well as an increase in local blood flow to support these activities. This increased blood flow is a result of both local vasodilatation as well as angiogenesis. We hypothesize that in tumors the normal signals that result in healing associated angiogenesis are overamplified, resulting in support for tumor growth and metastasis. Specifically, we hypothesize that the tumor cells themselves, or the infiltrating lymphocytes are capable of producing angiogenic factors.

In order to investigate this, we examined histologic sections of invasive, non invasive (in situ) cancers, as well as normal and non malignant breast tissues to determine if IL-8 antigen was present. In addition, we examined breast tumor homogenates to determine if IL-8 was present in the tissues. To further study the ability of breast cancer cells to produce angiogenic factors, breast cancer cells were grown in vitro under control and stimulated conditions. The production of IL-8 in the cell culture supernatant was measured.

Our Statement of Work is presented in the Appendix. Our original target was to complete all the immunohistochemical data gathering during the first year, and develop the in vitro model during the second year. We have completed accrual of the immunohistologic specimens and are in the process of analyzing our results. Our goal for the in situ hybridization was to directly demonstrate the production of IL-8 by the breast cancer cells. However, our early pilot in vitro data so clearly showed production of IL-8
by breast cancer cells, that we chose to pursue this direction first, and postpone the in situ hybridization. Thus, we have been quite successful in meeting the timeline specified.

**Body: Experimental Methods and Results**

**Specific Aim I- To characterize IL-8 expression in human breast cancer**

**Introduction:** The goals of Specific Aim 1 are to extend and expand our preliminary observations by: 1) demonstrating the presence of IL-8 in invasive breast cancer but not in non invasive cancer or fibrocystic disease, 2) establishing the production of IL-8 in invasive breast cancer by measuring mRNA; and 3) correlating neovascularization with IL-8 expression and patient outcome. These experiments will support our hypothesis that breast cancer cells produce angiogenic factors. Further studies will examine the regulation of this expression (Specific Aims II and III).

**Study IA- To characterize IL-8 antigen distribution in human breast biopsy specimens**

**Experimental Approach:** The purpose of Study IA is to confirm and extend our preliminary observations regarding IL-8 expression in invasive cancer, in situ cancer, and non malignant breast tissue. Tissue samples were obtained from patients undergoing breast biopsy. Where possible, clinical information was obtained, which included patient age, TNM stage, histologic grade, and survival information.

In order to further quantify the expression of IL-8 by breast cancer cells, we assayed the levels of IL-8 in tissue homogenates from tissue samples. Homogenates were also made from non malignant breast tissue when this was available. Although not included in the original Statement of Work, it was felt that this data might be used to quantify the level of expression seen in the tissues.

**Results**

**Immunohistochemistry**

The results of the immunohistochemical evaluation are presented in Table 1. and Figure 1. Only those specimens examined during Year 01 of the grant and reviewed by the PI’s are included in the Table. We have found that for consistency, slides are best processed and read in batches, with standards for staining being used. We have accumulated approximately 30 more specimens which are being processed. This will allow us to reach the target we set in the original proposal.

As observed in our preliminary data, IL-8 antigen expression is clearly seen in the majority of human breast cancer specimens evaluated. There is a lesser degree of inhomogeneous staining seen in the in situ cancer specimens. While some staining was
seen in the non malignant breast tissues, the pattern was luminal rather than cellular in contradistinction to that seen in the cancers.

Tissue Homogenates

Tissue homogenates were prepared, or obtained on 102 specimens (these specimens do not necessarily correspond with the specimens used for immunohistochemical analysis). IL-8 levels were determined by RIA for 52 cancers, 6 normal adjacent breast tissue, 1 fibroadenoma and 1 case of DCIS. The results of 15 representative specimens are presented as ng IL-8/mg protein (see Figure 2). As can be seen, elevated levels of IL-8 are seen in tissue homogenates obtained from some breast cancer specimens, but not from the benign tissues. The level in the single case of DCIS appears to be intermediate between the benign tissues and the invasive cancers. This is being further explored with the remainder of the samples.

Study IB- To demonstrate IL-8 mRNA expression in human breast cancer tissues

Experimental Approach: The purpose of Study IB is to establish the exact source of the IL-8 present in breast cancer specimens. Although the presence of IL-8 antigen on the surface of breast cancer cells in human tissue specimens strongly suggests that the breast cancer cells are producing the AF, direct identification using \( \text{in situ} \) RNA methods would further establish that the breast cancer cells are the source of the AF.

However, through the use of \( \text{in vitro} \) techniques using breast cancer cells in culture we will also be able to determine the source of AF (see Specific Aim II). Our early data suggested that breast cancer cells in culture can indeed produce IL-8. Therefore, for strategic reasons, based on the availability of breast cancer cell cultures, and the start up time anticipated for \( \text{in situ} \) hybridization, it was decided that this portion of the experiment be postponed to Year 02 or Year 03, depending on the \( \text{in vitro} \) results.

Study IC- To Correlate IL-8 expression with neovascularization in human breast biopsy specimens

Experimental Approach: Our central hypothesis is that breast cancer cells produce AF that result in neovascularization. Study IA established the presence of IL-8 in breast cancer tissue, the purpose of this Study is to correlate IL-8 expression with neovascularization. Breast tumor specimens obtained in Study IA were stained with antibody to vWF or CD-31 in order to identify blood vessels (Figure 3). In our experience the anti CD-31 better visualized the vessels and was associated with lower background staining thus facilitating vessel counting. For these reasons, anti CD-31 staining will be used for all future studies. Vessels were counted in the manner described by Weidner et al. Tumor specimens were reviewed by us and a staff pathologist using a dual head microscope. vessels were identified by specific staining. We did not require that a lumen be seen. The slides were scanned, and the area of highest vessel density selected. Both reviewers counted vessels.
Results

Blood vessel staining was identified in the breast cancer specimens. The level of angiogenesis varied between samples. We are currently correlating IL-8 antigen staining with angiogenesis. Clearly, relating the clinical data to the staining patterns will be critical to understanding the regulation of breast cancer growth.

Summary of Specific Aim I

Our results support our hypothesis. Specific staining for IL-8 antigen was identified in the breast cancer specimens. Not surprisingly, there is a lesser extent of IL-8 antigen in the non malignant breast tissue, although the pattern of staining is different. Angiogenic factors are a normal constituent of wound healing. Therefore, it is not unexpected that normal cells express IL-8. However, it is the exaggerated response and the regulation of the angiogenesis that is the focus of this project. Specific Aim II is directed towards understanding this regulation.

Goals and Obstacles for Year 02

Acquiring adequate tissue samples proved more difficult than anticipated. At this point we have established a more reliable tissue bank and access to more breast tissue. This has been organized in such a manner to allow us to obtain corresponding clinical information. This will allow us to better correlate the IL-8 expression with clinical information and angiogenesis (see Study IC). Our goal is to complete accumulation of tissue samples in the early part of Year 02, and complete both the IL-8 as well as the vessel staining. Correlation with clinical data should be completed during this year.

Specific Aim II- To characterize IL-8 expression by breast cancer cell lines in vitro

The studies in Specific Aim I were directed at establishing the presence and correlation of IL-8 with neovascularization in invasive breast cancer, in situ breast cancer and fibrocystic patients. These studies support our hypothesis on the role of IL-8 in neovascularization in breast cancer. We will clearly need to directly demonstrate that tumor derived IL-8 is involved in neovascularization and to identify those factors involved in regulation of this process. To achieve this we propose to utilize a xenograft model of IL-8 POS and IL-8 NEG human breast tumor cells in immunodeficient mice.

In our proposed in vivo model it is anticipated that IL-8 expression is regulated by a variety of factors (e.g. cytokines (IL-1/TNF), estrogens, etc.). Since the in vivo model is more complicated than an in vitro system, we first developed a parallel in vitro system to study the regulation of IL-8 expression by the cultured tumor cells. Selected studies were carried out to determine the ability of stimulants of IL-8 expression to regulate IL-8 expression from both the IL-8 POS and IL-8 NEG human breast cancer cells in vitro. Clearly, the first step in developing this model is identifying and characterizing IL-8 POS and IL-8 NEG human breast cancer cell lines. Initially, two well established cell lines, MCF-7 and BT-20 were used for these studies. These preliminary experiments were
expanded to include 5 additional breast tumor cell lines, and two lines derived from normal breast tissue. Thus, Specific Aim II will provide the foundations for the \textit{in vivo} model which will allow us to directly test our hypothesis.

\textbf{Study IIA- To characterize the expression of IL-8 antigen by cultured human breast cancer cell lines}

\textbf{Experimental Approach:} The breast cancer lines were obtained from the ATCC catalogue. The normal cell line HMEC was obtained from Clonetics (Clonetics Corp., San Diego, CA) Each cell line is grown in specific media as recommended by ATCC. Cell growth for the individual tumor lines is relatively constant between passages. The lines have distinctive morphological appearances which are confirmed visually to ensure healthy growth as well as identity. Cells are passed upon reaching confluence using trypsin.

In order to directly examine the ability of the breast cancer cells to produce IL-8, we performed immunocytochemical analysis. For these immunocytochemical studies, \(10^4\) breast cancer cells were plated on tissue culture slides. In selected studies, these cells were treated with the stimulants as described below. The cells become adherent after 24-48 hours. The slides are then exposed to 1cc of 10% formalin at 40°C for at least 2 hours. They are then stained for IL-8 as described in Study IA except that they do not go through the serial alcohol deparaffinization steps.

\textbf{Study IIB- To quantify IL-8 expression in cytokine stimulated breast cancer cells}

\textbf{Introduction:} Cytokine production by tumor cells has been demonstrated in multiple cancer models. Although in Study IIA we showed IL-8 antigen to be present at the cellular level in cultured breast cancer cells, we expect that IL-8 expression is influenced by other mediators either in an autocrine or paracrine fashion. Therefore we treated cultured breast cancer cells with a variety of agents to examine their ability to regulate AF expression. Completion of this study will allow us to proceed to \textit{in vivo} experiments using stimulants, and blockers of cytokine expression and hormonal manipulation.

\textbf{Experimental Approach:} Cells were prepared as described above, and plated on 6 well sterile culture plates. An adequate number of cells was plated in order to produce confluence at 24 hours. The number of cells used was recorded and varied between cell lines. Data is corrected for \(10^6\) cells. After 24 hours, the culture media was removed, and replaced with the test material in 3 cc of sterile media. Complete, serum containing culture media was used as control. The stimulants used were IL-1 alpha and beta (10 ng/ml), LPS 5ug/ml, TNF alpha and beta (100U/ml), TNF 500U/ml and fibrin. The cell cultures were exposed for 4, 24 or 48 hours. All incubations were carried out at 37°C, under 5% CO\textsubscript{2}. Cells and supernatants are harvested as described above and frozen at -70°C until analysis. Aliquots were analyzed for IL-8 by RIA.
Results

The results of Study IIB are presented in Table 2 and expressed as the stimulated index (ratio of stimulated over media control). The basal (unstimulated) levels in ng/ml are also included in the table. Baseline IL-8 expression was demonstrated by many of the cell lines. The response to the stimulating factors was somewhat variable between the cell lines. However, IL-1 alpha and beta were consistently found to be a potent inducers of IL-8 expression.

Summary of Specific Aim II

In Specific Aim II, we have demonstrated directly the production of IL-8 by breast cancer cells. Further, we have begun to explore the regulation of this expression. Clearly, cytokines such as IL-1 play a significant role in inducing angiogenic factor expression. This information will allow us to develop our in vivo model, and plan the studies that will explore inhibition of tumor growth in the immunodeficient mouse model.

Goals and Obstacles for Year 02

Our in vitro work is well ahead of schedule, and has been quite successful. As a result, we have been able to expand the number of cell lines being tested. In addition, if time and resources allow, we will expand our survey of angiogenic factors. Co-culture experiments are also planned. We are considering the feasibility of co-cultures with human vascular endothelial cells.

Specific Methods:

IL-8 Immunohistochemistry

Immunohistochemistry is performed by indirect immunoperoxidase staining. 8 um paraffin embedded tumor sections are deparaffinized in xylene and rehydrated in graded alcohol (100%, 95% and 50%). To inhibit endogenous peroxidases, the sections are immersed in methanol containing 0.01% hydrogen peroxide at 4°C for 15 minutes. Sections are allowed to air dry and then blocked with normal goat serum (Vector, Burlington, CA) at room temperature (RT) for 1 hour. The sections are then washed 3 times with phosphate buffer saline (PBS, pH 7.4) and affinity purified rabbit or chicken anti-human IL-8 (1/500 - 1/9000 dilution in PBS with 0.5% bovine serum albumin), chicken anti-human IL-8 preabsorbed with recombinant human IL-8 specificity control, or buffer control are applied to the sections overnight at 4°C.

After the incubation period, biotinylated goat anti rabbit, or rabbit anti-chicken antibody (Biotin SP Affinipure F(ab)2 fragment, (Jackson Immunoresearch Labs, Inc., West Grove, PA); 1/100 dilution in PBS with 0.5% BSA is applied to the sections and allowed to incubate for 1 hour at RT. Sections are washed 3x with PBS in between each
of the following steps. HRP-streptavidin (Zymed, San Francisco, CA) at a 1/250 dilution in PBS is applied to the sections and incubated at RT for 1 hour. The sections are incubated sequentially with 3 amino-9-ethylcarbazole in 0.1M sodium acetate buffer (pH 5.0) and 0.03% H$_2$O$_2$ for 30 minutes at RT. Sections are then counterstained in Mayer's hematoxylin (Sigma, St. Louis, MO) for 10 minutes, extensively washed in H$_2$O$_2$, and dipped in dilute ammonium hydroxide and mounted in crystal mounting solution (Biomeda, Foster City, CA).

The slides were reviewed by us and by a staff pathologist to confirm the histologic diagnosis, and to grade the degree of staining. Staining was graded as 0-4+. Nonspecific, species matched IgG was used as a negative control.

**Tissue Homogenates**

Tumor and control tissue samples were stored in a -70°C freezer until processing. They were then thawed, weighed, placed in 1 ml of phosphate buffer saline (PBS) per 0.25 grams of tumor or control tissue, and homogenized in a tissue homogenizer. Triton (0.01 percent final concentration) was then added to the supernatants, which were then re-homogenized. The homogenate was centrifuged twice at 4°C at 10,000 g. The supernatants were then aliquoted and frozen at -20°C.

In addition, residual tissue homogenates from specimens sent to pathology for ER/PR analysis were obtained. Aliquots were frozen at -70°C and then analyzed by RIA for IL-8, and for protein level using the Bradford reagent (see above). ER and PR values were available for these samples and will be correlated with IL-8 levels.

**Cytokine Analysis**

Cell culture supernatants are analyzed by an IL-8 specific RIA developed in our laboratory. Samples (100 ul) are incubated (250°C, 2h) with chicken anti-IL-8 (100 ul) diluted (1:4,000) in RB buffer (1% BSA, 0.1% Triton X-100 in PBS). Chicken antibody to Interleukin 8 (IL-8) was prepared by intramuscular injection of 100 ug of recombinant human IL-8 (77 amino acids, PreproTech Inc., Rocky Hill, NJ) prepared in Hunger's PÄ Titer Max (CytRx Corp., Norcross, GA). Egg yolks containing antibody were processed as previously described. Antibody titer and specificity were assessed by double immunodiffusion and immunoelectrophoresis, respectively. Human 125I-IL 8 (NEN products, Boston, MA) diluted in RB buffer (70-80,000 CPM/ml) is added (100 ul) and the reaction mix incubated overnight (40°C). Immune complexes are precipitated by adding (100 ul) affinity purified goat anti-chicken IgG coupled microspheres (Kirkegaard & Perry Lab Inc., Gaithersburg, MD) diluted in RB buffer (1:20). After incubation (250°C, 2h) beads are pelleted (2500g for 15 min.), blotted and counted (gamma counter 1 min.). Samples are quantified by reference to a standard curve constructed using rIL-8 standards (0.039-10 ng/ml). Samples were assayed in duplicate and results are expressed as the mean +/- S.D. All resulting data are expressed as pg of AF per 10^6 cells. Determination of statistical significance for AF expression between various culture conditions and times will be done by ANOVA analysis (JMP software program). For ease of analysis, the data were also expressed as cytokine index or CI = cytokine concentration (molar) divided by antagonist concentration (molar) x 10^3.
Conclusions

We have been extremely successful in pursuing the studies outlined in the application. Our immunohistochemical results to date have confirmed our preliminary data which showed IL-8 antigen expression in human breast cancer. Early neovascularization data confirms the presence of neovascularization in the breast cancer specimens. Clearly, correlating neovascularization with IL-8 expression will provide an important link in the data. Likewise, correlation with clinical data will be an important step in understanding the significance of our observations.

Our in vitro data has been extremely encouraging. Not only have we been able to validate our preliminary data demonstrating IL-8 production by tumor cells, but by expanding our number of cell lines and stimulants, we have shown that different cell lines respond differently to the various stimulants. IL-1 emerges a consistent and strong stimulant of IL-8 expression. This information will be key in establishing the in vivo model in Years 03 and 04. We have submitted our work to the Society of Surgical Oncology for presentation March 1996 (see Abstract 1 in Appendix).

We feel that the work done in the first year of this project has supported our central hypothesis. Breast cancer cells are capable of producing angiogenic factors. The implication of this observation is that development of agents that control angiogenesis will likely lead to treatment resulting in decreased tumor growth and metastasis.

During the latter part of the first year, we developed a more efficient and inexpensive way to obtain breast tumor specimens. This redistribution of resources allowed us to expand the in vitro model to include a number of additional breast cell lines. We have observed variability between the cell lines in the ability of cytokines to regulate IL-8 expression. In addition, the tumorogenicity of the various cell lines may vary in the immunodeficient mouse model. Therefore, having a larger group of cell lines to choose from will increase our ability to develop the in vivo model.

Further, although this project was directed at examining the role of IL-8 in particular, a variety of other AF exist. If time and resources allow, we plan to examine other angiogenic factors such as VEGF and basic FGF in our system. Our original proposal also did not look at the role of hormones or fibrin in our system. We are currently seeking additional support to extend our experiments in this area. Additionally, we have submitted a proposal on a related project examining the role of fibrin on angiogenic factors.

Our early successes have led to several “spin-off” projects. We are beginning to study other angiogenic factors such as VEGF in both the immunohistochemical studies as well as in the in vitro model. We have also generated preliminary data examining antagonists such as IL-1 receptor antagonists (IL-1 RA). If our hypotheses are correct that cytokines such as IL-1 are inducing expression of angiogenic factors, then the role of IL-1 RA will prove important.

Other workers in our laboratory have started similar projects similar to this one in the fields of prostate cancer and colo-rectal cancer. Preliminary work in prostate cancer has been submitted to the American Urologic Association (see Abstract 2 in Appendix). Additional independent funding is being sought for these projects.
Bibliography


Appendix

Statement of Work

Task 1 Year 1- To characterize IL-8 expression in human breast cancer

Study IA- To characterize IL-8 antigen distribution in human breast biopsy specimens
Study IB- To demonstrate IL-8 mRNA expression in human breast cancer tissues
Study IC- To Correlate IL-8 expression with neovascularization in human breast biopsy specimens

Task 2 Year 2- To characterize IL-8 expression by breast cancer cell lines in vitro

Study A- To characterize the expression of IL-8 antigen by cultured human breast cancer cell lines
Study B- To quantify IL-8 expression in cytokine stimulated breast cancer cells
Study C- To quantify IL-8 expression in co cultures of MCF-7 and BT-20 cells

Task 3. Years 3-4- To characterize IL-8 antigen expression and neovascularization in human breast cancer cells grown as tumors in nude mice

Study A- To demonstrate IL-8 antigen expression in subcutaneously implanted human breast cancers
Study B- To establish a dual tumor model using MCF-7 and BT-20 cells
### Table 1

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<th>4+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasive cancer</td>
<td>3</td>
<td>2</td>
<td>8</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>In situ cancer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benign breast</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table legend: Results of immunohistochemical staining for IL-8 on 29 samples. Rating scale: 0 = no staining; 1+ = minimal staining; 2+ = moderate staining; 3+ = strong staining; 4+ = intense staining.

### Table 2

Cytokine stimulation of IL-8 Expression in Human Breast Cancer Cells

<table>
<thead>
<tr>
<th></th>
<th>MCF-7</th>
<th>BT-20</th>
<th>MDA-231</th>
<th>ZR-75-1</th>
<th>T-47D</th>
<th>HBL-100</th>
<th>HS-578</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.30</td>
<td>1.84</td>
<td>12.20</td>
<td>0.73</td>
<td>0.91</td>
<td>0.32</td>
<td>9.80</td>
</tr>
<tr>
<td>TNF-β</td>
<td>1.00</td>
<td>1.06</td>
<td>1.59</td>
<td>0.68</td>
<td>0.67</td>
<td>0.09</td>
<td>4.10</td>
</tr>
<tr>
<td>IL-1α</td>
<td>0.95</td>
<td>1362</td>
<td>127</td>
<td>107</td>
<td>3.36</td>
<td>0.12</td>
<td>124</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.81</td>
<td>1292</td>
<td>144</td>
<td>137</td>
<td>1.00</td>
<td>0.24</td>
<td>119</td>
</tr>
</tbody>
</table>

**Basal IL-8 Expression (ng/ml)**

|       | Media | 0.45 | 0.31 | 1.5  | 0.15 | 0.15 | 0.40 |

Table 2: Stimulation index (IL-8 expression in ng/ml stimulated/IL-8 expression in ng/ml in media control). *The lower portion of the table lists the basal (unstimulated media control) IL-8 expression in ng/ml.
Figure 1

HUMAN BREAST TUMORS IMMUNOHISTOCHEMICALLY STAINED FOR IL-8 USING ANTI-IL-8 ANTIBODY

HUMAN BREAST TUMORS IMMUNOHISTOCHEMICALLY STAINED WITH NON IMMUNE IGG

IMMUNOHISTOCHEMICAL STAINING OF HUMAN BREAST TUMORS GROWN IN IMMUNODEFICIENT MICE AND STAINED WITH ANTI IL-8 ANTIBODIES

IMMUNOHISTOCHEMICAL STAINING OF HUMAN BREAST TUMORS GROWN IN IMMUNODEFICIENT MICE AND STAINED WITH NONIMMUNE IGG
Figure 2: RIA for IL-8 demonstrating expression of IL-8 antigen in a number of breast cancers, with no expression seen in non-malignant tissue. DCIS appears to be intermediate.
Figure 3: Demonstration of anti vWF (A) and control IgG (B) immunohistochemistry for identification of vessels in breast cancer specimen. As can be seen in this figure, both large and small vessels are easily visualized using the anti vWF antibody.
ABSTRACT 1

CYTOKINE REGULATION OF ANGIOGENESIS FACTOR EXPRESSION IN HUMAN BREAST CANCER. SH Kurtzman, L Miller, K Anderson Y Wang, DL Kreutzer, Univ of CT Dept of Surgery, Farmington, CT

Currently, little is known about the regulation of angiogenic factors (AF) expression in breast tumor cells. To fill this gap in our knowledge, we hypothesize that in breast cancer 1) breast cancer cells produce AF, and 2) this expression is regulated by cytokines. In order to investigate this 7 human breast cell lines were grown in continuous culture and exposed to the cytokines IL-1α, IL-1β, TNFα, TNFβ or control media. Resulting cell culture supernatants were harvested and assayed by RIA for IL-8, a known AF. The results are presented in Table 1 as the ratio of the levels of IL-8 in the stimulated over control condition.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>MCF-7</th>
<th>BT-20</th>
<th>MDA-231</th>
<th>ZR-75-1</th>
<th>T-47D</th>
<th>HBL-100</th>
<th>HS-578</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
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<tr>
<td>TNF-α</td>
<td>1.30</td>
<td>1.84</td>
<td>12.2</td>
<td>0.73</td>
<td>0.91</td>
<td>0.32</td>
<td>9.80</td>
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<tr>
<td>TNF-β</td>
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<td>0.67</td>
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<td>IL-1β</td>
<td>0.81</td>
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<td>144</td>
<td>137</td>
<td>1.00</td>
<td>0.24</td>
<td>119</td>
</tr>
</tbody>
</table>

These data clearly indicate that 1) IL-1 appears to be an extremely potent and consistent inducer of IL-8 in a number of breast cancer cell lines and 2) different tumor cell lines respond differently to individual cytokines (high vs. low responders.) Thus, human breast cancer cells are capable of producing AF in response to cytokines such as IL-1 and TNF. Since IL-1 and TNF are produced by tissue cells and leukocytes within the tumor microenvironment, they likely contribute to angiogenesis and resulting tumor growth and metastasis. This information will allow us to design future therapeutic agents targeted at AF expression and their receptors that will aid in the treatment of patients with breast cancer.

To be presented at the Society of Surgical Oncology Spring Meeting, Atlanta, GA March 1996
Abstract 2

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Cytokine Regulation of Angiogenesis Factors in Human Prostate Cancer. Fernando A. Ferrer, Lauri J. Miller, Ramez I. Andrawis, Peter C. Albertsen, Scott H. Kurtzman, Donald L. Kreutzler, Farmington, CT (Presentation by Dr. Ferrer)

Introduction Recently, we have demonstrated immunohistochemically the presence of the potent angiogenesis factor (AF), Interleukin-8 (IL-8) in human prostate cancer (PC). In these studies IL-8 antigen was noted to be consistently associated with tumor cells. Based on this observation, we hypothesized that PC tumor cells 1) produce AF, including IL-8, and 2) that their expression is regulated by cytokines present in the tumor microenvironment. To test this hypothesis, a human PC tumor cell line was exposed to the key cytokines Interleukin-1 (IL-1) and Tumor Necrosis Factor (TNF) in vitro and the resulting culture supernatants were assayed for IL-8 expression.

Methods The PC cell line DU-145 was grown in continuous culture and exposed to cytokines IL-1α, IL-1β, TNFα, TNFβ (10ng/ml), as well as a control media. Cell culture supernatants were harvested at 24 Hrs and analyzed by radioimmunoassay for IL-8. The resulting data was initially expressed as ng/ml and then normalized as a stimulation index (S.I.) which represents the ratio of stimulated divided by control IL-8 levels.

Results Our results indicate that IL-1α and IL-1β are potent and consistent inducers of IL-8 in the DU-145 PC cell line. Surprisingly, TNFα and TNFβ induced little to no significant IL-8 expression (see Table).

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Media</th>
<th>TNF-α</th>
<th>TNF-β</th>
<th>IL-1α</th>
<th>IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. L. mean (SEM)</td>
<td>n=9</td>
<td>n=9</td>
<td>n=9</td>
<td>n=9</td>
<td>n=8</td>
</tr>
<tr>
<td></td>
<td>1.00 ± (0.23)</td>
<td>1.90 ± (0.48)</td>
<td>1.13 ± (0.34)</td>
<td>16.73 ± (5.38)</td>
<td>11.85 ± (2.12)</td>
</tr>
<tr>
<td>p value ≤</td>
<td>0.1050</td>
<td>0.7540</td>
<td>0.0100</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>IL-8 range (ng/ml)</td>
<td>0.3 - 4.3</td>
<td>1.1 - 4.8</td>
<td>0.7 - 1.9</td>
<td>6.0 - 40.3</td>
<td>6.3 - 44.8</td>
</tr>
</tbody>
</table>

Conclusion In vitro, cytokines such as IL-1 are capable of regulating IL-8 expression in human PC. Cytokines such as IL-1 are known to exist within the tumor microenvironment and by inducing AF, likely contribute to angiogenesis, tumor growth and metastasis. Further in vivo studies will clarify the clinical significance of this observation and suggest treatment strategies targeted at AF and their inducers.
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