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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)* This year 01 annual report describes the study, to date, on the role of the cytokine Growth-Regulated Oncogene (GRO) protein in human breast cancer. **Key accomplishments:** 1) the establishment of a tumor bank, 2) Immunohistochemical analysis showing the expression of GRO and its receptor, CXC-R2 in human breast cancer patient samples, 3) Quantitation of GRO levels in human breast cancer tissue sample homogenates and correlation of these levels with the expression of nine other cytokines and cytokine receptors as well as the levels of the estrogen/progesterone receptor expression, and 4) using human mammary epithelial and tumor cell lines we described the constitutive and inducible expression of not only GRO, but the closely related cytokine IL8. **Conclusions:** 1) GRO is expressed by breast cancer tumor cell and its receptor is expressed not only on tumor cells themselves, but also on vascular endothelial cells. This suggests that GRO may be involved in cancer cell proliferation and may be important in promoting vascularization by vascular endothelial cell activation. 2) Analysis of breast cancer tissue homogenates shows a significant correlation with the closely related cytokine, IL8. Furthermore, GRO expression correlates with the GRO/IL8 inducer IL-1 and the IL-1Receptor 1. This suggests that tumor derived GRO and IL8 may result from tumor cell activation by IL-1. Additionally, GRO expression is inversely correlated to estrogen receptor expression, suggesting GRO expression is related to an unfavorable breast cancer patient disease outcome. 3) Our cell culture experiments support the data form the patient tumor samples showing IL-1 is a potent inducer of both GRO and IL8. **Summary:** These data clearly demonstrate the importance of the relationship between GRO and IL8, but more importantly, demonstrates the inductive ability of IL-1 to regulated expression og GRO and IL8. As such, we have expanded this study to not only define the role of GRO, but also to examine the association of GRO with IL8 and IL1. We have described manuscripts in preparation and grants applied for.

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INTRODUCTION

Background. It is now well recognized that tumor growth (i.e. proliferation) and angiogenesis (i.e. the formation of new blood vessels within tumors) are critical to tumor growth and metastasis, and therefore important in cancer disease progression. One of the key mechanisms for regulating tumor cell proliferation and tumor angiogenesis is by networks of chemical signals, which interact with specific receptors on cells. These chemical signals are referred to as cytokines. Cytokines and cytokine receptors can be loosely grouped based on their functional activity (i.e. mitogenic cytokines or growth factors induce proliferation and angiogenesis factors modulated new blood vessel formation). The cytokine Growth-Regulated Oncogene (GRO) protein is a member of the CXC family of cytokines (based on conserved cystine residues) of related cytokines which are best know for their ability to induce a migrational response for various cell types. These chemotactic cytokines (or chemokines) are now known to also be involved in the modulation/induction of proliferation and angiogesesis (see below). Members of this family include; GROα/MGSA, GROβ, GROγ, IL8, NAP-2, ENA-78 and granulocyte chemoattractant protein-2. The receptors for these chemokines were originally described as IL8-R1 and IL8-R2, but are newly designated CXC-R1 and CXC-R2. IL-8 binds to both receptors with high affinity, whereas GROα /MGSA, GROβ, GROγ, and NAP-2 bind with high affinity to IL8-R2/CXC-R2 (K_d 0.2-2.5nM) and low affinity to IL8-R1/CXC-R1 (K_d200-500nM), (1-5). Therefore, regulation of tumor cell proliferation and angiogenesis occurs *via* the expression and subsequent receptor interaction with cytokines. While very little has been described regarding the role of CXC cytokines in human breast cancer, there is evidence suggesting that this family of cytokines play an important role in other tumor systems.

CXC Cytokines (GRO and IL-8) and Receptors in Cancer: Recently, the CXC cytokine family members, Growth-Regulated Oncogene/Melanoma Growth Stimulatory-Activity – Protein (GRO/MGSA), and Interleukin 8 (IL-8) have been demonstrated in other tumor systems to be: ***a)*** an autocrine tumor cell growth factor for malignant melanoma, liver and pancreatic cancer and ovarian cancer (6-11), ***b)*** an in vitro (macrophage derived) and in vivo (the rat cornea model) angiogenesis factor (12-14), ***c)*** a potent *in vitro* migration factor for neutrophils and breast cancer cells (15-17), and ***d)*** able to support and augment melanoma and ovarian cancer tumor formation in the xenograft nude mouse model (11, 18-21). **Additionally IL8 is;** ***a)*** predictive indicator of therapeutic response and prognosis of patients with recurrent breast cancer (22), ***b)*** shown by ourselves, that IL8 is expressed by tumor cells and the receptors are expressed on both tumor cells and vascular endothelial cells from breast and neuroblastoma patient tissue samples (23-24). Furthermore, IL8 expression is inversely correlated with ER/PR expression in human breast cancer, suggesting IL8 expression is associated with poor prognosis (25), ***c)*** it has been shown that inhibition of the common GRO/IL8 receptor, CXC-R2 by antisense oligonucleotides inhibits tumor cell proliferation in vitro and in the xenograft nude mouse model (26).

This data suggests that tumor cell derived (TCD) CXC cytokines (GRO and/or IL8) may play a significant role in human breast cancer tumor growth and therefore understanding the role of these cytokines will potentially lead to exciting new therapeutic approaches in the fight against breast cancer.

Hypothesis: Based on our own preliminary data and a recent literature review, we hypothesize that in human breast cancer (HBC), the local expression of GRO and the GRO-Receptor (CXC-R2) by tumor cells supports tumor growth and metastasis by promoting tumor proliferation directly (as an autocrine growth factor), but also augments tumor growth indirectly by supporting tumor angiogenesis *via* expression of CXC-R2 on vascular endothelial cells (as a paracrine angiogenic factor). The duel activation of these essential protumorgenic pathways by CXC cytokines and the interaction/cooperativity between subpopulations expressing these cytokines and receptors is critical for successful tumor growth and metastasis.

Specific Aims [see Statement of Work (Verbatim) in Appendices]:

1) To characterize GRO expression and correlate GRO Receptor (CXC-R2) expression of human breast cancer tumor tissue with major diagnostic and prognostic indicators.

2) To characterize human breast cancer tumor cell activation (*i.e.*, GRO expression and proliferation) using *in vitro* models.

3) To determine the role of GRO/CXC-R2 expression in tumor growth and angiogenesis in a nude mouse xenograft model of human breast cancer.

BODY

The foundation of this training grant is to study the role of GRO in human breast cancer and importantly,

enhance the career development potential for ongoing breast cancer research by this post–doctoral fellow (A.G. Pantschenko). The research and training aspects of this grant for year 01 will be described in the sections to follow.

KEY RESEARCH ACCOMPLISHMENTS

1. EXPRESSION OF GRO AND CXC-R2 IN HUMAN BREAST CANCER PATIENT TUMORS (MONTH 1-12).

a. Established a breast tumor tissue bank for histology, immunohistochemisty (IHC), Western blot (WB) molecular biology (RT-PCR) and ELISA.

- Archival paraffin tissue blocks (n>60) with pathology report used for histology and IHC.
- Homogenized breast cancer tumor tissue clinical laboratory samples (n=171) measured for levels of estrogen and progesterone receptor were used to measure cytokine levels by ELISA and for WB to evaluate receptor expression in breast cancer patient samples.
- **Difficulties:** We have not been able to collect sufficient numbers of fresh (snap frozen and/or ethanol fixed) breast tumor tissues for our molecular quantitation studies (RT-PCR).
- **Resolution:** We established new protocols to obtain fresh and ethanol fixed tissues with Drs. Kurtzman, Deckers, and Zarfos (clinicians treating breast cancer patients at UCHC).

b. Immunohistochemistry for GRO, CXC-R2, VWF, and CD-31.

- GRO: We have processed approximately 60 slides to examine the expression of GROα, β, and γ.
- **Expanded Study:** In the grant, we proposed to only examine the expression of GROα, however we felt that the other two forms of GRO should be examined since the levels of the three forms may vary and the receptor affinities are different and potentially the function may be different depending on the cell/tissue type. We have experimented with antibodies that recognize each of the three forms individually, as well as antibodies that recognize all three forms collectively (GRO-Total).
- **Unexpected:** Reagents advertised by Santa Cruz Biotechnology as specific for the three different forms of GRO were not. We demonstrated to the company that the reagents cross-reacted to the peptide controls. We have also exhausted the commercially (Pharmigen and R&D) available reagents to distinguish between the three forms.
- **Resolution:** Based on these efforts, it is clear that the current sate of the art for IHC detection of GRO expression is based on using anti- GROα antibody, which we are currently using.
- CXC-R2: We have established the IHC assay to evaluate receptor expression.
- VWF and CD-31: We have established the IHC assay to measure microvessel density. We found better results using CD-31 versus VWF.

c. Measured levels of GRO in human breast cancer tissue homogenates by ELISA and correlated the levels of GRO with the expression of other cytokines and tumor markers.

- Developed ELISA to measure GRO_{TOTAL} and a separate protocol for an ELISA to measure GROα.
- ELISA for GRO_{TOTAL}: 97/171 patient tumor homogenates were assayed for expression of GRO_{TOTAL} **(See Table 1, Appendix)**. These same homogenates were also assayed for total protein, IL8, IL-1α, IL-1β, IL-1RA, IL-1R1, IL-1R2, TNF-R1, TNF-R2, ER, and PR. The levels of expression were examined by correlation analysis to compare association of expression levels. Importantly, we found a very high significance between IL8 and GRO expression ($p \leq 0.002$). This is not surprising since they both bind to CXC-R2 receptor. Other significant correlations for GRO include IL-1α ($p \leq 0.001$) and IL1-R1 ($p \leq 0.001$). Interestingly, like IL8, GRO expression is inversely correlated with ER expression suggesting poor prognosis.
- ELISA for GROα.: We have used the same commercially available reagents as described above for Immunohistochemical detection of GRO to develop our ELISA for GROα, β, and γ. We focused on developing an ELISA to distinguish the three forms of GRO by used specific peptides linked to BSA to enhance epitope presentation and thereby enhance sensitivity and specificity of the GRO

ELISA. **Unexpected:** as described above we discovered that reagents advertised by Santa Cruz Biotechnology were not specific for distinguishing the three GRO forms. **Resolution:** We have developed an ELISA protocol to measure GROα using reagents from R&D Systems. **Therefore, we are currently using an ELISA that measures GRO_{TOTAL} [GROα, β, and γ (PharMingen reagents)] and another ELISA that measures GROα (R&D reagents).**

d. Developed Western Blot protocol to evaluate expression of the GRO receptor (CXC-R2) in human breast cancer tissue homogenates and cell culture lysates.
   - Although we can demonstrate CXC-R2 expression by immunohistochemistry and specificity (no-reactivity using a control antibody) in the IHC assay, we need conformation of receptor expression by an alternate method. Western Blot of HBC tissue homogenates allows us to demonstrate CXC-R2 by immunoassay and can confirmed by molecular weight analysis. We have recently developed CXC-R2 Western Blot and are currently running HBC homogenates and cell line extracts.

2. EVALUATE GRO/CXC-R2 EXPRESSION (CONSTITUTIVE AND INDUCIBLE) IN HUMAN BREAST CELL LINES *IN VITRO* (MONTHS 2-10).

a. Human breast cell lines were evaluate for expression of GRO_{TOTAL} under unstimulated (media alone) and stimulated (TNF, LPS and IL-1) conditions **(see Table 2 and 3 in Appendix)**.
   - **Expanded Study:** Based on our observation (as shown in table 1) describing the strong correlation between GRO and IL8 expression in human breast cancer tissue homogenates and since both cytokines share the same receptor, we expanded these studies to include the characterization of IL8 as well as GRO (see Tables 2 and 3).

b. Assayed cell culture supernatants and cell lysates for expression of GRO_{TOTAL} and IL8 by ELISA.
   - Overall, constitutive expression (media) of GRO_{TOTAL} and IL8 was low (Table 2). However, when the cells were stimulated, in particular with IL-1, there was a marked increased expression for both GRO_{TOTAL} and IL8. Importantly, this increased expression tends to be related to the estrogen-independent cell lines (BT-20 and MDA-MB-231) suggesting that the more aggressive in vitro tumor cells are associated with increased expression of GRO_{TOTAL} and IL8. The induction of expression can best be observed by using the stimulation index [unstimulated (media) / stimulated (cytokine)] as shown in table 3. We are currently drafting a manuscript using this data**. Draft of Publication #1 in Appendix** "In Vitro Demonstration of Breast Cancer Tumor Cell Subpopulations Based on the Expression of Interleukin-8".
   - We have assayed cell lysates by ELISA for expression of IL8 and found little difference between the supernatant levels versus the levels in the cell lysates **(see draft of publication #1 in appendix)**. While we have generated, collected and stored the samples, we have yet to assay the lysates for GRO_{TOTAL} expression by ELISA.
   - This *in vitro* data is the foundation for our future continuing studies examining the role of GRO/IL8 in proliferation (year 02) and in tumorigenesis and angiogenesis with the nude mouse xenograft model (year 03).

b. Assay of cell culture supernatants and cell lysates for expression of CXC-R2 by Western Blot.
   - As described above, we have recently developed the Western Blot protocol for CXC-R2 and are currently using this method to characterize receptor expression.

c. Expanded Study:The function of the IL-1 family of cytokines in the regulation of GRO/IL8 expression.
   - Our studies of human breast cancer homogenates (table 1) and our in vitro studies using the human breast cell lines (tables 2 and 3), supports the hypothesis that IL-1 is an important regulator of GRO and IL8 induced expression in human breast cancer. Therefore, it is clear that a greater understanding of the cytokine networks that are involved in the regulation and expression of GRO/IL8 and their receptors is important, not only in the understanding of tumor growth and angiogenesis, but also may potentially lead to exciding new treatments in the fight against breast cancer. As such, we have also investigating the expression of the IL1 family of cytokines in human

breast cancer and are preparing a manuscript. **Draft of Publication #2 in Appendix** "Expression Of Interleukin 1 Family of Cytokines and Receptors In Human Breast Cancer"

KEY TRAINING ACCOMPLISHMENTS

1. ACADEMIC TRAINING ACCOMPLISHMENTS.

- Seminars, presentations, and lecture series. The University of Connecticut Health Center is a well recognized and active medical/basic research center with many opportunities for participation and interactions with scientists in various fields, including; immunology, cancer biology and cell biology. Attending these types of presentations is most helpful is maintaining a high level of knowledge required to successfully move forward research in human breast cancer and important in career development.
- Interaction with clinicians, faculty, and other scientists. The unique working environment at UConn is very supportive allowing for interaction with other members of the scientific community that are always willing to help. This is important for the discussion of scientific concepts, principals and techniques. As such, these assets make for a stronger ability to contribute to the area of breast cancer research.
- Research focus. My previous professional research experience and doctor dissertation was primarily related to immunology, normal mammary gland biology, and cell biology. This fellowship has allowed me to focus and gain knowledge in the field of human breast cancer.

2. TECHNICAL TRAINING ACCOMPLISHMENTS.

- Cancer biology and pathology. Research in human breast cancer has strengthened my background in the field of cancer biology. Similarly, a large focus of the study relies on the ability to distinguish between different types of human breast diseases and I have had to learn breast pathology and histopathology. Additionally, I have worked closely with pathologists and histotechnologists to learn clinical pathology and human tissue sample acquisition and preparation.
- Immunohistochemistry and cytokine biology. I have had to develop and use techniques to demonstrate cytokine presence in human breast cancer patient samples by IHC and techniques related to cytokine biology
- Molecular biology. Techniques utilizing molecular biology are a new addition to my list research tools that are being applied to my cancer studies. While we are still in the process of acquiring samples related to this study of human breast cancer using a molecular biology approach, I have been developing techniques to characterize cytokine induction and expression using RT-PCR. Along the way I have learned isolation of RNA, primer design, PCR, DNA quantitation, and electrophoresis. Additionally, we are developing methods for transfection of cDNA for cytokines and marker proteins [green florescent protein (GFP)] which will be useful for our year 03 studies using the nude mouse xenograft model.

3. CAREER/ADMINISTRATIVE SKILL DEVELOPMENT

- Publications/Grant Writing. As part of my post-doctoral position, I have been involved with the writing of manuscripts and grants. These efforts have improved by communication skills and hopefully, some of the grant proposals will attract funding which leads to career development.
- Administrative. As a post-doc, I have also had to work with administrative issues related to doing research. This would include writing reports for the Animal Care and Usage Committee, Institutional Review Board for using human anatomical substances, the grants/research office for grant submissions and preparing budgets. All these will surely prepare me for a successfully career as an academic researcher.

REPORTABLE OUTCOMES

1. MANUSCRIPTS.

- **The expression and distribution of GRO protein and receptor (CXC-R2) in human breast cancer.** Alexander G. Pantschenko, Lauri J. Miller, Kateri Fisher, and Donald L. Kreutzer. Manuscript in preparation.

- **Expression Of Interleukin 1 Family of Cytokines and Receptors In Human Breast Cancer** Alexander G. Pantschenko, Irina Pushkar, Kathleen H. Anderson, Yanping Wang, Lauri J. Miller, Scott H. Kurtzman George Barrows and Donald L. Kreutzer. To be submitted. See draft copy in appendix.

- **In Vitro Demonstration of Breast Cancer Tumor Cell Subpopulations Based on the Expression of Interleukin-8.** Alexander G. Pantschenko, Irina Pushkar, Lauri J. Miller, YanPing Wang, Kathleen Anderson, Ziv Peled, Scott H. Kurtzman, and Donald L. Kreutzer. To be submitted. See draft copy in appendix.

2. FUNDING APPLIED FOR.

- The Robert Leet & Clara Guthrie Patterson Trust " Pathogenesis of Ovarian Cancer "
- Mary Kay Ash Charitable Foundation " Role of Tumor Cell Cooperativity and Subpopulations in Ovarian Cancer: Role of CXC Cytokines and Receptors inControlling Tumor Growth and Angiogenesis"
- Komen Foundation " Role of Tumor Cell Cooperativity and Subpopulations in Human Breast Cancer: CXC Cytokines and Receptors in Controlling Tumor Growth and Angiogenesis.

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APPENDIX

STATEMENT OF WORK (VERBATIM)

Proposal Title: The Role of Growth-Regulated Oncogene (GRO) Proteins in Human Breast Cancer Growth.
PI's Name: Alexander G. Pantschenko, Ph.D.

TASK 1. Evaluate GRO and GRO-Receptor (CXC-R2) Expression in Fresh and Archival Human Breast Cancer Patient Tumor Specimens.
Experimental Approach: (months 1-12).
1) All HBC tumor specimens are coded and evaluated as a double-blind study.
2) HBC tumor tissues are stained with anti-GRO and anti-CXC-R2
3) HBC tumor tissues are stained with anti-VWF and anti-CD31 to quantitate microvessel density.
4) HBC tumor tissue homogenates are used to quantitate GRO and CXC-R2 by ELISA, Western blot, and Quantative PCR.
5) Clinical and laboratory data for patient specimens including; tumor stage, estrogen-receptor, and progesterone-receptor expression are compiled.
6) For archival specimens, invasive, ductal carcinoma *in situ* (DCIS) as well as benign breast disease tissue (fibrocystic) specimens are available from our previous studies and from archival specimens in the Department of Pathology, University of Connecticut School of Medicine. Clinical outcome is factored as a variable and the data compiled.
7) Data are segregated based on tumor stage and statistical analysis services are available at the University of Connecticut School of Medicine.

TASK 2. Evaluate GRO/CXC-R2 expression (constitutive and inducible) in human breast cell lines *in vitro.*
Experimental Approach: (months 2-10)
1) Established human breast cell lines (see Table 1) are evaluated for GRO/CXC-R2 expression under unstimulated (baseline) and stimulated (IL1 and TNF) culture conditions.
2) Cell culture supernatant and cell lysates are assayed for GRO/CXC-R2 by ELISA, Western Blot, and Quantitative PCR.
3) HBC cell lines (as above) are cultured onto glass chamber slides and evaluated for GRO/CXC-R2 expression by immunocytochemistry.

TASK 3. HBC cell line proliferative responsiveness to GRO and GRO inducers (IL-1 and TNF) are assayed *in vitro.*
Experimental Approach: (months 12-24)
1) Established human breast cell lines are cultured with GRO, IL8, TNF, or IL1 and proliferation is measured by ^{3}H-thymidine incorporation. Proliferation responsiveness to cytokines is confirmed by determining specificity. Confirmation of specific response is determined with the addition of blocking antibodies for ligand and/or receptor, or antagonists, or antisense oligonucelotides [unmodified phosphodiester oligonucleotides complimentary to GRO, CXC-R2, or nonspecific (negative control)].

TASK 4. To determine the relative contribution of GRO/CXC-R2 as a tumor growth factor compared to GRO as an angiogenesis factor using the xenograft nude mouse model.
Experimental Approach: (month: 24-36)
1) HBC cells lines characterized for varying levels of GRO and CXC-R2 expressors as described in Task 2 & 3 are implanted into the nude mouse mammary gland fat pad such that high and low ligand and/or receptor cell lines are compared. The resulting tumors (or lack of establishing tumors) are evaluated as follows:
    a) Tumor Growth
        - Rate
        - Size
        - Histology, (hematixylin and eosin stain)
    b) Cytokine Expression
        - Immunohistochemistry for GRO, CXC-R2, IL1 and TNF
        - Q-PCR, ELISA, Western blot to quantitate GRO, CXC-R2, IL1 and TNF
    c) Angiogesesis
        - Immunohistochemistry to determine microvessel density using anti-VWF and anti-CD31

IL-8 and GRO_{TOTAL} Expression in Human Breast Cancer Tumor Homogenates			
Tumor Cytokine Levels (pg/mgp)	**CYTOKINE**	**Correlation IL-8**	**GRO**
Mean ± S.E.M (n)		$p \leq$ (n)	$p \leq$ (n)
105.3 ± 30.36 (171)	**IL8**		**0.002** (97)
1.98 ± 0.48 (98)	**GRO**	**0.002** (97)	
17.75 ± 3.90 (82)	**IL-1α**	**0.01** (82)	**0.001** (30)
12.07 ± 1.42 (101)	**IL-1β**	**0.01** (101)	*n.s.*
18,740 ± 2,770 (65)	**IL-1RA**	*n.s.*	*n.s.*
15.37 ± 4.18 (70)	**IL-1R1**	*n.s.*	**0.001** (30)
58.34 ± 8.25 (70)	**IL-1R2**	*0.06* *(70)*	*n.s.*
197.13 ± 26.98 (60)	**TNF-R1**	*n.s.*	*0.09* *(26)*
241.58 ± 44.41 (35)	**TNF-R2**	*n.s.*	*n.s.*
89.37 ± 6.5 (223)	**ER**	**- 0.0002** (170)	**- 0.05** (98)
114.21 ± 1.75 (221)	**PR**	**- 0.02** (169)	*n.s.*

Table 1. IL-8 and GRO_{TOTAL} expression in human breast cancer tumor homogenates. Human breast cancer surgical samples collected at St. Francis Hospital and Medical Center, Hartford CT were homogenized and assayed by ELISA for cytokine/cytokine receptor levels and Estrogen receptor/Progesterone receptor (ER/PR) Expression.
Tumor homogenate cytokine levels are expressed as picograms per milligram of total protein and are represented as mean ± standard error or the mean. (n) = number of samples tested.
Correlation comparison of cytokine levels by linear regression analysis and Pearson correlation test. $p \leq 0.05$ is significant. *n.s.* is a non-significant relationship between the cytokine levels.

Table 2. Constitutive and inducible expression of IL-8 and GRO_{TOTAL} by human mammary epithelial and tumor cell lines. Human mammary epithelial and tumor cell lines were plated at 10^6 cells/well/ml^{-1} in 12-well flat bottom plates and incubated overnight at 37°C in saturated humidity. The following day, the media was aspirated and replaced with fresh culture media. Triplicate wells are then treated with 10ng/well/ ml^{-1} of either TNFα, TNFβ, IL-1α, IL-1β, or media alone, or 5μg/well/ml^{-1} LPS. The cells are incubated for an additional 24hrs and the supernatant is harvested are stored at -20°C until assayed by ELISA.
Each value represents the mean and standard deviation for three independent experiments. ELISA of the culture supernatants were assayed in duplicates.

IL-8 AND GRO_{TOTAL} Levels in Culture Supernatants						
Human Mammary Epithelial and Tumor Cell Lines	MEDIA		TNFα		TNFβ	
	IL-8 (pg/ml)	GRO (pg/ml)	IL-8 (pg/ml)	GRO (pg/ml)	IL-8 (pg/ml)	GRO (pg/ml)
NORMAL						
HMEC	2,300 ± 430	2,631 ± 543	3,010 ± 1240	1,516 ± 209	1,620 ± 280	1,856 ± 331
ESTROGEN-INDEPENDENT						
BT-20	240 ± 50	25 ± 0	290 ± 10	25.0 ± 0	260 ± 50	25 ± 0
MDA-MB-231	1,080 ± 360	58 ± 15	7,300 ± 2,750	125 ± 20	2,040 ± 790	44 ± 8
ESTROGEN DEPENDENT						
MCF-7	290 ± 80	46 ± 17	270 ± 50	53 ± 14	290 ± 80	14 ± 3
T-47-D	110 ± 10	71 ± 13	110 ± 10	62 ± 0	110 ± 10	62 ± 0
ZR-75-1	240 ± 40	20 ± 4	150 ± 30	14 ± 5	160 ± 30	15 ± 4

IL-8 AND GRO_{TOTAL} Levels in Culture Supernatants						
Human Mammary Epithelial and Tumor Cell Lines	LPS		IL-1α		IL-1β	
	IL-8 (pg/ml)	GRO (pg/ml)	IL-8 (pg/ml)	GRO (pg/ml)	IL-8 (pg/ml)	GRO (pg/ml)
NORMAL						
HMEC	3,390 ± 840	3,987 ± 445	25,060 ± 7,730	8,267 ± 1,106	15,080 ± 3,590	9,459 ± 765
ESTROGEN-INDEPENDENT						
BT-20	250 ± 40	25 ± 0	279,570 ± 45,510	2,379 ± 1,331	206,520 ± 12,500	3,953 ± 1,694
MDA-MB-231	136,210 ± 62,090	1,013 ± 130	358,830 ± 52,350	1,057 ± 128	356,960 ± 61,100	909 ± 122
ESTROGEN DEPENDENT						
MCF-7	330 ± 110	19 ± 4	320 ± 80	28 ± 5	400 ± 130	48 ± 18
T-47-D	100 ± 10	62 ± 0	310 ± 40	2,900 ± 774	350 ± 50	2,167 ± 1,194
ZR-75-1	1,980 ± 430	162 ± 22	29,260 ± 4,210	653 ± 50	32,600 ± 4,080	622 ± 131

Table 3. Stimulation index for induced expression of IL-8 and GRO_{TOTAL} by human mammary epithelial and tumor cell lines. Stimulation index (S.I.) = [unstimulated (media) / stimulated (cytokine)] for human mammary epithelial and tumor cell lines. This data clearly demonstrates that IL-1 is a potent inducer of both IL8 and GRO, in particular, for the estrogen-independent human breast cancer cells lines BT-20 and MDA-MB-231. for

IL-8 AND GRO_{TOTAL} Stimulation Index for Cell Culture Supernatants						
Human Mammary Epithelial and Tumor Cell Lines	MEDIA		TNFα		TNFβ	
	IL-8 (S.I.)	GRO (S.I.)	IL-8 (S.I.)	GRO (S.I.)	IL-8 (S.I.)	GRO (S.I.)
NORMAL						
HMEC	1	1	1	1	1	1
ESTROGEN-INDEPENDENT						
BT-20	1	1	1	1	1	1
MDA-MB-231	1	1	7	2	2	1
ESTROGEN DEPENDENT						
MCF-7	1	1	1	1	1	0
T-47-D	1	1	1	1	1	1
ZR-75-1	1	1	1	1	1	1

IL-8 AND GRO_{TOTAL} Stimulation Index for Cell Culture Supernatants						
Human Mammary Epithelial and Tumor Cell Lines	LPS		IL-1α		IL-1β	
	IL-8 (S.I.)	GRO (S.I.)	IL-8 (S.I.)	GRO (S.I.)	IL-8 (S.I.)	GRO (S.I.)
NORMAL						
HMEC	1	2	11	3	7	4
ESTROGEN-INDEPENDENT						
BT-20	1	1	1,138	95	841	158
MDA-MB-231	127	17	333	18	332	16
ESTROGEN DEPENDENT						
MCF-7	1	0	1	1	1	1
T-47-D	1	1	3	40	3	30
ZR-75-1	8	8	120	32	134	31

description of scientific method, see table 2.

In Vitro Demonstration of Breast Cancer Tumor Cell Subpopulations Based on the Expression of Interleukin-8.

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Key words: cell cooperativity, breast cancer, cytokines, *in vitro* models, cells

Abbreviations used: AF, Angiogenic Factor; bFGF basic Fibroblast Growth Factor; BEC, human breast epithelial cell lines; BCC, human breast cancer cell lines; IL, Interleukin; RIA, Radioimmunoassay; TNF, Tumor Necrosis Factor; VEGF, Vascular Endothelial Growth Factor.

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Summary/Abstract

The cytokine Interleukin 8 (IL-8) has recently been identified as an angiogenesis factor (AF) as well as a tumor cell mitogen. In human breast cancer (HBC), we have observed populations of tumor cells that express both IL-8 and the IL-8 Receptors, and we have also observed the expression of an IL-8 Receptor on vascular endothelial cells within patient samples. Together, these data suggest that IL-8 may act as a paracrine angiogenesis factor and as an autocrine mitogenic factor in breast cancer and that these subpopulations of IL-8 expressing tumor cells may be important in regulating tumor progression via a cytokine/cell subpopulation cooperativity network. Since proinflammatory cytokines, such as Interleukin 1 (IL-1) and Tumor Necrosis Factor (TNF) are known to induce IL-8 expression in a variety of cell types. We hypothesize that in vitro; 1) breast tumor cell lines produce IL-8, and 2) this IL-8 expression can be controlled (induced) by IL-1 and TNF. We further hypothesize, that the level of expression and responsiveness to induction for IL-8 expression will vary depending on the breast cell line. These differences in both constitutive and inducible IL-8 in vitro expression is representative of the IL-8 in vivo tumor cell subpopulations found within the tumor microenvironment. As such, this study is useful in the development of future models to determine the role of IL-8 and tumor cell cooperativity in human breast cancer progression. To test this hypothesis, we evaluated the ability of: 1) human breast cancer cell lines (BCC) and human breast epithelial cell lines (BEC) to produce IL-8; and 2) IL-1 or TNF to regulate the expression of IL-8. Five human BCC, and 3 BEC were stimulated with IL-1α, IL-1β, TNF-α or TNF-β. RIA of the culture supernatants and lysates measured basal and inducible IL-8 protein expression by the breast cells. Generally, baseline (unstimulated) IL-8 expression by BCC and BEC was extremely low. TNF-α and TNF-β induced a 3-24 fold increase in IL-8 protein expression of BEC, and a 2 - 8 fold increased IL-8 expression in estrogen-independent cell lines BCC. Neither TNF-α nor TNF-β induced significant IL-8 expression in estrogen-dependent cell lines. When BEC were stimulated with IL-1α or IL-1β, a 5 - 104 fold stimulation was demonstrated. Interestingly, the estrogen independent BCC showed a 330 - 1138 fold increase in IL-8 expression when stimulated with IL-1 cytokines. These observations demonstrate the ability of HBC cells to produce IL-8 in vitro, and further indicate that IL-1α and IL-1β are potent inducers of IL-8 expression by BEC and BCC. Furthermore, with the identification of breast cell lines with relatively higher or lower levels of IL-8, we can begin to develop an in vitro model to study the role of IL-8 in HBC tumor cell cooperativity.

Introduction

Tumor heterogeneity. Malignant solid tumors are known to consist of morphologically distinct heterogeneous populations of cells. This cellular heterogeneity includes not only phenotypically and genotypically distinct populations of tumor cells themselves, but is reflected by the normal or non-cancerous cell populations that are also found within the tumor microenvironment. Other cell that reside within, and populate the tumor tissue include; normal tissue cells (i.e. parenchyma), stromal cells (i.e. fibroblasts and myofibroblasts), the supporting vasculature (e.g. vascular endothelial cells and smooth muscle cells) and infiltrating inflammatory cells (e.g. histiocytes, granulocytes, and lymphocytes). Therefore, solid tumors consist of various cellular populations and subpopulations.

Tumor cell subpopulations, cellular cooperativity, and tumor progression. The term "tumor progression" was used by Foulds to describe the ability of individual tumor to change or evolve over time (Foulds, 1969) (Foulds, 1975) and reviewed in (Heppner and Miller, 1998). Today, tumor progression is use to describe the sequential appearance of subpopulations of phenotypically distinct cells (Cotran et al., 1999) that contribute to tumor aggressiveness and pathology. The process of tumor progression helps to explain differences in the clinical presentation among individuals with the same type of tumor and takes into consideration qualitative difference in tumor characteristics. Qualitative considerations, would include differences in growth rate, invasiveness, metastatic ability, and responsiveness to therapy. More specifically, progression can be considered as the acquisition of specific characteristics by cell subpopulations such as 1) enhanced tumor growth via mitogenic responsiveness to endocrine and cytokine growth factors, 2) induction and support of tumor vascularization via expression of angiogenesis factors, or 3) the ability to of a tumor to express matrix degrading proteinases to facilitate invasion and metastasis, to mention but a few examples. Therefore, as a tumor progresses, it acquires one or more of these quantitative changes which are driven by the appearance and interactions of cell subpopulations. Importantly, as explained by Heppner, progression deals with changes in a cancer, and not necessarily with specific changes in cancer cells, since cancer is a tissue and not a cellular phenomenon. The underlying processes that drive tumor progression rely on cellular heterogeneity and cell-to-cell cooperativity. As such, the contributing roles and interactions of the various cell types and subpopulations that make-up the neoplastic tissue, ultimately dictate the nature and course of tumor growth, invasiveness, metastasis, and therapeutic responsiveness (Heppner, 1991) (Shekhar et al., 1993) (Heppner, 1993) (Fidler, 1999).

Examples of cellular cooperativity in cancer progression. Tumor progression for breast cancer has been well described (Leslie and Howard, 1992) (Shay et al., 1993) (Ingvarsson, 1999) (Cotran et al., 1999). Sequential events associated with breast cancer progression include; the loss of normal cellular regulation, and genetic instability in multiple small subpopulations resulting in atypical hyperplastic morphology. With the progression to carcinoma, a long list of both genetic and phenotypic changes can be observed, including expression of oncogenes, inhibition of tumor suppressor genes, changes in expression of cytoskeletal, adhesion, and cell cycle regulatory proteins. Additionally, there are increases in expression of angiogenesis factors and proteinases. Not all of these changes occur in every tumor, nor does the occurrence of these changes follow a strict pattern. This suggests that multiple changes and interaction drive the phenotypic characteristic of a particular malignancy. While there is a great deal of information detailing cellular processes, such as the upregulation of a particular oncogene, little is known about the interaction of cell subpopulations and how they affect the progression process. Some of the better descriptions of cellular cooperativity in tumor progression would include 1) the role of stroma and stromal cells, 2) the influence of microenvironment on angiogenesis, and 3) the role of tumor associated macrophage (TAM) in tumor growth.

Tumor tissue, as with normal tissue, contains extracellular matrix and stroma. The extracellular matrix comprises the interstitial matrix and the basement membrane while the stroma consists mostly of fibroblasts and myofibroblasts.

Increasing evidence supports the importance of myoepithelial cells in mammary tumor progression. Myoepithelial cells produce inhibitors of proteinases which inhibit tumor cell invasion *in vitro* (Sternlicht and Barsky, 1997) and conditioned media produced by mammary myoepithelial cell cultures have been shown to have anti-proliferative and anti-invasiveness activity for breast carcinoma cells (Shao *et.al.*, 1998).

Immunohistochemically, we had previously described subpopulations of tumor cells expressing Interleukin-8 (IL-8) and the IL-8 Receptors in advanced human breast cancer patient samples (Kurtzman et al., 1996; Kurtzman et al., 1999; Kurtzman et al., 1994; Miller et al., 1998). We have also observed that the expression of IL8 is inversely associated with the estrogen/progesterone receptor (ER/PR) expression suggesting high IL8 levels is consistent with an unfavorable outcome for breast cancer patients (Kurtzman et al., 1996). These observations underscore the role of tumor cell cooperativity in tumor progression, metastasis and responsiveness to therapy.

Interleukin 8 (IL-8) and the IL-8 Receptors (CXC-R1 and CXC-R2) in human cancer. IL-8 is best recognized as a neturophil chemotactic factor, however it has recently been demonstrated as a) an autocrine human malignant melanoma tumor cell growth factor (Schadendorf et al., 1993), b) a potent vascular endothelial cell migration factor in vitro (Koch et al., 1992), c) an inducer of neovascularization in the rat cornea (Strieter et al., 1992), d) a correlate with melanoma metastatic potential in the nude mouse model (Singh et al., 1994), e) a predictive indicator of therapeutic response and prognosis of patients with recurrent breast cancer (Yokoe et al., 1997) and f) an autocrine ovarian carcinoma cell growth factor (Xu and Fidler, 2000). Furthermore, we have demonstrated the expression of IL-8 and IL-8 receptor in human breast cancer (Kurtzman et al., 1996; Kurtzman et al., 1999; Kurtzman et al., 1994; Miller et al., 1998). In these studies we found that there was intense IL-8 and IL-8 Receptor (IL-8RA and IL-8RB) staining of tumor cells as well as vascular endothelial cells (VEC) in human breast cancer specimens. We have also observed that IL-8 levels of human breast cancer tissue homogenates were inversely correlated with estrogen/progesterone receptor (ER/PR) expression, [*i.e.* homogenates with high IL-8 (>10pg/mg protein) were associated with negative ER and PR status], (Kurtzman et al., 1996). This evidence suggests that IL-8 and its receptors are present in the tumor microenvironment and may play a role in tumor growth, particularly in more aggressive breast cancer associated with negative hormone receptor status. Furthermore, these data demonistrate that IL-8 positive and IL-8 negative subpopulations exist within the breast cancer microenvironment. Importantly, IL-8 receptors are expressed on both breast tumor cells and vascular endothelial cells from breast cancer tissue samples (Miller et al., 1998). This further demonistrates the presence of IL-8 Receptor positive and negative subpopulations in breast cancer. This data suggests that tumor derived IL-8 could contribute to tumor growth directly by autocrine stimulation and indirectly, by paracrine modulation of vascular endothelial cells leading to angiogenesis of breast cancer tissue which subsequently contributes to growth and metastasis.

Angiogenesis in human cancer progression. Many factors beyond tumor size, histologic grade, tumor cell heterogeneity, and lymph node involvement have been examined as prognostic

indicators for metastatic breast disease and cancer (Donegan, 1997). Additionally, a considerable body of evidence has demonstrated that angiogenesis in invasive breast cancer is associated with poor outcome as demonstrated by a shorter disease free survival and an increased frequency of metastatic disease (Gasparini and Harris, 1995). Furthermore, the degree of intratumoral vascularization has also been shown to be a significant and independent prognostic factor in primary breast cancer (Bosari et al., 1992; Toi et al., 1993; Weidner et al., 1992). It is also well accepted that tumors can not grow beyond several millimeters in diameter without recruiting new blood vessels. Thus, angiogenesis is critical for tumor growth and metastasis. The processes associated with new vessel formation are thought to be controlled by a variety of chemical signals known as angiogenic factors (AFs). A number of well established AFs such as Vascular Endothelial Growth Factor (VEGF), basic Fibroblast Growth Factor (bFGF), and more recently, Interleukin 8 (IL-8) have been described and implicated in human cancer. (Schadendorf et al., 1993; Smith et al., 1994; von Biberstein et al., 1995). Therefore, based on the literature and our own studies, we hypothesize that IL-8 may not only be a cancer cell mitogen, but also an important angiogenic factor in human breast cancer.

Cytokine modulation of IL-8 expression. To fully appreciate the interaction and role of breast cancer cell subpopulations it is important to not only determine the expression of IL-8, but to examine the presence and distribution of known IL-8 inducers (IL-1 and TNF) in HBC tissues. Others and we have demonstrated IL-1 expression in human breast cancer tissue [Miller, 2000 #7; Kurtzman, 1999 #8; Jin, 1997 #36; Pantschenko, In preperation #53]. Jin *et.al.,* showed that 90% of invasive breast carcinomas were immuno-reactive for IL-1β and levels of this cytokine were significantly higher for invasive carcinoma than for ductal carcinoma *in situ* (DCIS) and benign lesions. The authors conclude that IL-1β is present in the tumor microenvironment and higher levels are associated with tumor invasiveness and with other pathologic parameters suggestive of tumor aggressiveness (Jin et al., 1997). We have recently demonstrated the presence of IL-1α and IL-1β in tumor cells from patients with invasive carcinoma and DCIS. Quantitative analysis of tissue homogenates by ELISA confirmed the presence and positive correlation of IL-1α and IL-1β to IL-8 in cancer specimens (Miller et al., 2000). As a result of our studies, we conclude that in the tumor microenvironment, IL-8 and the IL-8 inducer, IL-1 is present and associated with breast cancer cells. Since in inflammatory diseases, cytokines such as IL-1 and TNF are known to induce IL-8 expression. We therefore postulate that in the tumor microenvironment, subpopulations of human breast cancer cells can express IL-8 and that this IL-8 expression is under the control of IL-8 inducing cytokines [*i.e.* Interleukin 1 (IL-1) and Tumor Necrosis Factor (TNF)]. In order to directly test this hypothesis, we: 1) determined the ability of breast cancer cells (BCCs) and breast epithelial cells (BECs) to produce IL-8 *in vitro,* and 2) determined the ability of IL-1 and TNF to regulate IL-8 expression by these cells.

Materials and Methods

General reagents

Chicken antibody to IL-8 was prepared by intramuscular injection of 100 μg of recombinant human IL-8 (77 amino acids, Pepro Tech Inc., Rocky Hill, NJ) prepared in *Hunger's* Titer Max (CYTRX Corp., Norcross, GA). Egg yolks containing antibody were processed as previously described, and antibody titer and specificity were assessed as previously described (5). IL-1α and IL-1β were obtained from (R & D Corp., Minneapolis, MN), and TNF-α and TNF-β were purchased from (Pepro Tech Inc., Rocky Hill, NJ).

Cell culture and cytokine stimulation

All cell lines (HMEC, Hs578Bst, HBL-100, MCF-7, T-47D, ZR-75-1, BT-20, and MDA-MB-231) used for these studies were obtained from (ATCC, Manassas, VA) or (Clonetics Corp., Walkersville, MD). All cells were grown in recommended media containing 10% fetal calf serum (FCS), and maintained in humidified 5% CO_2 at 37°C. Estradiol was present in FCS at levels of 26 ng/ml. Cells were routinely cultured in T-75 flasks. Prior to an experiment,

preconfluent cells were detached from the flasks with 0.05% trypsin/0.04% EDTA in PBS and washed 3 times with PBS. Viability and cell count was determined by trypan blue exclusion method. Cells were plated at 10^6/2ml/well in 12-well tissue culture plates 24 hours prior to use. No cytokine-related cell toxicity was observed. Both the BEC and BCC were found to adhere and become confluent after overnight incubation in 12-well plates.

For the cytokine stimulation studies described below, culture media was aspirated from overnight BEC or BCC confluent cultures. The cells were immediately treated with control media, or media with 10 ng/ml cytokine (IL-1α, IL-1β, TNF-α or TNF-β) in triplicate cultures. Media used for each experiment was the same as that used for continuous culture of each cell line. Cell cultures were treated with media or media with cytokine for 24 hours. Then, the cell culture supernatants from each well was aspirated and frozen at -70°C until analyzed. The remaining adherent cells were treated with 0.1% Triton X-100 (Sigma, St Louis, MO) in PBS until cell lysis was observed microscopically. The resulting cell lysates from each well was aspirated and frozen at -70°C until analyzed.

IL-8 radioimmunoassay

BEC and BCC culture supernatants and lysates were analyzed by an IL-8 specific radioimmunoassay (RIA) developed in our laboratory (von Biberstein et al., 1995). IL-8 levels in samples were quantified using rh-IL-8 standards curve (0.039 - 10 ng/ml). Individual samples were assayed in duplicate. All cytokine stimulation studies were done in at least 3 independent studies and results are expressed as the mean ± S.D. of the three individual experiments.

Data Analysis

All IL-8 data was expressed as ng/ml, as well as stimulation index (SI). SI equals IL-8 level present in the cytokine stimulated cell supernatant divided by the IL-8 level in control media culture supernatants. The data was analyzed using the mean ANOVA or *t*-test, JMP statistical program (SAS Institute, Inc., Cary, NC). For our studies, $p < 0.05$ was considered statistically significant.

Results

Baseline IL-8 Expression: Breast Epithelial Cells

Basal IL-8 expression was initially determined in culture supernatants from normal breast cell lines (HMEC and Hs 578 Bst). As indicated in Table 1, HMEC cells produced 2.3 ± 0.43 ng/ml IL-8 as compared to 0.48 ± 0.07 ng/ml for the Hs 578 Bst. Parallel analysis of the cell lysates demonstrated low levels of cell associated IL-8 in both normal breast cell lines (*i.e.* <1 ng/ml). Interestingly, the SV-40 transformed BEC cell line, HBL-100 demonstrated significantly higher baseline IL-8 levels (*i.e.* 8.8 ± 1.43 ng/ml) in the cell culture supernatants when compared to either of the normal BEC lines.

Baseline IL-8 Expression: Estrogen Dependent Breast Cancer Cell Lines

After establishing baseline expression of IL-8 in the non malignant breast cell lines, we next examined the baseline levels in three human estrogen dependent breast cancer cell lines, MCF-7, T-47 D, and ZR-75-1 (see Figures 1 and Table 1). All three cell lines showed extremely low levels of baseline IL-8 expression in the cell culture supernatants (range: 0.1 - 0.3 ng/ml) and lysates (range: 0.07 - 0.15 ng/ml).

Baseline IL-8 Expression: Estrogen Independent Breast Cancer Cell Lines

Baseline IL-8 levels in cell culture supernatants and lysates were determined in the two estrogen independent human breast cancer cell lines, BT-20 and MDA-MB-231. Much like the estrogen dependent BCC, the BT-20 and MDA-MB-231 cell culture supernatants showed low levels of IL-8 expression in unstimulated conditions (0.24 - 1.08 ng/ml). The levels in the cell lysates were similarly low (<1.0 ng/ml).

TNF stimulation of IL-8 Expression

In order to test whether TNF-α or TNF-β could induce IL-8 expression from breast cells, we measured the levels of IL-8 in 24-hour cell culture supernatants and lysates from BEC and BCC treated with either TNF-α or TNF-β (see Figures 1 and 2, and Table 1). The results of these studies are presented below. Our data indicate that there is a heterogeneous response of breast cells to TNF stimulation. This phenomenon likely reflects the pattern seen in normal

breast tissue and in patients with malignancies.

In an effort to characterize the effects of TNF-α and TNF-β on IL-8 expression in normal breast epithelial cells, we utilized the cell lines HMEC and Hs 578 Bst. For Hs 578 Bst, TNF-a induced a 23.6-fold stimulation of IL-8 expression when compared to unstimulated (media) control supernatants [*i.e.* stimulation index (SI) = 23.6]. Interestingly, there was minimal stimulation of IL-8 by TNF-α with the HMEC cells (SI=1.31). Generally, a similar pattern of IL-8 stimulation was seen for TNF-β (see Figures 1 and 2, and Table 1). Thus, TNF-α and TNF-β stimulated the Hs 578 Bst BEC, but not the HMEC. Finally, the transformed BEC HBL-100 showed only modest stimulation of IL-8 expression by TNF-α (SI = 2.05) or TNF-β (SI = 1.35).

We next tested the ability of TNF-α to induce IL-8 expression in the estrogen dependent BCC. As can be seen in Figures 1 and 2, and Table 1, neither TNF-α nor TNF-β stimulation resulted in induction of IL-8 by any of the estrogen dependent cell lines. In fact, the ZR-75-1 BCC there was modest inhibition of IL-8 expression. Thus neither TNF-α nor TNF-β appear to be inducers of IL-8 expression for estrogen dependent BCC.

In parallel studies, we examined the ability of TNF-α and TNF-β to induce IL-8 expression in estrogen-independent BCC. We found that there was modest induction of IL-8 in the MDA-MB-231 BCC by TNF-α (SI = 6.78) and TNF-β (SI = 1.89). In contrast, there was no IL-8 stimulation seen in the BT-20 cells in response to either TNF-α or TNF-β.

IL-1 stimulation of IL-8 Expression

IL-1 cytokines are known to be potent inducers of IL-8 expression in leukocytes and normal tissue cells (6). Therefore, we extended our studies to investigate the ability of IL-1α and IL-1β to induce IL-8 expression in normal and malignant breast epithelial cells.

Both normal BEC lines showed marked increased expression of IL-8 when treated with either IL-1α or IL-1β (Figures 1 and 2, and Table 1). HS 578 Bst was consistently more responsive to IL-1α or IL-1β stimulation when compared to HMEC. Analysis of the induction of IL-8 expression in SV-40 transformed BEC line HBL-100 in response to IL-1α or IL-1β demonstrated that both IL-1α and IL-1β induced increased levels of IL-8 expression (IL-1α SI = 6.88, IL-1β SI = 5.24) in these cells. This data demonstrates that IL-1 is a potent inducer of IL-8 in non malignant BEC. This observation also emphasizes the heterogeneous responsiveness between cell lines, as well as between various cytokines.

Evaluation of the ability of IL-1α or IL-1β to induce IL-8 expression in estrogen-dependent cell lines demonstrated a clear heterogeneity in IL-8 response (see Figures 1 and 2, and Table 1). For example, MCF- 7 cells showed no response to stimulation (IL-1α SI = 1.08, IL-1β SI = 1.37). The T-47 D BCC showed intermediate responses to IL-1 stimulation (IL-1α SI = 2.89, IL-1β SI = 3.23), and the ZR-75-1 BCC demonstrated profound stimulation (IL-1α SI = 120.26, IL-1β SI = 133.99).

In order to evaluate the ability of IL-1 cytokines to induce IL-8 expression in estrogen independent BCC, we measured the IL-8 expression in cell culture supernatants and lysates at 24 hours post-stimulation in response to IL-1α or IL-1β stimulation. As can be seen in Table 1, Figures 1 and 2, IL-1 cytokines were extremely potent inducers of IL-8 expression in both estrogen independent BCC. IL-1α and IL-1β induced SI of over 300-fold in the MDA-MB-231 cells, and approximately 1,000-fold in the BT-20 BCC. This data not only emphasizes the differential expression of IL-8 by benign and malignant breast cells in response to various cytokines *in vitro*, but also demonstrates that a marked heterogeneity exists in individual breast cell responsiveness to the different cytokines (*i.e.*, IL-1 or TNF) *in vitro*.

Discussion

Angiogenesis and Breast Cancer

Angiogenesis has long been known to be an important aspect of tumor growth and metastasis. Angiogenesis is not only critical in allowing the influx of gases and nutrients into the

rapidly proliferating tumor microenvironment, but also provides a route for dissemination of the tumor cells to distant sites within the body. Recently, it has been demonstrated that patients whose tumors have a high degree of angiogenesis have a shorter disease-free survival, and that these patients are more likely to develop distant metastases (Weidner et al., 1991); (Weidner et al., 1992).

The development of angiogenesis is under the regulation of molecules called angiogenic factors (Folkman and Klagsbrun, 1987). AF are known to control angiogenesis by inducing vascular endothelial cell proliferation and migration. Interestingly, while the identity of these AF has been described, and the importance of the process well accepted, little is known about the regulation of AF expression by human cancers. Well known AFs include: IL-8, VEGF, and bFGF. Understanding the underlying 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.

IL-1 and Cancer

IL-1 is a term for two polypeptides, IL-1α and IL-1β, that possess a wide spectrum of inflammatory, metabolic, physiologic, hematopoietic, and immunologic properties (Dinarello, 1991). Although the term interleukin means "between leukocytes," IL-1 is synthesized by many different cells, including leukocytes; macrophages; astrocytes; epithelial cells, endothelial cells; smooth muscle cells; fibroblasts; synovial lining cells; dermal dendritic cells; keratinocytes; intestinal, gingival, and cervical epithelium; natural killer cells; and maternal placental cells (Wuyts et al., 1998). IL-1 is generally considered a "prime controller/inducer" cytokine in the hierarchy of pro-inflammatory and pro-tumorigenic cytokines because 1) it is rapidly synthesized by nearly every nucleated cell when stimulated, 2) is present in many different organs, 3) is associated with numerous biological effects *in vitro* and *in vivo*, and 4) is known to induce a wide variety of other cytokines, such as IL-8. Interestingly few studies have investigated the relationship of IL-1 in cancer. Smith *et al* detected IL-1 in tumor tissue and speculated on the role of IL-1 in tumor growth and metastasis (Smith et al., 1992). More recently, studies in our laboratory have demonstrated elevated levels of IL-1α, IL-1β and IL-1 receptor antagonist in head and neck squamous cell carcinoma (von Biberstein et al., 1996). Thus, IL-1 is present in tumor tissue and likely influences the level of AF such as IL-8 in the tumor microenvironment.

TNF and Cancer

TNF-α and TNF-β are known to have both pro-tumorigenic and anti-tumorigenic activities *in vitro* and *in vivo*. The beneficial versus detrimental effects of tumor necrosis factors on tumor growth and metastases remain controversial (Mannel et al., 1993). There is considerable evidence that TNF plays a role in cancer. Recently, we have demonstrated the presence of TNF-α and TNF-β, as well as IL-8 in head and neck squamous carcinoma (HNSCC) (Cohen et al., 1995). This observation supports the hypothesis that TNF promotes tumor growth and metastasis by playing a regulatory role in the expression of AF such as IL-8.

IL-1 and TNF Induction of IL-8 Expression in Breast Cancer

We have previously demonstrated elevated IL-8 levels associated with HNSCC tumor cells (Cohen et al., 1995). IL-8 is a member of a family of 8-10 kDa cytokines that are involved in proinflammatory and reparative processes (Koch et al., 1992; Oppenheim et al., 1991; Wuyts et al., 1998). Cytokines in this family are basic heparin binding proteins that display chemotactic activities for vascular endothelial cells (VEC) *in vitro* and angiogenesis *in vivo*. It has been shown that IL-8 is also a growth factor for human melanoma cells (Mueller et al., 1994; Schadendorf et al., 1993), and that it mediates the angiogenesis and inflammation in bronchogenic carcinoma tissue (Smith et al., 1994). Additionally, we have demonstrated that *in situ*, IL-8 is a prominent immunohistologic feature of human breast cancer cells (Kurtzman et al., 1994; Miller et al., 1998). These data, together with our previous observations of IL-8 production by head and neck cancer cells (Cohen et al., 1995), suggests that IL-8 may be an important marker for the invasive properties of human tumors and likely contributes to angiogenesis. These studies not only underscore the importance of IL-8 in cancer, but raise the question of its regulation by cytokines such as IL-1 and TNF. Recent work done in our

laboratory has shown that IL-1 is present in homogenates of human breast cancer [Pantschenko, In preperation #53; Kurtzman, 1999 #8; Miller, 2000 #7] and supports the hypothesis that IL-1 may be involved in the regulation of IL-8 expression in human breast cancer.

Our present data clearly demonstrates that human breast cells are capable of producing angiogenic factors such as IL-8. We found that unstimulated breast cells (both benign and malignant) grown in culture, produce low levels of IL-8. Interestingly, the virally transformed cell line HBL-100, expressed the highest levels of IL-8 expression in the unstimulated state compared the other breast cells tested. More importantly, we found that cytokines are potent stimulants for IL-8 production in human breast cells *in vitro* with IL-1 being a major inducer of IL-8 production. Further, this induction may be related to the estrogen dependence of the breast cells; *i.e.*, both of the estrogen independent breast cancer cells tested were highly responsive to IL-1 stimulation, where two of the three estrogen dependent breast cancer cell lines did not increase production of IL-8 in response to cytokine stimulation.

Tumor cell cooperativity.

To understand the role of cytokines and cytokine receptors in tumor progression it is important to The interaction of heterogenous subpopulations of cells within the tumor microenvironment must take into consideration modulation of individual tumor cells within and between their societies as well as the interaction of tumor cells with the normal tissue and inflammatory cell populations.

These difference in both constitutive and inducible IL-8 in vitro expression is representative of IL-8 in vivo tumor cell sub-populations found within the tumor microenvironment and would be a useful model to determine the role of IL-8 in human breast cancer progression by understanding cooperativity among the various tumor cell types.

This data supports our general hypothesis that in the tumor microenvironment, breast cancer cells regulate their own growth through the production of angiogenic factors such as IL-8. In our earlier studies we demonstrated that IL-8 antigen, and more recently IL-1 antigens are present in breast cancer tissue [Pantschenko, In preperation #53; Kurtzman, 1999 #8; Miller, 2000 #7 Kurtzman, 1994 #6; Kurtzman, 1996 #52; Kurtzman, 1999 #8; Miller, 1998 #9]. This present study demonstrates that breast cancer cells themselves are the likely source of IL-8 seen in the tissues. Further, these observations are consistent with our hypothesis that *in vivo,* production of IL-8 is possibly under the influence of cytokines such as IL-1 and TNF. Future studies targeting inhibition of IL-1 and/or TNF may be useful in suppression of AF expression by breast cancer cells and therefore impair tumor growth and metastasis.

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Legends

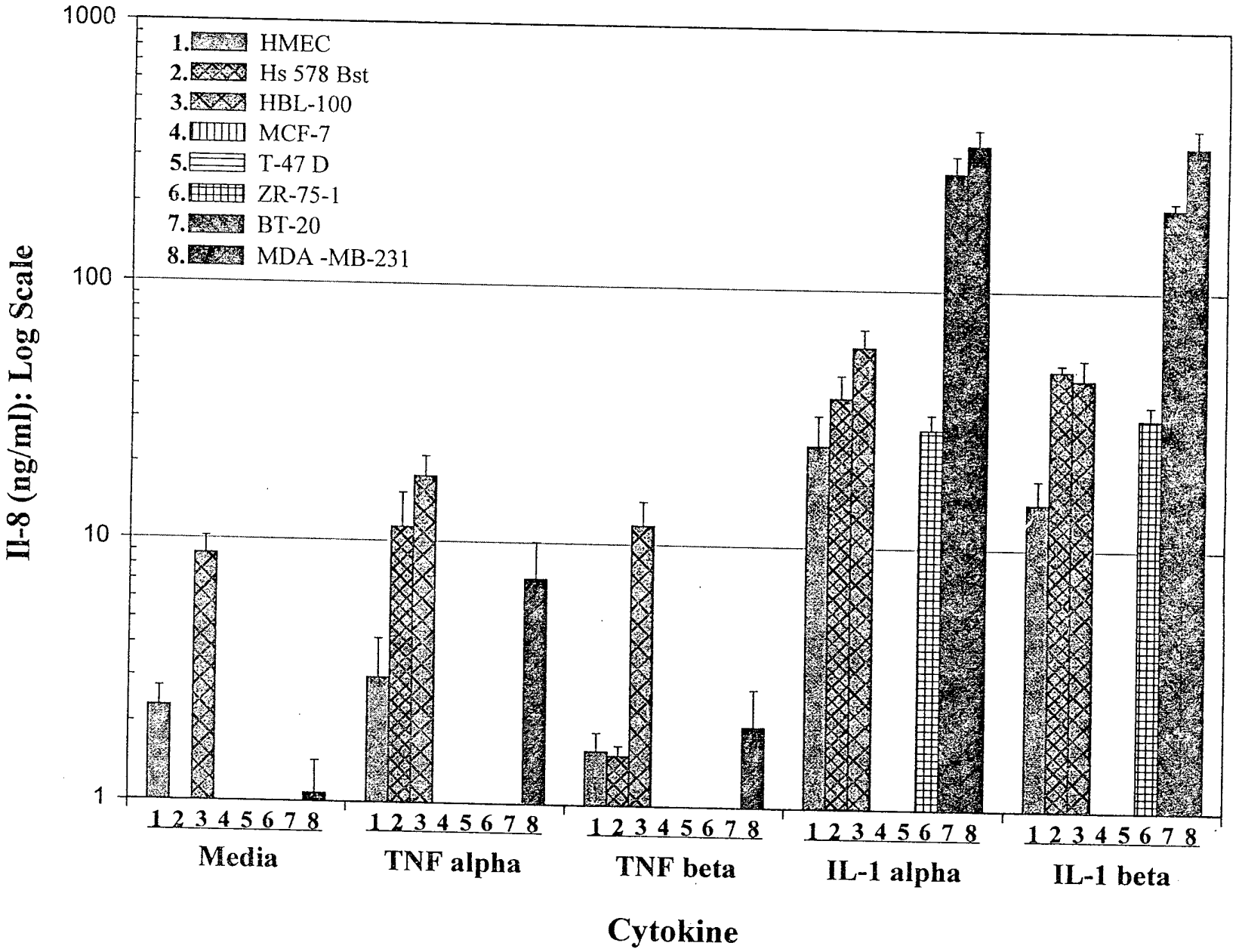
Table 1: Cell culture supernatants from various human breast cell lines were assay by RIA for IL-8 expression. Constitutive expression was for cells cultured in media alone, while inducible expression was for cells cultured with 10 ng/ml of TNF or IL-1. Values represent means and standard deviation. The p value compares stimulated to unstimulated levels. For all experiments n = 9, except for Hs 578 Bst where n = 5 and BT-20 where n = 3.

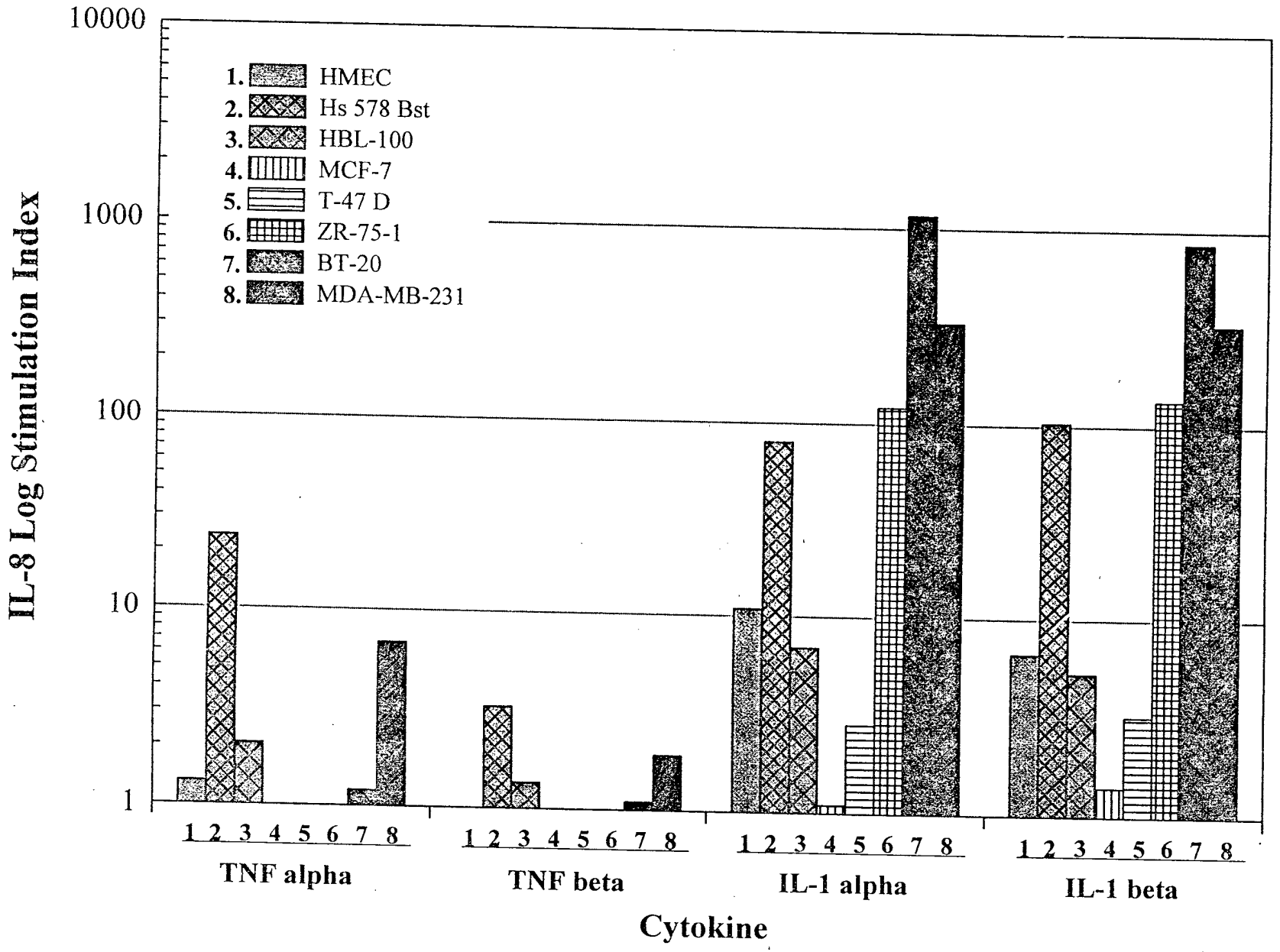
Figure 1: IL-1 and TNF induction of IL-8 expression in human breast cell lines. Breast cell line culture supernatants were assayed for IL-8 expression under unstimulated (media alone) condition or after treatment with 10ng/ml TNF or IL-1. Normal BEC cell lines are shaded light gray, estrogen dependent BCC are white, and estrogen independent BCC are shaded dark gray.

Figure 2: IL-8 stimulation indexes for IL-1 and TNF stimulation of human breast cell lines. Stimulation index = cytokine / media control presented on log scale.

TABLE 1 - CONSTITUTIVE AND INDUCIBLE EXPRESSION OF IL-8 BY HUMAN BREAST CELL LINES.

	Media	**TNF-alpha**		**TNF-beta**		**IL-1 alpha**		**IL-1 beta**	
	IL-8 ng/ml	IL-8 ng/ml	**p ≤**	IL-8 ng/ml	**p ≤**	IL-8 ng/ml	**p ≤**	IL-8 ng/ml	**p ≤**
Normal BEC									
HMEC	2.30 ± 0.43	3.01 ± 1.24	**0.59**	1.62 ± 0.28	**0.02**	25.06 ± 7.73	**0.009**	15.08 ± 3.59	**0.003**
Hs 578 Bst	0.48 ± 0.07	11.36 ± 4.14	**0.04**	1.56 ± 0.14	**0.00**	38.51 ± 8.63	**0.004**	49.96 ± 2.74	**0.0001**
HBL-100	8.80 ± 1.43	18.02 ± 3.59	**0.03**	11.88 ± 2.86	**0.35**	60.58 ± 10.52	**0.0002**	46.12 ± 9.05	**0.001**
Estrogen Dependent BCC									
MCF-7	0.29 ± 0.08	0.27 ± 0.05	**0.82**	0.29 ± 0.08	**0.96**	0.32 ± 0.08	**0.83**	0.40 ± 0.13	**0.50**
T-47 D	0.11 ± 0.01	0.11 ± 0.001	**0.85**	0.11 ± 0.01	**0.96**	0.31 ± 0.04	**0.0001**	0.35 ± 0.05	**0.0005**
ZR -75-1	0.24 ± 0.04	0.15 ± 0.03	**0.06**	0.16 ± 0.03	**0.09**	29.26 ± 4.21	**0.0001**	32.60 ± 4.08	**0.0001**
Estrogen Independent BCC									
BT-20	0.24 ± 0.05	0.29 ± 0.01	**0.38**	0.26 ± 0.05	**0.84**	279.57 ± 45.51	**0.004**	206.52 ±12.53	**0.0001**
MDA-MB-231	1.08 ± 0.36	7.30 ± 2.75	**0.04**	2.04 ± 0.79	**0.29**	358.00 ± 52.35	**0.0001**	356.96 ±61.10	**0.0001**


1000
100
10
1
1. HMEC
2. Hs 578 Bst
3. HBL-100
4. MCF-7
5. T-47 D
6. ZR-75-1
7. BT-20
8. MDA -MB-231
Il-8 (ng/ml): Log Scale
1 2 3 4 5 6 7 8
Media
TNF alpha
TNF beta
IL-1 alpha
IL-1 beta
Cytokine


10000
1000
100
10
1
IL-8 Log Stimulation Index
1. HMEC
2. Hs 578 Bst
3. HBL-100
4. MCF-7
5. T-47 D
6. ZR-75-1
7. BT-20
8. MDA-MB-231
1 2 3 4 5 6 7 8
TNF alpha
TNF beta
IL-1 alpha
IL-1 beta
Cytokine

EXPRESSION OF INTERLEUKIN 1 CYTOKINES AND RECEPTORS IN HUMAN BREAST CANCER

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ABSTRACT

Based on the importance of the interleukin (IL)-1 family of cytokines and receptors in other diseases, our previous studies, and the existing literature, we have develop the following hypothesis: 1) human breast tumor cells express the IL-1 family of cytokines (IL–1α, IL-1β and IL-1Ra) and receptors (IL-1RI and IL-1RII) *in vivo* and; 2) the local expression of the IL-1 family of cytokines and receptors within the tumor microenvironment can control tumor expression of protumorogenic cytokines such as the angiogenic factor interleukin-8 (IL-8). To begin to test this hypothesis we characterized the *in vivo* and *in vitro* expression of IL-1α, IL-1β, IL-1Ra, as well as the IL-1 receptors RI and RII by human breast cancer (HBC) cells. Immunohistochemical studies of archival specimens from HBC patients demonstrated that IL-1α, IL-1β, IL-1Ra as well as IL-1RI and IL-1 RII were expressed on both DCIS (n=10) and invasive (n=32) HBC tumor cells, with IL-1RII dominating IL-1RI based on intensity of staining. Interestingly, IL-1RI receptors appear to be expressed at higher levels in invasive breast tumor cells when compared to DCIS tumor cells and benign breast disease. It was also noted that vascular endothelial cells, fibroblasts and smooth muscle cells in the tumor microenvironment also expressed IL-1 receptors. Parallel quantitative studies (ELISA) of the tumor homogenates demonstrated not only detectable levels of IL-1RI (15.37 +/- 4.18 pg/mg total protein) and IL-1RII (58.34 +/- 8.25) in the tumor tissue, but that IL-1α, IL-1β, IL-1Ra and IL-8 were also present.

In these tumor homogenates, IL-1RI and IL-1RII levels correlated directly with IL-1α levels. Our studies also demonstrated that IL-1α and IL-1β levels correlated directly with IL-8 levels in HBC tissue. Parallel studies using these same immunoassays on tumor tissue from tumor cell lines (MCF 7, ZR 75, and MDA) grown in a nude mouse xenograph model of HBC, showed that the tumor cells also expressed the same IL-1 family of cytokines and receptors as well as IL-8 *in vivo*.

To directly demonstrate the ability of these HBC cell lines to express IL-1 and IL-8 we initiated *in vitro* studies. Our *in vitro* studies demonstrated that human breast cancer cell lines (MCF 7, ZR 75 and MDA) not only expressed IL-1α, IL-1β IL-1Ra, IL-1RI and IL-1 RII, but that these tumors cells can be induced by IL-1α or IL-1β to express the protumorgenic cytokine IL-8.

Add in vitro receptor data

Our data clearly demonstrates the presence and distribution of IL-1 cytokines and receptors in HBC and suggests that the local expression of IL-1 likely results in the activation of a population of cells within the tumor microenvironment. This activation of the IL-1/IL-1R cytokine family *via* autocrine and/or paracrine mechanisms leads to a cascade of secondary protumorgenic cytokines. These secondary signals induce the expression of numerous protumorgenic activities such as the expression of IL-8, and subsequently contribute to angiogenesis, tumor proliferation, and tumor invasion. These studies also suggest that targeting of the IL-1RI receptor may provide new approaches in HBC therapy.

We have previously demonstrated breast cancer tumor-association expression of interleukin (IL)-1a and IL-1b, and correlated IL-1 levels with the expression of the angiogenic/protumorigenic cytokine IL8. We have also shown that the expression of the IL-1 and the IL-1 receptor antagonist (IL-1ra) correlated with estrogen receptor (ER) status, a well known breast cancer prognosticator. Here, we extent and confirm the relationship between IL-1, IL-1 Receptors, prognostic indicators, and present *in vivo* and *in vitro* data supporting our hypothesis that IL-1 is an important regulator of human breast cancer tumor growth and angiogenesis.

We IL-1a inversely correlated with ER levels, whereas IL-1b and the IL-1 receptor antagonist (IL-1ra) correlated directly with ER levels in human breast cancer tissues. Furthermore, an increased IL-1ra/IL-1 ratio is associated with low ER status and poor prognosis.

These data support our hypothesis that the IL-1 family of cytokines plays a pivotal role in the growth and metastasis of human breast cancer.

INTRODUCTION

It is estimated that 180,000 new cases of breast cancer will be diagnosed in the United States for the year 2000 and breast cancer will cause 44,000 deaths annually. While surgery has been the primary therapeutic modality for these patients, a substantial number of women will die of metastasis despite adequate operations and adjuvant treatments. Many factors beyond tumor size and lymph node involvement have been examined in an effort to identify those patients who are likely to have a poor outcome. Among the current prognostic indicators in human breast cancer is estrogen receptor (ER) and progesterone receptor (PR) status and tumor vessel counts [1]. Recent efforts in our laboratory, as well as others, have focused on dissecting the role and prognostic value of tumor cell derived cytokines in human breast cancer. The interest in the role of cytokines in cancer is a result of the growing body of literature that indicates that tumor growth and metastasis is likely controlled by a variety of cytokines such as, growth factors and angiogeneic factors [2-8].

Cytokines are low molecular weight glycoproteins secreted by tissue, inflammatory, and tumor cells, which can regulate cell functions in an autocrine or paracrine fashion. The cytokine interleukin 1 (IL-1) is one of the early cytokines released during the inflammatory process, and is also known to stimulate the expression of a wide variety of cytokines including interleukin-8 (IL-8). Previous studies in inflammation and trauma have demonstrated *in vitro* that angiogenic factors such as IL-8 and vascular endothelial cell growth factor (VEGF) can be induced by specific cytokines such as IL-1 [9]. Interestingly, both IL-8 and VEGF have been implicated as major angiogeneic factors in a number of human tumors. Additionally IL-1 has been shown to increase protumorgenic activities in tumor cells *in vitro* including metalloprotease activity [10].

To date a considerable volume of literature exists demonstrating the *in vitro* ability of IL-1 to regulate a variety of cellular functions in tumor cells [11-17]. Interestingly, previous studies have demonstrated that IL-1 cytokines have diverse and sometimes contradictory effect on the growth of tumor cells. In some instances IL-1 exerts inhibitory activity, yet in other situations IL-1 has stimulatory effects [18]. Surprisingly little information exists on the *in vivo* expression, distribution and interrelationships of IL-1 in humans [18-20], and currently, nothing is known about the existence or role of IL-1 receptors on human tumor cells *in vitro* or *in vivo*. Interestingly, *in vivo* studies have demonstrated that in models of malignant disease, the administration of the naturally occurring IL-1 antagonist [interleukin-1 receptor antagonist (IL-1Ra)]: 1) reduces the number and size of metastasis; 2) reduce tumor growth and 3) reduce tumor-mediated cachexia [21]. These studies suggest that IL-1 likely plays a key role in tumor growth and metastasis. These studies raise the question of whether the IL-1 family of cytokines through their receptors control the expression of IL-8 and other pro-tumorigenic cytokines within the tumor microenvironment (TME) of breast cancer. These observations have led us to hypothesize that: a) IL-1 cytokines (IL-1α, IL-1β, IL-1Ra) and receptors (IL-1RI and IL-1RII) are present in the tumor microenvironment (TME) of human breast cancer (HBC); b) IL-1α, IL-1β and IL-1Ra are produced by tumor cells; c) IL-1RI and IL-1RII are expressed on tumor cells; and d) the local expression of the IL-1 family of cytokines and receptors within the tumor environment is pivotal in controlling the expression of protumorigenic factors such as IL-8. Since we hypothesize that IL-1 and IL-1 receptors are the key regulators of the expression of

pro-tumorigenic factors such as IL-8 in the TME/tumor cells, it is essential that we demonstrate the presence, distribution, levels and correlation of IL-1 family of cytokines and receptors as well as IL-8 in HBC tissue. In our present study we demonstrate that IL-1 family of cytokines and receptors are expressed by human breast cancer cells *in vivo* and *in vitro*, and that the expression of these factors correlate with expression of protumorgenic factors such as IL-8 expression and prognostic indicators such as ER/PR status. Demonstration of the expression of IL-1 family of cytokines by tumor cells is the first step in understanding the role of the IL-1 family in HBC.

MATERIALS AND METHODS

PATIENT POPULATION

Patients for this study represent individuals who underwent surgical procedures for benign and malignant breast disease at the John Dempsey Hospital (Farmington, CT) and Saint Francis Hospital and Medical Center (Hartford, CT).

HUMAN BREAST CANCER (HBC) TISSUE SPECIMENS

Breast cancer tissue [32 invasive, 10 ductal carcinoma in situ (DCIS)] as well as 7 benign breast disease tissue specimens were obtained from archival specimens in the Department of Pathology, University of Connecticut School of Medicine. These formalin fixed paraffin-embedded specimens were cut into 4-μm sections and mounted on slides for pathologic and immunohistochemical evaluation. Fresh tumor tissue obtained from patients undergoing surgery (either lumpectomy or mastectomy) for breast cancer was also used in this study to determine tissue levels of the various cytokines. The fresh tumor tissue was homogenized as described below, aloquoted and frozen at $-70^{\circ}C$ until assay. IL-1α, IL-1β, IL-1Ra, IL-1RI, IL-1RII, IL-8 and protein analysis was performed on the tumor tissue homogenates as described below.

IMMUNOHISTOCHEMICAL AND IMMUNOCYTOCHEMICAL TECHNIQUES

Immunohistochemical and immunocytochemical analysis of the tissue and cell specimens was performed by indirect immunoperoxidase staining as previously described [22], using commercially available antibodies to human IL-1α, IL-1β, IL-1Ra, IL-1RI, IL-1RII and IL-8 (Santa Cruz Laboratories, Santa Cruz, CA, and R&D Systems, Mineapolis, MN). The specificity of staining for each antibody was confirmed using specific antigens related to the antibody to block immunoreactivity or uses of non-immune serum/immunoglobulin. The stained slides were evaluated. For the malignant diagnoses, (invasive and DCIS) the presence of staining on the tumor cells, VEC, [both small vessels (SVEC) and large vessels (LVEC)], along with the smooth muscle surrounding the arterial large vessels was recorded. In samples where areas of adjacent normal or hyperplastic ductal morphology was present, the staining pattern of these ducts was noted. On the benign specimens, the staining pattern of the ductal epithelial cells (DEC) was noted, as well as the staining of the SVEC, LVEC and the surrounding arterial smooth muscle. The immunohistochemical analysis was performed by two of the authors. Staining was graded from 0 to 4+, 0 = no staining; 1+ = faint staining; 2+ = light staining; 3+ = moderate staining; and 4+ = intense staining. Staining 1+ or higher was considered positive.

The immunohistochemical staining results was analyzed by comparing the mean grade for each group (invasive breast cancer, DCIS, or benign) using the one-way ANOVA analysis from the JMP 3.0 statistical package (SAS, Cary, NC). A linear regression analysis was also determined to ascertain the relationship between staining for each patient. A p value of <0.05 was considered statistically significant.

HBC TISSUE HOMOGENATES

Tumor tissue samples were obtained and stored at -70°C until processing. To prepare tumor homogenates the frozen tissue was placed in 2.5 ml of phosphate buffered saline (PBS) and homogenized in a tissue homogenizer. The resulting homogenates were centrifuged at 40,000 RPM for 30 minutes at 4°C. These homogenates were then aliquoted and frozen at -70°C until analysis. Protein levels for each specimen were determined spectrophotomically (215nm vs.

225nm) and compared to protein standards. Estrogen receptor (ER), and progesterone receptor (PR) levels were determined for each specimen using Abbott ER-EIA and PR-EIA commercial ELISA kits. Specimens containing ER and PR levels < 15 fmoles/mg total protein (TP) were considered ER or PR negative, while levels ≥ 15 fmoles/mg TP were considered ER or PR positive.

To analyze the cytokine levels in the tumor homogenates, the data was transformed into natural log to achieve a normal distribution. The transformed values were then analyzed using a linear regression model (JMP Software). A p value of <0.05 was considered statistically significant.

HUMAN BREAST CANCER-MOUSE XENOGRAFT MODEL

Reference numbers for mouse model: [23, 24]

For our xenograft model human breast cancer cells (MCF-7, ZR-75 or MDA-MB-231) were injected subcutaniously into athymic nude mice (Harlan Sprague Dawley). Specifically, tumor cells were cultured *in vitro* and removed from the tissue culture flask by scraping. The resulting cell suspension was centrifuged at 1000 rpm for 8 minutes, and the resulting cell pellet was resuspended in 150 ul of media. Next, an equal volume of Matrigel (Becton Dickision, Bedford, MA) was added to the cell suspension, and mixed at 4°C and maintained on ice until injection. The resulting cell-Matrigel mixture was then injected in the right and left mammary fat pads of mice. Tumor grow was monitored weekly. Sixty days post injection, the tumors were harvested. One half of the tumor mass was fixed in 10% buffered formalin and the remaining tissue was frozen at –70°C. The formialin fixed tissue was paraffin embedded and cut into 4 μm sections for immunohistochemical studies.

HBC CELL CULTURE

All human breast cancer cell (HBC) lines (MCF-7, ZR-75-1, MDA-MB-231) used for these studies were obtained from ATCC. All cells were grown in recommended media containing 10% fetal calf serum (FCS), and maintained in humidified 5% CO_2 at 37°C. Estradiol was present in FCS at levels of 26 ng/ml. In addition, phenol red was present in all media. The cells used in these studies were initially propagated in T-75 flasks, and then plated in 12-well tissue culture plates at approximately 10^6 cells per well, 24 hours prior to use. No cytokine-related cell toxicity was observed. The HBC cells were found to adhere and become confluent after overnight incubation in the 12-well plates.

CYTOKINE STIMULATION OF HBC CELLS *IN VITRO*

For the cytokine stimulation studies, the HBC cells were allowed to adhere to the to 12-well plates overnight. Then the individual culture media was aspirated from the plates, and the cells were immediately treated with control media, or media containing the individual test cytokine; IL-1α or IL-1β (10 ng/ml; Peprotech Labs, Rocky Hill, NJ) in triplicate cultures. Media used for each cell stimulation study was the same media used to keep the cells in continuous culture. Cell cultures were exposed to media or media plus IL-1 cytokines for 24 hours. At the end of the exposure period, the cell culture supernatants were aspirated, and frozen at -70°C until analyzed. The adherent cells were treated with 0.1% Triton-X in PBS until cell lysis was demonstrated microscopically. The resulting cell lysates were then collected, and frozen at -70°C until analyzed.

All IL-8 data was expressed as ng/ml, as well as stimulation index (SI). The SI equals the IL-8 level present in the cytokine stimulated cell supernatants divided by the IL-8 levels in control media culture supernatants. The data was analyzed using the mean ANOVA or *t*-test from the JMP statistical program. For our studies, $p < 0.05$ was considered statistically significant.

CYTOKINE ASSAYS AND ANALYSIS

Immunoassays of HBC tumor homogenates, HBC cell culture supernatants and lysates were performed using IL-1α, IL-1β, IL-1Ra, IL-1RI, IL-1RII, and IL-8 enzyme linked immunoabsorbant assay (ELISA) that was developed in this laboratory using commercially available matched antibody pairs and cytokine standards (Endogen, Cambridge, MA and Pharmagen and R&D Systems), and ELISA kits from R & D Systems (Minneapolis MN). All cytokine stimulation studies were done in at least 3 independent studies and results are expressed as the mean ± S.D. of the three individual experiments.

RESULTS

IL1-α, IL-1β AND IL-1Ra EXPRESSION IN HBC TUMOR TISSUE *(fig 1/table 1)*

To begin our studies on the role of the IL-1 family of cytokines and receptors in HBC we initially determined the presence and distribution of IL-1 agonists (IL-1α, IL-1β) and antagonists (IL-1ra) in HBC tumor tissue using standard immunohistochemical techniques. IL-1α was consistently expressed in the tumor cells of all of the specimens from patients with both invasive (32/32) and DCIS (10/10) (see figure 1 and table 1A). When histologically normal ducts within these specimens were examined they also appeared consistently IL-1α positive (32/32). IL-1α was also expressed by 100% of the ductal epithelial cells in the benign specimens (7/7). Other tumor stroma cells including vascular endothelial cells, smooth muscle, and fibroblasts expressed IL-1α in about one half of the specimens tested, however, the overwhelming majority of the IL-1α antigen present in the tumor microenvironment was expressed by the tumor and ductal epithelial cells. Immunohistochemical analysis of the human breast specimens for IL-1β indicated that tumor cells showed a consistent cytoplasmic staining pattern for IL-1β, invasive 88%, (23/26) DCIS 78% (7/9). (figure 1 and table 1A). Analysis of normal ducts found in the specimens indicated that ductal epithelium in 77% (20/26) of the invasive, but only 56% (5/9) of the DCIS, samples showed positive staining for IL-1β. Ductal epithelial expression of IL-1β was evident in 77% (20/26) of the benign specimens tested. Again IL-1β was present in some of the tissue cells, but the majority of the staining was associated with the tumor and ductal epithelial cells. Finally, All of the malignant specimens expressed IL-1ra 100% [invasive (23/23) and DCIS (5/5)]. A number of malignant specimens contained areas of normal or hyperplastic ductal morphology adjacent to the tumor. A majority of the adjacent ductal epithelial cells of the invasive [86%(20/23)] and DCIS [75%(4/5)] specimens expressed IL-1ra. Again IL-1ra was present in some of the stroma cells, but the majority of the staining was associated with the tumor and ductal epithelial cells.

Analysis of the intensity of staining for the tumor cells and ductal epithelial cells (Table 1B) indicated that tumors cells consistently staining more intensely than ductal epithelial cells for all IL-1 cytokines, and that this difference was statistically different (Table 1C). These IHC studies demonstrate that the IL-1 family of cytokines is not only present in the tumor microenvironment, but that tumor cells themselves are likely the major source of these cytokines within the tumor microenvironment.

IL-1 RECEPTOR RI AND RII EXPRESSION IN HBC TUMOR TISSUE *(fig1/ table 1&2)*

Clearly the data presented above demonstrates the presence of IL-1 cytokines (agonists and antagonists) in HBC tissue, but this data also raises the question of what cells in the tumor microenvironment express the IL-1 receptors. To begin to answer this question we next conducted IHC analysis of the human breast disease (HBD) tissue for the IL-1 receptors IL-1 RI and IL-1RII. HBC tumor cells consistency stained positive (87-100%) for both IL-1RI and IL-1 RII in both DCIS and invasive breast cancer (figure 1 and Table 1A). The ductal epithelial cells in both malignant and benign breast tissues also stained positive for IL-1 RI and IL-1 RII (table 1A). Interestingly, when the intensity of staining for the IL-1 receptors was compared on the ductual epithelial cells and tumors cells, the DCIS and invasive cancer tumor cells had a statistically higher level of expression of both IL-1RI and IL-1RII than the benign breast disease

specimens (see Tables 1B and 1C). Further analysis indicated that both small and large vessel endothelium in all specimens stained positively for both IL-1RI and IL-1RII, as well as the smooth muscle cells surrounding large vessels (Table 2A and Table 2B). Interestingly, fibroblasts in invasive cancer specimens consistently stained positive compared to fibroblasts in DCIS and benign specimens as well as staining statistically more intensely then fibroblast in DCIS or benign specimens (Table 2A and Table 2B). IL-1RI staining of the stroma matrix was seen in all three types breast tissue, but no staining for IL-RII was seen in the stroma matrix (Table 2A and Table 2B). This data clearly demonstrates that the tumor cells and vascular endothelial cells both express IL-1 receptors, thus supporting our hypothesis that IL-1 cytokines (agonists and antagonists) can regulate both tumor cells and vascular cells within the tumor microenvironment (TME). Since IL-1α and IL-1β are known to induce expression of the angiogenic factor IL-8, our immunohistochemical observations raises the question of whether the tumor cells also express IL-8. To begin to answer this question we next conducted IHC studies of IL-8 expression in the same HBD specimens and well as *in vitro* studies described below.

INTERLEUKIN 8 EXPRESSION IN HBC TUMOR TISSUE *(fig 1, table 1a 1b 1c)*

IHC of the same human breast tissue for IL-8 antigen indicated that IL-8 antigen was consistently expressed by HBC tumor cells and ductal epithelial cells (figure 1 and table 1A). When the intensity of IL-8 staining in these breast tissue were compared, IL-8 staining was consistently higher in the tumor cells in Invasive and DCIS when compared to the IL-8 staining in epithelial cells (Table 1B). Our IHC studies clearly demonstrate that *in vivo* tumor cells appear to be the major sources of both IL-1 and IL-8 cytokines and that staining for these cytokines was most intense in tumor cells. To extend these observations quantitatively, we next evaluated tissue levels of IL-1 cytokines and receptors as well as IL-8 cytokines in fresh human breast tissue.

IL1-α, IL-1β, IL-1Ra and IL-8 EXPRESSION IN HBC HOMOGENATES *(table 3)*

To begin our quantitative studies we initially analyzed HBC tumor homogenates for IL-1α, IL-1β, and IL-1 Ra cytokine levels (Table 3A). Analysis of breast tumor homogenates indicated that 71 of 82 (87%) cancer specimens had detectable levels of IL-1α (i.e. >5 pg./ml). The levels of IL-1α ranged from 0.94 pg./mg TP to 236.20 pg/mg TP, with a mean for the 82 samples of 17.6 ± 3.90 pg./mg TP. Analysis of tumor homogenates for IL-1β antigen indicated that 96 of 101 (95%) cancer specimens had detectable levels of IL-1β (i.e., > 5 pg./ml). The IL-1β levels ranged from 0.67 pg./mg TP to 96.3 pg./mg TP. The mean IL-1β value for the 101 samples was 12.02 ± 1.42 pg./mg TP. ELISA analysis of the HBC homogenates demonstrated the presence of IL-1α and IL-1β in HBC tumor homogenates in approximately equal concentrations (17.65 ± 3.9 pg./mg TP and 12.02 ± 1.42 pg./mg TP,) respectively. These data clearly demonstrate the presence of significant levels of IL-1α and IL-1β in HBC tissue.

Since we hypothesize that IL-1α and IL-1β induce IL-8 and it is known that the naturally occurring antagonist IL-1Ra can block IL-1 binding to receptors. We next determined IL-1Ra levels in the same HBC homogenates. IL-1Ra levels were determined in 65 tumor homogenates. 65 of 65 (100%) number of the homogenates had detectable levels of IL-1Ra, with a range of 1,118.0 - 108,114.0 pg./mg TP. The mean level of IL-1Ra in the homogenates was 18,741.0 +/- 2768.7 pg./mg TP. Since IL-1Ra is a naturally occurring competitive antagonist for the binding of IL-1α and IL-1β to IL-1 receptors, we also calculated the molar ratios for IL-1Ra for IL1α, IL-1β and IL-1α+IL-1β. As can be seen in table 3B, IL-1Ra consistently dominated IL-1α and IL-1β within the HBC breast tumor microenvironment. Specifically, IL-1Ra displayed between a 31.1 to over 800,000 fold higher ratio over the IL-1 cytokines (Table 3B). Since we hypothesize that IL-1 cytokines regulate IL-8 expression in HBC, we next determined the IL-8 levels in these HBC homogenates (Table 3A). Analysis of the tumor homogenates for IL-8 antigen indicated that 72 of 103 (70%) cancer specimens had detectable levels of IL-8 (i.e. > 40 pg./ml) ranging from 4.6 pg./mg TP to 3175.7 pg./mg TP. The mean IL-8 value for the 103

samples was 106.43 ± 38.72 pg./mg TP. Clearly, these data raise the question of the correlation's of the IL-1 family with each other (see below) as well as with IL-8, thus we next determined the IL-8 levels in these HBC tumor homogenates.

IL1-RI and IL-1RII EXPRESSION IN HBC HOMOGENATES (TABLE 3)

INSERT INFO HERE

IL-1, IL-8 AND ER/PR CORRELATIONS IN HBC HOMOGENATES *(table 4)*

Although above studies clearly demonstrate the presence of these IL-1 and IL-8 cytokines in the tumor microenvironment (i.e. homogenates), they do not directly demonstrate a relationship between these cytokines *in vivo*. To begin to investigate these relationships we conducted a correlation analysis of IL-1 and IL-8 cytokines and known prognostic indicators in HBC, i.e. ER/PR levels in the homogenates (Table 4). IL-1β levels correlated directly with IL-1Ra levels, and IL-1Ra directly correlated with ER levels in the HBC homogenates, i.e. low levels of IL-1Ra correlated with low levels of ER. Although IL-1Ra did directly correlate with IL-1β levels, IL-1α did not correlate with IL-1β levels in the tumor homogenates. Analysis of the IL-8 data indicated that IL-8 correlated with all cytokine and ER/PR levels in the HBC homogenates (table 4). Specifically IL-8 inversely correlated with ER, PR and IL-1Ra, and correlated directly with IL-1α, IL-1β and IL-1α+IL-1β. These studies not only demonstrate the presence of the IL-1 family of cytokines in HBC tissue, but also demonstrate that there is a relationship between the levels of the angiogenic factor IL-8 and the levels of IL-1 related cytokines in the tumor tissue. Although this data does support the hypothesis of the role of IL-1 family of cytokines and receptors in the progression of HBC, further invivo and invitro studies using established HBC cell lines were undertaken.

IL-1RI AND IL-1RII CORRELATIONS IN HBC HOMOGENATES (TABLE 4)

Insert info here

IL-1A, IL-B, IL-1Ra AND IL-8 EXPRESSION IN HBC-MOUSE XENOGRAFT MODEL

(INSERT immunohisto DATA ON THE 3 CELL LINE STUDIES)

IL-1 RECEPTOR EXPRESSION IN HBC-MOUSE XENOGRAFT MODEL *(figure 2)*

(INSERT INFO AND DATA)

IL-1α, IL-1β AND IL-1Ra EXPRESSION BY HBC CELLS IN VITRO

IMMUNOHISTO DATA
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IL-1 RECEPTOR RI AND RII EXPRESSION IN HBC TUMOR CELLS INVITRO *(fig 3)*

INSERT INFO AND DATA

IL-1 STIMULATION OF IL-8 EXPRESSION BY HBC CELLS INVITRO *(figure 4)*

All the data to this point supports our hypothesis that tumor expression of IL-1 results in the autocrine expression of protumorigenic factor such as IL-8 from the tumor cells themselves. To test this part of the hypothesis directly we next conducted *in vitro* studies to determine the ability of IL-1α or IL-1β to directly induce IL-8 expression from HBC tumor cells. IL-1 cytokines are known to be potent inducers of IL-8 expression in leukocytes and normal tissue cells [25]. Therefore, we initiated our invitro studies by investigating the ability of IL-1α and IL-1β to induce IL-8 expression in malignant breast epithelial cells. For these studies we utilized the estrogen dependent cell lines MCF 7 and ZR-75, as well as the estrogen independent cell line MDA-MB-231. Evaluation of the ability of IL-1α or IL-1β to induce IL-8 expression in estrogen-dependent cell lines demonstrated a clear heterogeneity in IL-8 responses (see figure 4). For example, the estrogen dependent cells MCF- 7 showed no response to IL-1 stimulation (IL-1α: SI=1.08, IL-1β:SI=1.37) but the ZR-75-1 BCC demonstrated profound IL-8 stimulation (IL-1α:SI=120.26, IL-1β:SI=133.99). IL-1 cytokines were extremely potent inducers of IL-8 expression in the estrogen independent BCC line MDA-MB-231. IL-1α or IL-1β induced SI of over 300-fold in the MDA-MB-231 cells. This data not only emphasizes the ability of IL-1 to induce IL-8 expression from human breast tumors, but also that a marked heterogeneity exists in individual breast cell responsiveness to IL-1. The heterogeneity of responsiveness of the different tumor cell lines may be a result of differential expression of the IL-1 receptors. To investigate this possibility we next conducted studies to characterize the expression of IL-1RI and IL-1RII in the BCC lines.

INVITRO EXPRESSION OF IL-1 BY HBC CELLS (FIGURE 3 &TABLE 3 AND 4)

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DISCUSSION

Cytokines such as IL-1 and TNF are considered to be key regulators of tissue cells and leukocytes in chronic inflammatory diseases such as rheumatoid arthritis [26-32], inflammatory bowel disease [33, 34], and interstitial lung disease [35] to name but a few. It has been further postulated that in the case of acute and chronic inflammation, cytokines such as IL-1 and TNF are present in the tissue microenvironment in quantities sufficient to control both the inflammatory and repair processes. Thus, the key role of cytokines in general, and IL-1 specifically, in inflammation and wound healing is clearly established. Since tumors can be considered "wounds that never heal", do to their ever expanding tissue invasion and injury, It like highly likely that cytokines such as IL-1 play a central in tumor growth and metastatetis. Thus we hypothesize that tumor derived proinflammatory cytokines such as IL-1 play a key role in cancers by regulating the functions of tumor, tissue and immunologic cells within the tumor microenvironment via expression IL-1 receptors on these cells. The prior studies that are the foundations of our hypothesis on the role of IL-1 and IL-1 receptors in human breast cancer are presented below.

Interleukin 1 Cytokine Family

Currently the IL-1 family of cytokines and receptors encompasses two agonist polypeptides (IL-1α and IL-1β), a competitive antagonist (IL-1Ra) and two receptors (IL-1RI and IL-1RII). Interleukin-1 (IL-1) designates two distinct proteins, IL-1α and IL-1β. These forms are structurally related and show approximately 25% amino acid homology. IL-1α is the acidic form while IL-1β is the neutral form [23]. Both IL-1α and IL-1β are synthesized as 31kD precursors, which are cleaved into 17kD proteins. Interestingly, these cytokines lack classical signal peptides (for secretion) yet while IL-1α remains intracellular; IL-1β is secreted out of the cell [24]. IL-1α and IL-1β exert their physiological effects by binding to specific receptors. There are two IL-1 receptors, IL-1 receptor I (IL-1RI) and IL-1 receptor II (IL-1RII). IL-1RI is a 80kD

membrane bound receptor while IL-1RII is 68kD's. They are both members of the Ig superfamily. The two receptors share 28% homology in their extracellular domains but differ in their cytoplasmic regions. Where IL-1RI has a 213 amino acid cytoplasmic domain, IL-1RII contains only 29 amino acids in this region. IL-1RI is the signal transducing receptor and IL-1RII does not transduce signal when IL-1 is bound to it. Generally, IL-1α binds with higher affinity to IL-1RI while IL-1β binds better to IL-1RII. IL-1RI is found on endothelial cells, hepatocytes and T lymphocytes while IL-1RII can be found on B lymphocytes, monocytes and neutrophils [36].

Interleukin-1β (IL-1β) is considered a key or prime inflammatory/immunologic regulatory cytokine. It is produced very rapidly during the inflammatory response and it is known to induce the expression of a wide number of cytokines [31]. *In vitro* studies have demonstrated that IL-1 activity can be inhibited by the naturally occurring receptor antagonist designated IL-1 receptor antagonist (IL-1Ra) [32]. These studies have further demonstrated that the ratio of IL-1Ra to IL-1 in the range of 100:1 to 1000:1 can inhibit IL-1 activity [28, 29]. Investigators have studied the role of IL-1 and IL-1Ra in wound healing, sepsis and chronic inflammatory diseases such as arthritis [35] and inflammatory bowel disease [28]. In these studies, a decrease in the ratio of IL-1Ra to IL-1 was associated with a more severe disease state (i.e. more inflammation and tissue destruction). Thus the multifunctional roles of the IL-1family of cytokines in inflammation and immunology are becoming increasingly clear, but the role of IL-1 in cancer is less well understood.

IL-1 Family and Cancer

A considerable volume of literature exists demonstrating the in vitro ability of IL-1 cytokines to regulate a variety of cellular functions in tumor cells [11-17]. Interestingly, IL-1 cytokines have been shown to have diverse and sometimes contradictory effects on the functions and growth of tumor cells. For example, in some instances IL-1 exerts growth-inhibitory activity, yet in other situations IL-1 stimulates tumor growth [18]. In the case of breast cancer, In vitro studies have shown that IL-1 can induce a variety of factors and functions in breast cancer cells. For example, malignant human breast tumors are known to contain high levels of prostaglandins. In a recent study [37], IL-1β induced PGE2 production in breast fibroblasts. In that study, only two breast cell lines, MDA-MB-231 and Hs578T demonstrated increases of PGE2 in response to IL-1βs, several other BCC lines did not respond. IL-1 is also known to inhibit the growth of cultured BCC. For example, insulin and insulin like growth factor I (IGF-I) induced BCC proliferation was inhibited by the presence of IL-1α and IL-1β in MCF-7 cells. In that study, insulin receptor protein and mRNA were increased in the presence of IL-1β. Additional data in that study suggests that IL-1 antagonizes insulin and IGF-I mitogenic effects in MCF-7 by blocking tyrosine kinase [33]. Danforth et al, examined the ability of IL-1 and IL-6 to inhibit BCC growth *in vitro*. These investigators found that both IL-1 and IL-6 inhibited growth of MCF-7 BCC. IL-1 alone had a greater effect than IL-6 alone. When the two were combined, the effect was synergistic. Further, IL-1 and IL-6 decreased the estradiol stimulated growth of the BCC. Additionally, their studies also demonstrated that IL-1, but not IL-6, caused increased secretion of TGF-b by the BCC (34[38]. Interestingly, studies by Speiser et al found that IL-1 up-regulated HLA class I and HLA Class II (DR) antigen expression on the cell surface of ZR-75-1 cells. This up-regulation of antigens was associated with increased TNF expression [34]. Thus, IL-1 appears to play an important distribution in the regulation of breast cancer cell function and growth in vitro. Surprisingly little is known about the presence, distribution and role of IL-1 in human breast cancer *in vivo* [19, 20], and nothing is know about IL-1 receptors in cancer. Recent efforts in our laboratory are intended to begin to fill this gap in our knowledge regarding the presence and localization of IL-1 receptors in human breast cancer. For example in

a recent study, we have demonstrated the expression of IL-1α and IL-1β in malignant and non-malignant human breast tissue [39]. These cytokines were found to be associated with breast tumor epithelial cells within the tumor microenvironment. Non-malignant breast epithelial cells also expressed IL-1. Our studies also demonstrated an inverse relationship between IL-1α and ER existed, i.e. as the levels of IL-1α increased the ER level decreased. This would suggest that elevated IL-1α levels are associated with poorer clinical outcome. Interestingly, Few studies have investigated the invivo or invitro relationship of IL-1 and it's naturally occurring antagonist IL-1Ra in human cancer.

Expression of IL-1Ra has been reported in endometrial cancer [40], bronchogenic carcinoma [41], and head & neck squamous cell carcinoma [42]. In the studies by Van Le [40] and Smith [41] increased levels of IL-1Ra was found in tumor cells when compared with normal tissue. In the study by von Biberstein [42] a lower levels of IL-1Ra was seen in the tumor versus normal tissue. Interestingly, while some believe that IL-1Ra has the role of blocking IL-1 induced inflammation and disease, others have hypothesized that IL-1Ra actually can support the malignant growth by blocking the growth-inhibiting autocrine loop of IL-1. A recent study by Oelmann et al [18] found that glioblastoma cell lines over expressed IL-1Ra. Needless to say, this conflicting data indicates the complexity of the network of IL-1, its receptors, and its antagonist and the need for further study in this area.

Recently, we have investigated the expression of IL-1 in HBC [39].

Based on these observations we have constructed a hypothetical model describing the role of IL-1 cytokines and receptors within the breast cancer microenvironment. In this model, we hypothesize that HBC tumor cells express both IL-1 angonists (IL-1α and IL-1β) and antagonists (IL-1Ra). Theses IL-1 cytokines then act in both an autocrine and paracrine fashion via IL-1 receptors (IL-1RII and IL-1RII) to: 1) regulate tumor and tissue cell proliferation; and 2) control production of protumorigenic factors such as angiogenic factors, growth factors, metalloproteases, etc. Interestingly, and in support of our hypothesis, tissue levels of both IL-1α and IL-1β both correlated with tissue levels of IL-8, a known angiogenic factor.

IL-1 RECEPTORS

WHAT THEY ARE HOW THEY WORK ETC

SEE H/N PAPER

IL-1 RECEPTORS IN HUMAN DISEASE

SEE H/N PAPER

IL-1 RECEPTORS IN CANCER

SEE H/N PAPER

IL-1 RECEPTORS IN BREAST CANCER

LITERATURE SUMMARY

OUR DATA

CONCLUSION AND FUTURE DIRECTIONS

Therefore, future in vivo studies to evaluate our hypothetical model are needed to directly demonstrate the pivotal role that the IL-1 family of cytokines plays in regulating tumor growth and metastasis.

ACKNOWLEDGEMENTS

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FIGURES

FIGURE 1: IMMUNOHISTOCHEMICAL ANALYSIS OF THE EXPRESSION OF THE INTERLEUKIN 1 FAMILY OF CYTOKINES (IL-1α, IL-1β, IL-1 RA) AND RECEPTORS (IL-1RI, IL-1RII) IN TUMOR AND DUCTAL EPITHELIAL CELLS HUMAN BREAST DISEASE TISSUE

FIGURE 2: IMMUNOHISTOCHEMICAL ANALYSIS OF THE EXPRESSION OF INTERLEUKIN 1 RECEPTORS (IL-1RI AND IL-1RII) IN HUMAN BREAST CANCER-MOUSE XENOGRAFTS

FIGURE 3: IMMUNOCYTOCHEMICAL ANALYSIS OF THE EXPRESSION OF INTERLEUKIN 1 RECEPTORS (IL-1RI AND IL-1RII) IN HUMAN BREAST CANCER CELL LINES INVITRO

FIGURE 4: INTERLEUKIN 1 INDUCED EXPRESSION OF INTERLEUKIN 8 IN HUMAN BREAST CANCER CELL LINES INVITRO

TABLES

TABLE 1A : IMMUNOHISTOCHEMICAL ANALYSIS OF INTERLEUKIN 1 FAMILY OF CYTOKINES (IL-1α, IL-1β, IL-1 RA) AND RECEPTOR (IL-1RI, IL-1RII) EXPRESSION BY TUMOR AND DUCTAL EPITHELIAL CELLS IN HUMAN BREAST CANCER: PERCENT POSITIVE STAINING

TABLE 1B: IMMUNOHISTOCHEMICAL ANALYSIS OF INTERLEUKIN 1 FAMILY OF CYTOKINES (IL-1α, IL-1β, IL-1 RA) AND RECEPTOR (IL-1RI, IL-1RII) EXPRESSION IN HUMAN BREAST DISEASE SPECIMENS: STAINING INTENSITY

TABLE 1C: CORRELATION OF INTERLEUKIN 1 FAMILY OF CYTOKINES (IL-1α, IL-1β, IL-1 RA) AND RECEPTOR (IL-1RI, IL-1RII) EXPRESSION IN HUMAN BREAST DISEASE SPECIMENS: STAINING INTENSITY

TABLE 2A: IMMUNOHISTOCHEMICAL ANALYSIS OF INTERLEUKIN 1 RECEPTOR (IL-1RI, IL-1RII) EXPRESSION IN HUMAN BREAST DISEASE TISSUE: PERCENT POSITIVE STAINING

TABLE 2B: IMMUNOHISTOCHEMICAL ANALYSIS OF INTERLEUKIN 1 RECEPTOR (IL-1RI, IL-1RII) EXPRESSION IN HUMAN BREAST DISEASE TISSUE: STAINING INTENSITY

TABLE 3A: INTERLEUKIN 1 FAMILY OF CYTOKINES (IL-1α, IL-1β, IL-1 RA) AND RECEPTOR (IL-1RI, IL-1RII) LEVELS IN HUMAN BREAST CANCER TISSUE HOMOGENATES

TABLE 3B: INTERLEUKIN 1 FAMILY OF CYTOKINES (IL-1A, IL-1β, IL-1 RA) AND RECEPTOR (IL-1RI, IL-1RII) RATIOS FOR IL-1 FAMILY IN HUMAN BREAST CANCER TISSUE HOMOGENATES

TABLE 4: CORRELATION OF INTERLEUKIN 1 FAMILY OF CYTOKINES (IL-1A, IL-1β, IL-1 RA) AND RECEPTOR (IL-1RI, IL-1RII) LEVELS IN HUMAN BREAST CANCER TISSUE HOMOGENATES

TABLE 1A

IMMUNOHISTOCHEMICAL ANALYSIS OF TUMOR AND DUCTAL EPITHELIAL CELLS IN HUMAN BREAST DISEASE FOR EXPRESSION OF THE INTERLEUKIN 1 FAMILY OF CYTOKINES (IL-1α, IL-1β, IL-1 ra) RECEPTORS (IL-1RI, IL-1RII).

Specimen	Tissue Cells – Percent Positive Staining											
	Tumor Cells						Ductal Epithelial Cells					
	IL1α	IL1β	ILRA	IL1RI	IL1RII	IL8	IL1α	IL1β	ILRA	IL1RI	IL1RII	IL8
Invasive	100% (32/32)	88% (23/26)	100% (23/23)	96% (28/29)	100% (25/25)	97% (35)	100% (32/32)	77% (14/18)	86% (20/23)	66% (6/9)	83% (10/12)	62% (13/21)[a]
DCIS	100% (10/10)	78% (7/9)	100% (5/5)	87% (7/8)	100% (8/8)	100% (7/7)	90% (9/10)	56% (5/9)	75% (4/5)	75% (6/8)	38% (3/8)	100% (6/6)
Benign	---	---	---	---	---	---	100% (7/7)	86% (6/7)	100% (5/5)	71% (5/7)	86% (6/7)	100% (8/8)

(#) = number of specimens

[a] = not all slides contained normal ducts

TABLE 1B
IMMUNOHISTOCHEMICAL ANALYSIS OF TUMOR AND DUCTAL EPITHELIAL CELLS IN HUMAN BREAST DISEASE FOR EXPRESSION OF THE INTERLEUKIN 1 FAMILY OF CYTOKINES (IL-1α, IL-1β, IL-1RA) AND RECEPTOR (IL-1 RI, IL-1RII): STAINING DENSITY

Tissue	Cell Type	IL-1α (Mean Values ± SEM)	IL-1β (Mean Values ± SEM)	IL-1RA (Mean Values ± SEM)	IL-1RI (Mean Values ± SEM)	IL-1RII (Mean Values ± SEM)	IL-8 (Mean Values ± SEM)
Invasive	Tumor	2.5 ± 0.11[a]	1.86 ± 0.20	2.6 ± 0.18	2.21 ± 0.16	3.04 ± 0.15	2.5 ± 0.17
DCIS	Tumor	2.5 ± 0.22	2.11 — 0.32	1.8 ± 0.38	2.0 ± 0.37	2.7 ± 0.37	2.8 ± 0.40
Benign	Epithelial	1.5 ± 0.36	0.75 ± 0.25	0.88 ± 0.38	1.0 ± 0.37	1.2 ± 0.35	1.0 ± 0.37

TABLE 1C
CORRELATION OF INTERLEUKIN 1 FAMILY OF CYTOKINES (IL-1α, IL-1β, IL-1RA) AND RECEPTORS IL-1 RI, IL-1RII) IN HUMAN BREAST DISEASE SPECIMENS: STAINING INTENSITY

Tissue	IL-1α (p values)	IL-1β (p Values)	IL-1RA (p Values)	IL-1RI (p Values)	IL-1RII (p Values)	IL-8 (p Values)
Invasive vs. Benign	0.002[b]	0.04	0.0002	0.004	0.000	0.0007
DCIS vs. Benign	0.006	0.025	0.095	0.046	0.0004	0.0015
Invasive vs. DCIS	NS[c]	0.52	0.054	NS	NS	NS

[a]Mean Values (tumor mean ± SEM)
[b]p Value
[c]NS – Not Significant.

TABLE 2A

IMMUNOHISTOCHEMICAL ANALYSIS OF HUMAN BREAST DISEASE TISSUE FOR INTERLEUKIN 1 RECEPTOR (IL-1RI, IL-1RII) EXPRESSION: PERCENT POSITIVE STAINING

Tissue Type	Tumor or Epithelial		Normal Ducts		Small Vessel Endothelium		Large Vessel Endothelium		Smooth Muscle		Fibroblasts		Stroma	
	IL-1RI	IL-1RII	IL-1RI	IL-1RII	IL-1RI	IL-1RII	IL-1RI	IL-1RII	IL-1RI	IL-1RII	IL-1RI	IL-1RII	IL-1RI	IL-1RII
Benign	NA	NA	5/7 (71%)	6/7 (86%)	5/7 (71%)	3/7 (43%)	4/7 (57%)	3/7 (43%)	7/7 (100%)	4/7 (57%)	1/6 (14%)	0/7 (0%)	6/7 (86%)	0/7 (0%)
DCIS	7/8[a] (87%)[b]	8/8 (100%)	6/8 (75%)	3/8 (38%)	6/7[d] (86/%)	3/8 (38%)	4/7[d] (57%)	3/8 (38%)	7/7[d] (100%)	7/8 (88%)	3/7[d] (42%)	0/8 (0%)	8/8 (100%)	0/8 (0%)
Invasive	28/29 (96%)	25/25 (100%)	6/9[c] (66%)	10/12 (83%)	21/29 (72%)	19/25 (76%)	12/29 (41%)	24/29 (82%)	21/25 (84%)	21/25 (84%)	16/24[e] (66%)	12/23[e] (52%)	23/28[e] (82%)	3/25 (12%)

[a]Number of slides staining positive/all slides stained.
[b]( ) Percent of slides staining positive for indicated cell type.
[c]Not all slides contained normal ducts.
[d]Small vessels, large vessels and fibroblasts not found in 1 sample.
[e]Fibroblasts not found in all samples.

TABLE 2B

IMMUNOHISTOCHEMICAL ANALYSIS OF HUMAN BREAST DISEASE TISSUE FOR INTERLEUKIN 1 RECEPTOR (IL-1RI, IL-1RII) EXPRESSION IN: STAINING INTENSITY

Tissue Type	Tumor or Epithelial		Normal Ducts		Small Vessel Endothelium		Large Vessel Endothelium		Smooth Muscle		Fibroblasts		Stroma	
	IL-1RI	IL-1RII	IL-1RI	IL-1RII	IL-1RI	IL-1RII	IL-1RI	IL-1RII	IL-1RI	IL-1RII	IL-1RI	IL-1RII	IL-1RI	IL-1RI
Benign	1.0±0.37[b]	1.2±0.35	1.0±0.38	1.2±0.35	1.0±0.38	0.71±0.36	0.86±0.40	0.43±0.20	1.29±0.18	0.71±0.29	0.16±0.16	0 ± 0	1.9±0.28	0
DCIS	2.0±0.37 (p<0.046)	2.7±0.16 (p<0.0004)	1.25±0.37 (NS)	0.37±0.18 (p<0.004)	1.0±0.22 (NS)	0.50±0.27 (NS)	0.57±0.20 (NS)	0.38±0.18 (NS)	2.29±0.36 (p<0.057)	1.0±0.19 (NS)	0.43±0.20 (NS)	0 ± 0 (NS)	1.81±0.33 (NS)	0
Invasive	2.21±0.16 (p<0.004) [NS][d]	3.04±0.15 (p<0.000) [NS]	1.12±0.30 (NS) [NS]	0.87±0.15 (p<0.055) [NS]	1.29±0.17 (NS) [NS]	1.24±0.19 (NS) [p<0.050]	0.56±0.13 (NS) [NS]	0.80±0.17 (NS) [NS]	1.77±0.19 (NS) [NS]	1.6±0.21 (p<0.029) [NS]	1.0±0.17 (p<0.010) [p<0.008]	0.59±0.14 (p<0.012) [p<0.008[	1.5±0.14 (NS) [NS]	0

[a]Immunohistochemical staining grading scale: 0 = stain; 1 = faint stain; 2 = light stain; 3 = moderate stain; 4 = intense stain.
[b]Mean ± SEM for intensity of staining.
[c]( ) = p value for comparison of diseased vs. benign breast tissue.
[d][ ] = p value for comparison of DCIS vs. Invasive Breast Cancer (IBC).
[e]Statistical significance considered to be $p < 0.05$.

TABLE 2C

Summary of Positive Immunohistochemical Staining For IL-1 Receptor RI and IL-1 RII in Human Breast Disease

Tissue Type	Benign		DCIS		Invasive	
	IL-1rI	IL-1rII	IL-1rI	IL-1rII	IL-1rI	IL-1rII
Tumor Cells	NA	NA	++++	++++	++++	++++
Normal Ducts	+++	++++	+++	++	+++	++++
Small Vessel Endothelium	+++	++	+++	++	+++	++++
Large Vessel Endothelium	+++	++	+++	++	++	+++
Smooth Muscle	++++	+++	++++	++++	++++	++++
Fibroblasts	+	+	++	+	+++	+++
Stroma	++++	+	++++	+	++++	+

[a] Percent of total staining positive:
+ = 0-25%
++ = 26-50%
+++ = 51-75%
++++ = 76-100%.

TABLE 3A

INTERLEUKIN 1 FAMILY OF CYTOKINES (IL-1a, IL-1B, IL-1 RA) AND RECEPTOR (IL-1RI, IL-1RII) LEVELS IN HUMAN BREAST CANCER TISSUE HOMOGENATES

Cytokine	Number of HBC Homogenates Assayed	Cytokine mean ± sem (pg/mg TP)[a]	Cytokine Range (pg/mg TP)[a]
IL-1α	82	17.6 ± 3.9	0.9 - 236.2
IL-1β	101	12.0 ± 1.4	0.7 - 96.3
IL-1RA	65	18,741.0 ± 2768.7	1,118.0 - 108,114.0
IL-8	103	106.4 ± 38.7	4.6 – 3175.7

a. pg/mg TP: picograms cytokine per mg total protein

TABLE 3B

INTERLEUKIN 1 FAMILY OF CYTOKINES (IL-1A, IL-1B, IL-1 RA) AND RECEPTOR (IL-1RI, IL-1RII) RATIOS FOR IL-1 FAMILY IN HUMAN BREAST CANCER TISSUE HOMOGENATES

Cytokine Ratio[b]	Number of HBC Homogenates Assayed	Cytokine Index[c] mean ± sem	Cytokine Index Range
IL-1RA / IL-1α	54	33,771.0 ± 15,946.1	59.3 - 809,248.7
IL-1RA / IL-1β	64	1,990.6 ± 474.9	35.0 - 24,896.2
IL1-RA / IL-1α + β	55	963.4 ± 317.3	31.1 - 3835.3

b. cytokine ratio = Index
c. indexes calculated on molar basis