Effects of Two Cancer Genes, HTLV-1 Tax and E-Cadherin, on Cancer Development and Progression

Dissertation

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By

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Abstract

The research presented in this dissertation focuses on the effects of expression of two different genes in cancer. First is a study on the loss of expression of the tumor suppressor gene, E-cadherin, in canine mammary cancer. The loss of E-cadherin has been associated with increased tumor invasiveness and poor prognosis in a number of neoplasias including human pancreatic, colorectal and breast cancer. This loss is most frequently attributed to mutations, deletions and hypermethylation of the E-cadherin promoter. The mechanism for the loss of E-cadherin expression in canine mammary cancer is not well understood. To examine the role of methylation in E-cadherin gene silencing, bisulfite sequencing was used. First, expression of E-cadherin in normal, hyperplastic, benign and malignant canine mammary tissue was determined using immunohistochemistry. The mammary gland was then isolated using laser capture microdissection and the DNA was purified. The purified DNA underwent bisulfite treatment and subsequent bisulfite sequencing to determine the degree of methylation within each sample. The immunohistochemistry confirmed a correlation between tissue diagnosis and the level of membranous
staining for E-cadherin. A correlation between methylation and this difference in gene expression could not be determined, however.

The second gene examined in this dissertation is the viral ongogene, Tax. Tax is encoded in the regulatory pX region of the Human T Lymphotrophic Virus Type-1 (HTLV-1) genome. HTLV-1 is the causative agent of adult T-cell leukemia/lymphoma (ATL) and HTLV-1 Tax has been shown to be necessary and sufficient for viral oncogenesis. To study the effect of Tax in oncogenesis and cancer progression, we examined transgenic mice that express HTLV-1 Tax under regulation of the human granzyme B promoter as well as double transgenic mice that express HTLV-1 Tax and are also deficient in IFN-γ (Tax+/IFN-γ−/−). Through gross examination, histology, flow cytometry, immunohistochemistry and imaging, we determined that these mice develop histiocytic sarcomas that arise from tendon sheaths and invade bone resulting in osteolysis.

To further understand the function of HTLV-1 Tax in tumor formation and progression, three cell lines were derived from tumors that spontaneously formed in Tax+/IFN-γ−/− mice. These cell lines, T94, 501 and Tom3 were all immortal in culture. T94 cells were determined to be a poorly differentiated sarcoma and 501 and Tom3 cells were determined to be macrophage/monocyte origin based on flow cytometry. All three cell lines formed tumors when injected subcutaneously, intratibially and bone adjacent in nude mice. Mice injected with T94 and 501 cells developed humoral hypercalcemia of malignancy (HHM) which correlated well
with increased expression of PTHrP in these cell lines. Mice injected with Tom3 cells developed splenomegaly as the result of increased myelopoiesis and marked neutrophilic infiltration into the tumors. This finding corresponded to a marked increase in granulocyte-colony stimulating factor expression by this cell line compared to the other two. Finally, an increase in ex vivo osteolysis was found in 501 and Tom3 cells. This finding correlated with increased expression of IL-1α and IL-1β in these cell lines.

In summary, in this dissertation research, we found a correlations between loss of E-cadherin expression and invasive mammary cancer in dogs, and increased expression of PTHrP, IL-1 and G-CSF that correlated to the development of HHM, osteolysis and neutrophilic infiltration in Tax-induced malignant cells in vivo and in vitro. These findings support further use of these model systems for mechanistic and therapeutic studies of breast cancer and ATL.
Dedicated to my parents Kathleen, Timothy and Melanie; my husband, Tim and my daughters, Molly and Kathleen.
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Dr. Steven Weisbrode and Dr. Paul Stromberg have been instrumental in developing and shaping my skills as a veterinary pathologist. Their dedication and skill in performing and teaching veterinary pathology are skills I aspire to follow in my own career.

I would like to thank my family. My husband, Tim has been a constant source of support throughout my residency and PhD training. Without his unwavering support and encouragement, I could not have completed this program. To my parents, Tim and Melanie, thank you for pushing me to follow my dreams and supporting me through all of these years of education. I will be forever grateful.
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FIELD OF STUDY

Major Field: Veterinary Biosciences
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Chapter 1: Methylation of the E-Cadherin Tumor Suppressor Gene in Canine Mammary Cancer

Abstract

Loss of E-cadherin expression in epithelial cancers correlates with increased tumor invasiveness, metastasis and poor prognosis in humans and animals. Loss can be attributed to mutations, deletions, and/or promoter methylation. Little is known about the mechanism(s) for the loss of E-cadherin expression and subsequent neoplastic progression in canine mammary tumors. We hypothesized that promoter methylation of E-cadherin contributed to loss of E-cadherin gene expression in canine mammary tumors and that loss was correlated with increased invasiveness. In order to test this hypothesis, immunohistochemistry for E-cadherin was performed on histological sections of normal, hyperplastic and neoplastic mammary tissues to assess E-cadherin expression. Epithelial cells from mammary tissues were isolated from formalin-fixed, paraffin-embedded sections using laser capture microdissection. DNA was extracted from the cells, modified by bisulfite treatment, and sequenced to identify methylated cytosines. Fourteen tissue samples were sequenced. For
each sample, at least 4 alleles were sequenced. The alleles in all of the samples showed an all-or-none pattern of methylation, where each allele was either >95% or <5% methylated. The only exception was an apparent methylation boundary following a poly-A segment between the 6th and 7th methylation site. The results of the bisulfite sequencing demonstrated a poor correlation between the presence of methylation in the alleles and tumor invasiveness. Immunohistochemistry for E-cadherin was performed on 31 tissue samples. Although a correlation was not present between methylation and protein expression, the malignant mammary tumors had decreased E-cadherin expression.

**Introduction**

Canine mammary tumors have an approximately 40% malignancy rate and rank second only to skin tumors as the most common canine neoplasms, and are the most common tumor type in intact female dogs.\(^\text{10, 15}\) Mammary tumors are frequently classified based on their histologic and morphologic characteristics. Carcinomas frequently metastasize through lymphatics to regional lymph nodes with distant metastasis occurring most commonly in the lungs.\(^\text{18}\) Although the routes of metastasis are understood, the mechanisms of cancer progression and metastasis in canine mammary cancers are not. A greater understanding of the pathogenesis of carcinogenesis and metastasis will lead to adjunct treatment options which are not available at this time.
One possible mechanism for the induction of metastasis is loss of epithelial cell-cell adhesion, which allows cells to dissociate and form distant metastases. Previous studies have shown that reduction or loss of expression of E-cadherin, a transmembrane glycoprotein involved in cell-cell adhesion, correlated with increased tumor invasiveness, metastasis, and poor prognosis in humans, dogs and cats.\textsuperscript{3, 23, 26} The mechanisms for decreased E-cadherin expression in human breast cancers have been attributed to inactivating mutations, deletions, or DNA methylation.\textsuperscript{16} The reversibility of the epigenetic silencing of E-cadherin marks an important distinction between DNA methylation and irreversible mutations and deletions. Previous studies have shown that loss of E-cadherin expression may be transient, with down-regulation detected in primary tumors and subsequent up-regulation of E-cadherin in distant metastases.\textsuperscript{5, 6} The loss of E-cadherin expression also has been implicated as a basis for deregulation of cell growth and a primary step in epithelial-mesenchymal transition, both of which are indicators of cancer progression. Sarli et al. used immunohistochemistry to demonstrate decreased membrane expression of E-cadherin in canine mammary carcinomas compared to normal mammary tissue. In the same study, increased expression of E-cadherin was seen in metastatic cells in regional lymph nodes in comparison to the primary mammary tumor.\textsuperscript{23} This finding was suggestive of a reversible change such as DNA methylation rather than an irreversible genetic alteration such as mutation or deletion.
DNA methylation is an epigenetic modification that can alter the expression of a gene without modifying the DNA sequence. Methylation occurs when a methyl group is added to the 5’ carbon of a CpG dinucleotide (a cytosine 5’ to a guanine separated by a phosphodiester bond) by DNA methyltransferase enzymes. As a regulatory mechanism, methylation occurs in areas near a gene promoter with high CpG densities referred to as CpG islands. A CpG island is defined as an area greater than or equal to 200 base pairs with a GC content above 50% and a CpG ratio (observed/expected) above 0.6.\textsuperscript{2} The methylation of CpG islands has been correlated to gene transcription silencing. In cancer, hypermethylation of a tumor suppressor gene can be one of the “two-hits” in the Knudson two-hit hypothesis which describes loss of transcription of a tumor suppressor gene by loss of function of each copy of the gene.\textsuperscript{7} In this study, we hypothesized that promoter methylation was responsible for transcriptional inactivation of E-cadherin in canine mammary dysplasias and tumors.

**Materials and Methods**

*CpG island location*

Homology between the human and canine E-cadherin genes was analyzed. Within the 5’ region and exon 1, an area with increased frequency of CG dinucleotides (CpG island) corresponding to an area of interest in the human gene was identified. This region was targeted for primer design for bisulfite PCR and DNA sequencing.
Sample selection

Formalin-fixed, paraffin-embedded sections of normal, hyperplastic and neoplastic mammary tissues as well as metastatic tumors were selected from the Ohio State University College of Veterinary Medicine pathology archives. H&E sections were evaluated by LGL and LJR to confirm the histopathologic diagnosis. Classification of tumors were based on the World Health Organization classification of breast cancer.\textsuperscript{27} Additionally, normal mammary tissue was obtained from the local dog shelter following humane euthanasia.

Immunohistochemistry staining and scoring

Immunohistochemistry for E-cadherin was performed on 31 paraffin-embedded tissue sections using the avidin-biotin complex (ABC) method as described by Ramos-Vara.\textsuperscript{19} The primary antibody was a monoclonal mouse anti-E-cadherin (BD Biosciences, Rockville, MD) and was used at a 1:200 concentration, the biotinylated secondary antibody was horse anti-mouse (Vector Laboratories, Burlingame, CA) and was also used at 1:200. The slides were evaluated and scored by LGL and board-certified veterinary pathologist, LJR. Immunohistochemical scores were based on degree and cellular location of staining using a scoring system adapted from Fisher et al. 1994.\textsuperscript{1,11} The scoring consisted of strong, moderate, weak and negative membrane as described in Table 1.1. Examples of the staining scores can be seen in Figure 1.1.
**Laser capture microdissection and DNA isolation**

Laser capture microdissection (LCM) was used to isolate mammary epithelial cells from the surrounding stroma (PixCell II, Applied Biosystems®). The technique uses an infrared laser to select the desired formalin-fixed, paraffin-embedded epithelial cells and pull them free from the surrounding tissue without causing damage to the cellular DNA. An example laser capture microdissected tissue is in Figure 1.2.

DNA was isolated from the LCM tissues. Fifty µL of proteinase K digestion buffer consisting of 1 mg/ml proteinase K (Roche, Indianapolis, IN) and 1% Tween 20 (Sigma, St. Louis, MO) in an 8.0 pH TE (Invitrogen, Carlsbad, CA) buffer was used to remove the cells from the laser capture cap. Further digestion of the tissue was performed by adding 4µL of pure proteinase K (Qiagen, Valencia, CA) to the samples and incubating at 65°C overnight for 2 consecutive nights. Following tissue lysis, DNA was extracted using the QIAamp DNA Kit (Qiagen) according to the manufacturer’s instructions. Two elutions were used for each sample and were combined for a final volume of 100µL per sample. DNA was stored at 4°C prior to bisulfite treatment. DNA from normal liver, spleen and kidney for use as positive control was isolated using the DNeasy Kit (Qiagen).
Bisulfite treatment and Bisulfite Sequencing PCR

In order to differentiate methylated from unmethylated DNA, bisulfite treatment was used. Using this procedure, all unmethylated cytosines were converted to thiamines with the methylated cytosines remaining unchanged. Each 100 µL sample of microdissected DNA was concentrated by heating and centrifugation to a final volume of 45 µL prior to bisulfite treatment. Five µg yeast tRNA and 5.5 µL of fresh 3M NaOH were added to the samples followed by 20 min. incubation at 42°C. Six hundred µL bisulfite /hydroquinone solution consisting of 3.9-4.3 M sodium bisulfite (Fisher) and 10 mM hydroquinone at a pH of 5.0 was added followed by incubation at 55°C for 4 hr in the dark. The DNA was isolated using the QIAquick Gel Extraction Kit (Qiagen) and treated further with 5.5 µL fresh 3M NaOH and 25 min incubation at 37°C prior to further purification with the QIAquick Gel Extraction Kit (Qiagen). The final elution of 30 µL was stored at -20°C prior to bisulfite sequencing PCR. Ten µg of each positive control DNA sample underwent in vitro methylation using SSsI (New England Biolabs). Both the in vitro methylated and normal unmethylated DNA underwent bisulfite treatment according as previously described.

The forward primer (5’-ATT GGA TTT TTG GAG TTT AGG AGT A-3’) and reverse primer (5’-TAA AAC CTA CAA CAA CAA CAA C-3’) used were designed using MethPrimer (http://urogene.org/methprimer/index1.html) to amplify both methylated and unmethylated DNA. Bisulfite sequencing PCR (BS-PCR) was performed using 20 µL of the eluted, bisulfite-treated DNA samples
and 1 µL of positive control bisulfite-treated DNA. The amplicons were extracted from an agarose gel using the QIAquick gel extraction kit (Qiagen). Following extraction, the amplicons were cloned using the TOPO TA Cloning Kit (Invitrogen). Five colonies were randomly selected for isolation of plasmid DNA (Qiagen) and sequencing with the M13 R and F primers at the Genotyping and Sequencing Unit of the Ohio State University Comprehensive Cancer Center.

**Sequence analysis**

The resulting sequences were expected to be 534 base pairs in length and contain 65 CpG dinucleotides. Each allele sequenced was compared to a DNA sequence which reflected bisulfite treatment and 100% CpG cytosine methylation. This sequence consisted of the published DNA sequence which was modified to reflect bisulfite treatment changes (all non-CpG cytosines converted to thiamines; all CpG cytosines remained unchanged to indicate DNA methylation).

**Results**

*Immunohistochemistry for E-cadherin*

The 31 samples stained for E-cadherin consisted of 7 normal, 2 hyperplastic, 6 benign and 11 malignant mammary glands as well as 5 metastatic tumors. A strong correlation between diagnosis and degree of staining was seen (Figure 1.3). All normal and hyperplastic tissue had strong, membranous
staining for E-cadherin. The frequency of strong membranous staining decreased dramatically from normal mammary tissue to malignant tumors. Additionally, negative staining scores were only present in one benign tumor, but three malignant and metastatic tumors had negative staining. Interestingly, three of the five metastatic tumors had a higher IHC score at the site of metastasis compared to the primary tumor. Two of these were metastases to lymph nodes and one was a metastasis to liver.

**Laser Capture Microdissection and Bisulfite Sequencing**

A total of 14 tissues underwent laser capture microdissection and bisulfite sequencing. These tissues consisted of 1 normal sample each of liver, skin and mammary gland, 2 hyperplastic mammary glands, 3 mammary gland adenomas and 6 mammary gland carcinomas. At least 4 alleles were sequenced for each sample. The CpG island methylation results for each sample are shown in Table 1.3. This table lists samples by diagnosis and gives the percent methylation, which is the total number of methylated CpGs for all alleles examined/the total number of CpGs examined. Interestingly, in almost all sequenced samples, the sequenced allele showed a pattern of either fully unmethylated (<5% methylated) or fully methylated (>75% methylated) with the exception of an apparent methylation boundary between the 6th and 7th methylation site (Figure 1.4). This boundary followed a poly-A segment and it was frequent for the first 6 CpGs to have no methylation and the CpG dinucleotides 7-65 were completely
methylated. Another unexpected finding was the presence of methylated and unmethylated alleles within the same sample. The number of alleles that were greater than 75% methylated over the total number of alleles was reported as well as the number of alleles examined that had methylation beginning after the apparent methylation boundary. As the table shows, samples often had several alleles from each category. There was no correlation between histological diagnosis or immunohistochemical score and DNA methylation.

**Discussion**

This investigation demonstrated that strong, membranous expression of E-cadherin was consistently present in normal and hyperplastic canine mammary tissue. As mammary gland tumors progress from benign to malignant, E-cadherin expression at the plasma membrane becomes weaker and may be lost entirely. The molecular mechanisms for the loss of expression of E-cadherin are not fully understood. In this study, there were several metastatic mammary cancers that had increased E-cadherin expression demonstrated by IHC at metastatic sites compared to the primary malignancy. This finding suggests a reversible, epigenetic modification of E-cadherin expression as opposed to permanent gene silencing due to a loss of heterozygosity.

Previous studies have shown a link between the loss of E-cadherin expression and more aggressive tumor types and shorter survival times.\(^{12, 20, 28}\)
This connection was attributed to a transition from strongly adherent epithelial cells to less differentiated, non-adherent mesenchymal cells in a process called epithelial-to-mesenchymal transformation (EMT). EMT is commonly believed to be one of the first steps of malignant transformation and metastasis in many tumor types including breast, lung and colorectal carcinomas.\textsuperscript{4, 9, 24}

As in women, mammary tumors are the most common neoplasms that occur in female dogs. Approximately 50\% of mammary tumors are malignant in dogs similar to women. Morbidity and mortality in dogs are the result of distant metastases, predominantly to the lymph nodes and lungs.

Maintenance of cell-cell adhesion in mammary epithelial cells is dependent on the cadherin-catenin adhesion complex, of which, E-cadherin is an integral component. Disruption of this complex as the result of loss of expression of one or more components will result in a loss of normal cellular adhesion.\textsuperscript{25} E-cadherin is localized in the basolateral portion of the cell membrane and functions to define apicobasal polarity of the cells.

The goal of this study was to further demonstrate the frequency of down-regulation of E-cadherin in canine mammary cancer and explore methylation of a CpG island located in the 5’ region of Exon 1 of the E-cadherin gene as a possible mechanism for the loss of expression. Laser capture microdissection was used to purify the epithelial component of the tumors from the surrounding stroma. Overall, there was no correlation between tumor type, E-cadherin IHC score and degree of methylation in the samples examined. There are several
possibilities for this finding. First, in addition to hypermethylation, several other mechanisms for the loss of E-cadherin expression in human breast cancer have been proposed including loss of heterozygosity and overexpression of transcription factors such as Slug/Snail resulting in repression of E-cadherin expression.\textsuperscript{8,14} As demonstrated in this study, metastases expressing E-cadherin at the cell membrane can arise from malignant tumors that express little or no E-cadherin. This finding suggests that loss of E-cadherin expression in a subset of canine mammary cancers is the result of reversible changes such as hypermethylation.

Another possibility for the lack of correlation between E-cadherin expression and degree of methylation in this study is PCR bias in the bisulfite sequencing reaction. Following bisulfite treatment, methylated and unmethylated alleles have fundamentally different sequences. The conversion of unmethylated cytosine to uracil and the non-reaction of 5-methylcytosine to the treatment results in a T-rich sequence in unmethylated DNA and a C-rich sequence in methylated DNA. Previous studies have reported that this difference in sequence can result in differences in amplification of one of the sequences.\textsuperscript{29} The use of laser capture microdissection, which reduced the amount of stromal contamination, resulted in a low number of cells isolated for analysis and a subset of alleles were sequenced for each sample, therefore, any PCR bias that favors the cytosine-rich methylated DNA could significantly alter the overall findings. In addition, recent studies have asserted the presence of aggressive or
malignant cell populations within a larger, benign tumors. This heterogeneity of cells within a single mass cannot be accounted for when selecting the epithelial component of neoplasms with laser capture microdissection.\textsuperscript{17} Due to the limitations of sample size, tumors were classified in broad categories including malignant and benign tumors. A larger sample size which allows for subclassifications may have also resulted in differences in methylation to be observed.

In this study, the frequency of methylation was greater than expected in most samples. Interestingly, the liver, which had mainly E-caderin-positive cells with very little stroma, had minimal methylation. In addition, normal skin that had only a single methylated allele. This pattern of methylation suggests possible stromal contamination or PCR bias. A mosaic pattern of methylation with a predominance of methylated or unmethylated DNA was expected in individual samples. Instead, entire alleles were either completely unmethylated or methylated. This pattern supports the possibility that differences in methylation between individual alleles are a reflection of different cell types sampled.

This investigation demonstrated both the loss of E-cadherin expression in canine mammary cancer and possible re-expression following metastasis. These findings correlate closely with previous studies in human breast cancer progression and metastasis.\textsuperscript{13} The frequent occurrence of spontaneous mammary tumors in dogs, the similar rates of malignancy and metastasis and the loss of E-cadherin expression in malignant mammary cancer as demonstrated by
IHC in this study as well as previous studies indicate that dogs are a useful model for human breast cancer for both mechanistic studies and therapeutic trials.\textsuperscript{21, 22}
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15


(27) Tavassoeli F, Devilee P. *Pathology and Genetics Tumours of the Breast and Female Genital Organs*. No. 4 ed. IARC; 2003.


<table>
<thead>
<tr>
<th>Grade</th>
<th>Diagnostic Criteria</th>
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<tr>
<td>Strong</td>
<td>Dark membrane staining that widespread and visible with a low (4x) objective and was in &gt;50% of cells</td>
</tr>
<tr>
<td>Moderate</td>
<td>Focal darkly staining areas involving &lt;50% of cells or moderate membrane staining of &gt;50% of cells</td>
</tr>
<tr>
<td>Weak</td>
<td>Focal moderate staining in &lt;50% of cells, or pale membrane staining in any proportion of cells not seen with a low (4x) objective.</td>
</tr>
<tr>
<td>Negative</td>
<td>Tumor cells lack membrane staining</td>
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Table 1.1 Immunohistochemistry Scoring for E-Cadherin
Table 1.2 Results of Immunohistochemistry (IHC) and Bisulfite Sequencing.

Multiple samples from the same patient are given alpha numeric designations.

* denotes metastases that have a higher IHC score than primary tumors, -- denotes samples that did not undergo bisulfite sequencing
<table>
<thead>
<tr>
<th>ID</th>
<th>Diagnosis</th>
<th>IHC score</th>
<th>% Methylated</th>
<th>Alleles Methylated</th>
<th>Methylation Boundary</th>
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<tbody>
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<td>1</td>
<td>Normal Skin</td>
<td>Strong</td>
<td>32</td>
<td>2/6</td>
<td>1/6</td>
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<td>2</td>
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<td>0/5</td>
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<td>80.6</td>
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<td>0/5</td>
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<td>5</td>
<td>Cystic ductular</td>
<td>Strong</td>
<td>1.8</td>
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<td>20</td>
<td>1/5</td>
<td>0/5</td>
</tr>
<tr>
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<td>Normal mammary gland</td>
<td>Strong</td>
<td>--</td>
<td>--</td>
<td>--</td>
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<tr>
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<td>Weak</td>
<td>57.8</td>
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<td>1/5</td>
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<td>5.8</td>
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<td>7c</td>
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<td>Strong</td>
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<td>8</td>
<td>Complex Adenoma</td>
<td>Strong</td>
<td>97.6</td>
<td>7/7</td>
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<td>11a</td>
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<tr>
<td>11b</td>
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<td>--</td>
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<td>0/5</td>
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<tr>
<td>12b</td>
<td>Normal mammary gland</td>
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Table 1.2
Figure 1.1 Immunohistochemistry for E-cadherin. Strong cell membrane staining in a hyperplastic mammary gland (A); simple carcinoma with moderate membrane staining (B); metastatic mammary cancer cells in a hepatic vessel with weak membrane staining (C); and negative membrane staining in a simple carcinoma (D), although there is positive staining for E-cadherin, it is not located at the plasma membrane.
Figure 1.2 Laser capture microdissection of a simple mammary carcinoma. 

Prior to laser microdissection, the H&E stained tissue is intact on a non-coverslipped slide (A). Following laser capture microdissection, only fibrous stroma remains on the slide (B) and the epithelial tissue has been isolated and is ready for DNA purification (C).
Figure 1.3 Immunohistochemistry for E-cadherin. Thirty-one mammary tissue samples were stained for E-cadherin; 7 were normal, 2 hyperplastic, 6 benign, 11 malignant, and 5 were from metastatic sites. All normal and hyperplastic mammary glands stained strongly for E-cadherin. Benign tumors had strong (2), moderate (3) or negative (1) membrane staining for E-cadherin. Malignant tumors had strong (2), moderate (4), weak (4), or negative staining (1), and metastatic lesions had strong (1), moderate (2), weak (1), or negative membrane staining (1) for E-cadherin.
Figure 1.4. Examples of methylation patterns for E-cadherin. Typically, unmethylated samples had very few methylated CpGs (A), methylated samples frequently had one or two alleles that were not methylated (B) and a methylation boundary between the 6th and 7th CpG was present in multiple samples (C).
Chapter 2: Histiocytic Tumors of Tail Tendon Origin in Transgenic Mice Expressing the Human T Lymphotrophic Virus-1 Tax Viral Oncogene

Abstract

Human T lymphotrophic virus-1 (HTLV-1) is responsible for adult T-cell leukemia and is commonly associated with hypercalcemia and osteolysis, major contributors to morbidity. There is no effective treatment and the mechanisms by which the neoplastic lymphocytes induce osteolysis are not fully understood.

A transgenic mouse expressing the HTLV-1 oncoprotein, Tax, under control by the human granzyme B promoter consistently develops osteolytic tumors. The goal for this study was to investigate the histogenesis, development and progression of the bone-invasive malignancies.

The tumor morphology, osteolysis and the cellular components of the complex malignancies were investigated using flow cytometry, gross pathology, histopathology, immunohistochemistry, high-resolution radiography, and microcomputed tomography (µCT). Groups of wild-type, Tax+ and Tax+/Interferon-γ− mice, which develop lesions similar to Tax+ mice at a faster rate, were sacrificed at 3-month intervals over a 15-month period. Tissue
collection and Faxitron radiography was performed on all mice and µCT images, complete blood counts and flow cytometry were completed on a subset of mice. Necropsies of 80 mice revealed malignancies in the tail, ears and feet in transgenic mice consisting of poorly differentiated round to spindle-shaped cells with prominent neutrophilic infiltrates that occurred primarily after initial tumor growth. All transgenic mice developed tail tumors originating from tendon sheath cells expressing histiocytic antigens and with frequent invasion into adjacent bone. Splenomegaly in transgenic mice was due to marked extramedullary hematopoesis. Immunohistochemistry and flow cytometry of the malignancies revealed that the predominant cell type was F4/80 and Mac-1-positive, with infiltration of non-neoplastic neutrophils. Based on these findings, the tumors in Tax+ mice were tumors of tendon sheath expressing histiocytic antigens with invasion into adjacent bone.

**Introduction**

Adult T-cell leukemia/lymphoma (ATL) due to Human T-cell leukemia virus type 1 (HTLV-1) infection is an aggressive lymphoproliferative malignancy of helper T-lymphocytes and is associated with hypercalcemia and osteolytic bone tumors.\(^2, 12, 31, 32\) ATL develops in 2-4% of HTLV-1 carriers and usually emerges after a 20-40 year latency; however, cases in HTLV-1-infected children developing ATL have also been reported.\(^28, 44\) Bone invasion and osteolysis are
relatively uncommon characteristics of most lymphomas and leukemias. However, ATL, along with multiple myeloma, is distinctive for its high rate of osteolytic tumors and induction of humoral hypercalcemia of malignancy with increased osteoclastic bone resorption.\textsuperscript{34} HTLV-1, a delta retrovirus, expresses the viral oncogene Tax. Tax has multiple transcriptional \textit{trans}-activating effects that function through transcription factors, including the cAMP-response element binding proteins (CREB), nuclear factor kappa B (NF-kB), serum response factor, and zinc-finger proteins Sp1 and Egr1, as well as \textit{trans}-repressing effects through basic helix-loop-helix proteins.\textsuperscript{16, 17, 26, 45} Tax also has post-transcriptional effects through interactions with the cell cycle inhibitor p16, cyclin D3, and mitotic arrest protein MAD1, and by activation of protein kinase(s) that phosphorylate and inactivate the tumor suppressor protein p53.\textsuperscript{5, 16, 23} Additionally, Tax cooperates with Ras to transform primary rat embryo fibroblasts.\textsuperscript{29} Tax expression by herpes or retroviral vectors can lead to immortalization of primary human CD4+ lymphocytes.\textsuperscript{6} Furthermore, deletions of the HTLV-1 genome occur frequently in ATL patients, but the Tax gene is retained.\textsuperscript{40, 43}

The most convincing evidence that Tax acts as an oncoprotein comes from studies of murine transgenic models.\textsuperscript{9, 22} Several murine Tax transgenic models have been established. Expression of Tax from the HTLV-1 LTR results in mesenchymal tumors, bone abnormalities, exocrinopathy, growth retardation, thymic atrophy, inflammatory arthropathy, and premature mortality.\textsuperscript{1, 7, 19, 25, 35, 36} Mesenchymal tumors were also found with expression of Tax under the control of
the Thy1.2 promoter.\textsuperscript{25} Thymic atrophy was observed with Tax expression from the mouse mammary tumor virus promoter and the SV40 promoter fused to the immunoglobulin enhancer.\textsuperscript{3} Arthritis developed in mice with Tax expressed from the CD4 promoter.\textsuperscript{10}

In this report, we demonstrate that C57BL6/SJL mice expressing the viral oncoprotein, Tax, under control of the human granzyme B promoter (Tax\textsuperscript{+}) as well as Tax\textsuperscript{+} mice that have been crossed with interferon gamma-deficient mice (Tax\textsuperscript{+}/IFN-\textgamma\textsuperscript{−}/IFN-\textgamma\textsuperscript{−}) develop marked splenomegaly due to myelopoesis and widespread multifocal, bone-invasive sarcomas of histiocyte origin. These mice were developed by collaborators at Washington University (Dr. Lee Ratner) and preliminary phenotyping revealed the presence of splenomegaly and bone-invasive tumors on the distal extremities.\textsuperscript{8, 46} Granzyme B was used as the promoter for HTLV-1 Tax in these mice because it is known to be expressed in cytotoxic T-lymphocytes and natural killer (NK) cells. Initial examination of these lesions suggested a lymphocytic morphology. Further characterization of the tumor cells was performed to better understand the specific histogenesis of the tumor cells and examine the pathogenesis of tumor formation. The origin of the tumors was determined to be a histiocytic sarcoma based on pathology, immunohistochemistry, flow cytometry, radiography and microcomputed tomography (\textmu CT).
Materials and Methods

Animals

Mice utilized for this study included wild-type control mice (WT), mice that express HTLV-1 Tax under the control of the human granzyme B promoter (Tax\(^+\))\(^8\), and mice that express HTLV-1 Tax and are deficient in interferon-\(\gamma\) (Tax\(^+/IFN-\gamma^-\)).\(^46\) All mice were on a C57BL6/SJL background. Animals were maintained in specific pathogen-free housing and all experiments were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee. Mice were genotyped by polymerase chain reaction (PCR) using ear pinna genomic DNA that was isolated using the DNAeasy kit (Qiagen, Valencia, CA). Mice were monitored twice weekly for the development of tumors or illness characterized by a ruffled hair coat, dyspnea or weight loss. Animals reaching a designated termination time point, having tumors 15 mm in diameter or signs of illness including hunched posture and/or ruffled hair coat were humanely euthanized.

Gross and Histologic Examination

Mice were euthanized by 100% CO\(_2\) asphyxiation. Following euthanasia, a complete post-mortem examination was performed on each mouse. Gross lesions were noted and all tissues were placed in 10% neutral-buffered formalin for 48-72 hours at 4°C followed by 70% ethanol. Following fixation, bones were
decalcified in 10% EDTA (pH 7.4) for 10-14 days. Fixed soft tissues and
decalcified bones were embedded in paraffin, sectioned at 4 μm, mounted on
positively-charged glass slides, and stained with hematoxylin and eosin (HE) for
histologic examination.

Immunohistochemistry
Immunohistochemistry was performed on select paraffin-embedded mouse
tissues. Table 2.1 shows a list of the specific antibodies and tissue specificities.
All stains were performed using the avidin-biotin complex (ABC) method as
described by Ramos-Vara.30

Flow cytometry
Flow cytometry was performed on cells isolated from peripheral tumors as well
as spleens from both wild-type and transgenic mice. Cell isolation was achieved
using a 70 μM nylon mesh screen. Following isolation, cells were washed and
resuspended in FACS buffer (PBS with 2% FCS, 0.01% NaN₃). Half of the cells
were stimulated for 1 hour at 37°C with 50 ng/mL PMA and 750 ng/mL
ionomycin. Cytokine secretion was inhibited with a 4-hour incubation at 37°C with
2 μM GolgiStop (BD Biosciences, San Jose, CA). For extracellular staining, cells
were incubated for 30 min at 4°C with a combination of the following fluorescent
anti-mouse antibodies: anti-CD11b (Mac-1), anti-CD8, anti-B220 (CD45R), anti-
CD3e, anti-CD4 (BD Biosciences). For intracellular staining, cells were fixed with
Cytofix/Cytoperm (BD Biosciences) solution for 20 minutes at 4°C, and permabilized by washing three times with 1x PermWash (BD Biosciences). Cells were incubated for 30 min at 4°C with anti-human granzyme B antibody (BD Biosciences). Cells were then washed in FACS buffer and analyzed by flow cytometry (C6 flow cytometer, Accuri Cytometers, Ann Arbor, MI).

**Complete Blood Count**

Complete blood counts and blood cell differentials were performed on eight Tax⁺ and nine WT mice, all aged 12 months, to measure differences in red and white blood cell counts as well as to determine the presence of circulating neoplastic cells. Briefly, following euthanasia of mice, whole blood was collected by percutaneous cardiac puncture. Complete blood counts with white blood cell differential counts were performed on a portion of whole blood with EDTA anticoagulant (FORCYTE Autosampler 10, Oxford Science, Inc., Oxford, CT). Additionally, a drop of blood from each mouse was used to make a blood smear for direct examination at 1000x magnification.

**Digital Radiography**

Radiographs were taken of whole mice and isolated tails from 3 mice randomly selected from each genotype at 3, 6 and 12 month time points. These radiographs were obtained using the Faxitron LX-60 Digital Radiography System.
Whole mouse radiographs were completed at 40 kVp for 30 seconds and tails were radiographed at 32 kVp for 11 seconds.

Variations in caudal vertebral bone density were quantitatively assessed using μCT imaging. Tail images were acquired by μCT (Inveon Preclinical CT scanner, Siemens AG, Munich, Germany). Images were generated in 400 projections over 360 degrees at 100 kVp, 200 MA, 1250 millisecond exposure, Bin 2, and a medium-high system magnification with a pixel width of 19.4 μm. Image data were 3D reconstructed (COBRA, Exxim Computing Corp., Pleasanton, CA) and the lengths of individual vertebrae were measured (Inveon Research Workplace 3-D Image Software, Siemens Preclinical). Vertebral metaphyses were defined as 20% of the overall length of the bone adjacent to the physis. This defined region was cropped to generate a volume of interest (VOI). The volume of bone within the metaphyseal VOI, the density of bone within the metaphyseal VOI, the overall volume of the entire metaphyseal VOI, and the overall density of the entire metaphyseal VOI were measured. The percentage of the overall metaphyseal VOI that was bone was calculated. Segmentation thresholds were kept constant for all measurements.

**Plasma Calcium**

Total plasma calcium concentrations were measured in five, twelve-month-old mice from each group. Heparinized blood for this assay was collected following euthanasia by percutaneous cardiac puncture. Plasma calcium concentrations
were measured using the QuantiChrom™ Colorimetric Calcium Assay Kit (BioAssay Systems, Hayward, CA).

**Statistical Analysis**

Results are displayed as mean and standard deviation unless otherwise indicated. Data were analyzed using Student’s *t*-test or one-way ANOVA. Data with P values less than 0.05 were considered statistically significant. All statistical comparisons were performed using JMP, Version 9 (SAS Institute Inc., Cary, NC).

**Results**

**Mice expressing HTLV-1 Tax developed histiocytic malignancies around tendon sheaths and perichondrium, and splenomegaly due to myelopoiesis.**

A total of 80 mice were used to characterize tumors. This included 28 wild-type (six 6-month, four 9-month, twelve 12-month, and six 15-month-old mice), 22 Tax⁺ (three 6-month, four 9-month, six 12-month, and nine 15-month-old mice) and 30 Tax⁺/IFN-γ⁻ mice (ten 6-month, ten 9-month, and ten 12-month-old mice). Mice that died spontaneously as well as mice that met early removal criteria (ERC) due to ulcerative dermatitis, a common background lesion in C57/B6 mice, were excluded from the final evaluation. Soft tissue masses were
grossly visible on the tails, feet and nose of Tax⁺/IFN-γ⁻ mice as early as 3 months with mice meeting ERC at 6 months due to the onset of ulceration and lameness. Tax⁺ mice, on the other hand, did not have external evidence of tumor formation until 6 months and mice did not meet ERC until 15 months. In the case of Tax⁺ mice, ERC was due to marked splenomegaly resulting in dyspnea and loss of body condition (Figure 2.1). The spleens of Tax+ mice were often grossly enlarged compared to wild-type mice with marked splenomegaly observed in 9 of 19 Tax⁺ and 10 of 20 Tax⁺/IFN-γ⁻ mice 9 months and older. A summary of the most common gross post-mortem findings and their incidence in each group is shown in Table 2.2.

With the exception of the spleen, there were no significant histologic lesions in any soft tissues examined of 12 and 15 month old mice. The marked splenic enlargement observed in transgenic mice was the result of marked extra-medullary hematopoiesis characterized by widespread tissue expansion with a mixed population of myeloid precursors, megakaryocytes and, less frequently, erythroid precursors (Figure 2.2).

Multiple bones of transgenic and wild-type mice were examined histologically including the skull, radius, ulna, tibia/fibula, metatarsals and metacarpals in 12- and 15 month-old mice as well as multiple sections of tail vertebrae of mice at all time points. Tumors on the ear pinna were also evaluated histologically and had cellular infiltrates surrounding the pinnal cartilage of similar histologic and immunohistochemical properties as the bone tumors. Tumors seen
in the tail were identical to those in the appendicular skeleton; however, the tail was chosen for further histologic evaluation at multiple time points for uniformity of sectioning and comparisons of histologic with radiographic and µCT imaging. Although tumors were not observed grossly in all transgenic mice, all tail sections of transgenic mice had some degree of cellular proliferation around tendon and nerve sheaths. Cellular infiltrates in Tax+ mice at the 6- and 9-month time points were mild, multifocal and increased in severity in an age-dependant manner. Evidence of bone invasion was not typically present in the early tumors. The cellular infiltrates were more severe in Tax+/IFN-γ−/− mice compared to Tax+ mice. Peritendinous tumors were composed of histiocytic cells that were characterized by large, round to spindle-shaped with oval to round nuclei and coarsely stippled chromatin. The intercellular matrix consisted of a lightly basophilic granular material interpreted as mucin. About half of the cells in the tumors were non-neoplastic, well-differentiated, mature neutrophils (Figure 2.3 & 2.4). On longitudinal section, histiocytic tumors were consistently within the soft tissue adjacent to the metaphysis and frequently invaded from the soft tissue into the bone, especially in the advanced tumors that occurred in Tax+ mice at 12 months and Tax+/IFN-γ−/− mice at 9 months (Figure 5). The site of bone invasion was consistent among the groups of mice and occurred at the cortical bone adjacent to the metaphysis (so called ‘cut back’ zone of the bones). The tumor cells in the bone marrow were more spindle-shaped and the matrix was collagenous (Figure 6). The neoplastic nature of the tumor cells was confirmed by derivation of
immortalized cell cultures and subsequent tumor formation in athymic nude mice inoculated subcutaneously with cultured cells (data not shown).

In addition to the osteolytic tumors in the Tax^+/IFN-γ^- mice, separate regions of metaphyseal osteosclerosis were frequent in the vertebrae and appendicular bones. The increased trabecular bone was independent of the histiocytic tumors and associated bone lysis. There was an increase of both the number and thickness of trabeculae in the regions of medullary osteosclerosis. Frequent cement lines and regions of retained cartilage suggested there was increased abnormal modeling and remodeling of the bone. Osteosclerosis was present only in the Tax^+/IFN-γ^- mice and not the Tax^+ mice, and may be related to the lack of interferon-γ (Figures 7-9). The transgenic mice also had increased cellularity of the bone marrow compared to wild-type mice, which consisted of hematopoetic cells in expected proportions.

Immunohistochemistry for macrophage-specific antigen, F4/80, and HTLV-1 Tax were consistently positive in the histiocytic tumor cells. All other immunohistochemical stains performed were negative. As expected, the staining for F4/80 was largely restricted to the cell membrane with a small amount of cytoplasmic staining. The HTLV-1 Tax staining was more diffusely cytoplasmic and weakly nuclear with less clearly defined cellular margins (Figure 10). The histiocytic phenotype of the tumor cells was confirmed by flow cytometry, which demonstrated that the majority of cells within tumors flowed were positive for Mac-1 (CD11b), a marker for macrophages and dendritic cells (Figure 11).
Additionally, expression of granzyme B in the Mac-1 positive cells was demonstrated with intracellular staining for granzyme B both before and after stimulation with PMA and ionomycin. Figure 12 demonstrates the increased co-expression of granzyme B and Mac-1 following stimulation.

**Mice expressing HTLV-1 Tax had leukocytosis and mild anemia.**

Blood smears revealed no evidence of circulating tumor cells in either the wild-type or Tax+ mice. However, there was a significant increase in white blood cell count in Tax+ mice. This increase was attributable to significant increases in neutrophils and lymphocytes in Tax+ mice compared to the wild-type (Table 2.3).

**Bone invasion and osteolysis, but not hypercalcemia, were features of tumor-bearing mice**

Histopathology, radiography and μCT revealed loss of bone in Tax+ transgenic mice and were characterized by increased radiolucency and osteoclastic bone resorption (Figures 13&14). The bone loss was more severe in Tax+/IFN-γ−/− mice and occurred at an earlier time point and advanced to greater bone lysis by 12 months. The typical area of bone invasion was the cortex adjacent to the metaphysis (near the ‘cut back’ zone).

Quantification of bone volume by μCT was performed on 6 caudal vertebral metaphyses from 12-month-old wild-type and Tax+ mice at sites of tumor invasion. The previously described osteosclerosis in the Tax+/IFN-γ−/- mice
precluded meaningful quantification of bone in this group. Metaphyseal bone volume in Tax+ mice (64.2 ±14.7%) was significantly lower than wild-type controls (86.1±8.7%) (p<0.0001; Figure 2.15)

Despite multifocal regions of bone lysis and a significant decrease in bone volume in the Tax transgenic mice, there was no evidence of hypercalcemia in the mice. Groups of five 12-month-old mice were used to measure total plasma calcium concentrations from each group. There was no significant difference in plasma calcium between the groups and none of the mice in any group showed had plasma calcium above the normal range (Figure 16). Calcium concentrations were 12.5 mg/dL ± 0.6 in WT, 11.2 ± 0.4 in Tax+ and 12.1 ± 1.1 in Tax+/IFN-γ-/- mice.

**Discussion**

In this study, we investigated the pathogenesis of tumor progression in mice expressing HTLV-1 Tax under regulation of the human granzyme B promoter. Using gross and histopathology, immunohistochemistry, imaging and flow cytometry, it was demonstrated that histiocytic sarcomas originated around tendon sheaths and perichondrium with invasion into the bone resulting in lytic bone tumors. In addition, mice had increased hematopoiesis both within the bone marrow and spleen, resulting in splenomegaly and leukocytosis with anemia. It was previously reported that tumors in human granzyme B promoter-Tax
transgenic mice were large granular lymphocyte (LGL) leukemia/lymphoma as well as sarcomas of tendon sheath origin (JM Ward, personal communication).\(^8\)

The differentiation of histioocyte-rich lymphomas and histiocytic sarcomas in mice is challenging and may require examination with multiple modalities. The abundance of F4/80+ cells in the tumors of this study had decreased cytoplasmic staining as cells transitioned from the differentiated round cells to the less differentiated spindle-shaped cells, which is typical for murine histiocytic sarcomas.\(^11\)

It is more common to find histiocytic sarcomas in soft tissues invading into bone compared to lymphomas. Also, it would be unexpected for lymphoma to occur in the distal extremities without involvement of lymph nodes, spleen, thymus or the distributed lymphoid system. Granzyme B was initially described in cytotoxic T-lymphocytes and NK cells, but has also been shown to be expressed in other cell types including plasmacytoid dendritic cells, mast cells, basophils and macrophages, particularly in the presence of inflammation.\(^18, 27, 33, 42\)

Flow cytometry experiments from this study showed that there was an increase in intracellular granzyme B expression in Mac-1-positive histiocytic tumor cells following stimulation with PMA and ionomycin. This finding correlates with previous reports of granzyme B expression in macrophages in the presence of inflammation. Interestingly, previous transgenic models expressing HTLV-1 Tax under control by human CD4 promoter as well as the HTLV-1 LTR frequently developed inflammation around the joints of distal appendages.\(^10, 14\)

The alteration of inflammatory cytokine expression in the presence of HTLV-1 Tax is
well documented and most commonly associated with activation of NF-κB pathway. It is likely that the development of neutrophilic infiltrates within the tumors as well as splenomegaly seen in Tax+ mice is the direct result of increased expression of inflammatory mediators such as GM-CSF and G-CSF due to transactivation by Tax. Elevation of plasma GM-CSF has been previously demonstrated in studies with these mice by our collaborators.  

Both exogenous and endogenous Tax have been shown to promote tumor formation in multiple cell lineages, including T-lymphocytes and rat fibroblasts. Tax expression within dendritic cells has been reported to induce both replication and maturation. The stimulation of dendritic cell replication by Tax has been implicated in the pathogenesis of human T-lymphotrophic virus-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) as well as other inflammatory lesions associated with HTLV-1 infection.

Currently an animal model of ATL that mimics all stages of the disease, from viral infection through oncogenesis, is not available. In order to study various aspects of the disease, multiple animal models must be utilized. Previous studies have utilized rabbits to model HTLV-1 infection and immune response. Human cells infected with HTLV-1 have been used for in vitro experiments that explore gene expression related to viral transformation and osteolysis. Most HTLV-1-infected cells utilized for in vitro experiments do not consistently grow as xenografts in vivo. Conversely, many cell lines grow as xenografts with limited survivability in culture. These cell lines are useful for therapeutic studies, but
have limited usefulness in mechanistic experiments. Although they cannot serve as a model for viral transformation and oncogenesis by HTLV-1, non-virally induced lymphoma cell lines such as the human Jurkat cell line, that mimics ATL \textit{in vivo} and grows well \textit{in vitro}, have been used to study gene function both \textit{in vitro} and in xenograft transplant mice.

Adult T-cell leukemia is a devastating disease that, in the acute form, has a poor survival with limited treatment options. Due to the long latency period and low percentage of HTLV-1-infected patients developing ATL, there is a lack of a thorough understanding of the progression of CD4+ lymphocytes from viral integration to ATL development and progression. Viral oncogenesis, specifically retroviral oncogenesis, is not unique to HTLV-1. A number of retroviruses are associated with the onset and progression of specific tumors due to the expression of proto-oncogenes, insertional mutagenesis and expression of auxiliary oncogenes, as seen with HTLV-1 and Tax expression. Previous studies have demonstrated that expression of the viral oncogene, Tax, is both necessary and sufficient for oncogenesis.\textsuperscript{6,7,13,41} Examination of the oncogenesis and progression of ATL using animal models is vital to further understand the disease in humans and develop effective preventions and therapeutics for HTLV-1 and ATL.

Transgenic mice expressing HTLV-1 Tax on the granzyme B promoter serve as a useful model for HTLV-1-Tax-induced malignancy. In addition, the mice can be used to study the basic mechanisms of cancer cell interaction with
bone and the bone marrow microenvironment in the osteolytic and bone-invasive malignancies. The consistent formation of bone-invasive tumors in the tails of the mice and the ability to derive transplantable cell lines from these tumors will permit both *in vivo* and *in vitro* research into both the mechanism of HTLV-1 oncogenesis and therapeutics that specifically target HTLV-1 Tax, a protein known that is both necessary and sufficient for development of ATL.
References Cited


(10) Habu K, Nakayama-Yamada J, Asano M et al. The human T cell leukemia virus type I-tax gene is responsible for the development of both inflammatory polyarthritis resembling rheumatoid arthritis and


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Table 2.1 Summary of Antibodies Used for Immunohistochemical Stains
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Table 2.2 Summary of Gross Pathological Findings
Table 2.3. Complete Blood Counts for 12-month-old Wild-type (n=9) and Tax+ (n=8) Mice (mean ± standard deviation, *p<0.05 was considered statistically significant)
Figure 2.1 Marked splenomegaly in a 12-month-old, female, Tax⁺ mouse due to marked myelopoiesis. Splenomegaly was a common feature in Tax⁺ (A, bottom) and Tax⁺/IFN-γ⁻/⁻ mice at 12-15 months of age compared to wild-type controls (A, top). Histology and flow cytometry demonstrated that splenomegaly was due to marked myelopoiesis. Panel B shows multiple cell types present within the spleen of transgenic mice. Specifically, there were increased numbers of megakaryocytes and myeloid precursors. H&E
Figure 2.2 Multifocal areas of histiocyte proliferation surrounding tendons and nerves in the tail with osteolysis and extension into the marrow cavity.

The low magnification image demonstrates multifocal histiocyte proliferations surrounding tendon bundles adjacent to the caudal vertebrae (A). The proliferative histiocytes were consistently observed throughout the tail of Tax\(^+\) mice as early as 6 months and in Tax\(^+\)/IFN-\(\gamma^{-}\/-\) mice as early as 3 months. Panel B is a high magnification image of proliferative histiocytes surrounding tendon (*) and nerve (black arrow). Cells are a mixture of proliferative pleomorphic histiocytes (white arrow) with a myxomatous stroma and well-differentiated neutrophils. A low magnification image of a histiocytic tumor in a 12-month-old, Tax\(^+\)/IFN-\(\gamma^{-}\/-\) mouse (C) demonstrates the invasion of neoplastic cells into the bone with osteolysis and subsequent loss of cortical bone at the level of the metaphysis. Despite the presence of neoplastic cells surrounding tendon along the length of the vertebral bodies, this region was the typical site of bone invasion. After invading the bone, the histiocytic tumor cells become more spindle-shaped and the intercellular matrix was collagenous (D). Osteoclastic bone resorption is apparent (*). H&E.
Figure 2.2
Figure 2.3 Caudal vertebral metaphysis of 12-month-old wild-type, Tax\(^+\) and Tax\(^+\)/IFN-γ\(^-\) mice. The metaphyseal bone of a wild-type mouse (A) has evidence of age-related osteoporosis and reduced bone marrow cellularity. The Tax\(^+\) mice (B) had typical age-related osteoporosis as seen in the wild-type mice. Unlike the wild-type group, mice expressing HTLV-1 Tax had increased hematopoietic cells within the bone marrow. This correlated well with the leukocytosis present in this group. Mice expressing HTLV-1 Tax and deficient in interferon-γ had increased bone marrow hematopoiesis similar to mice expressing only HTLV-1 Tax, as well as increased number and thickness of trabecular bone within the metaphysis (C). Trabeculae have frequent cement lines consistent with increased bone remodeling.
Figure 2.3
Figure 2.4 Immunohistochemistry of a tumor along the pinna cartilage of a 6-month-old Tax+/IFN-γ⁻⁻ mouse. Immunohistochemistry for macrophage-specific antigen F4/80 (A). F4/80 was consistently positive in the neoplastic cells. The round histiocytic tumor cells had more intense staining compared to the spindle-shaped tumor cells. The immunohistochemistry for HTLV-1 Tax (B) demonstrated widespread cytoplasmic with less-frequent nuclear staining in the neoplastic cells.
Figure 2.4
Figure 2.5 Flow cytometry for select surface leukocyte markers in the spleen of wild-type and Tax+ mice and a tail tumor from a Tax+ 12-month-old mouse. The predominant cell type in the spleen of a wild-type mouse was B-cells. The majority of cells in the tail tumor were Mac-1 (CD11b)-positive with few B (B220) or T lymphocytes (CD4 and CD8). This demonstrated that the predominant tumor cell population was monocytic in origin. The spleen from the Tax+ mouse had increased Mac-1-positive cells with few B and T cells. Note that the majority of the splenic cells (myelopoietic cells) and the tumor neutrophils in the Tax+ mice were not stained by the antibody panel.
Figure 2.6 Change in Granzyme B expression in tumor cells after PMA and ionomycin stimulation. Flow cytometry of a representative tail tumor from a Tax+ mouse shows co-expression of granzyme B and Mac-1 in the tumor cells was increased from 12.1% in unstimulated cells (top row) to 19.9% following stimulation with PMA and ionomycin (bottom row). The increase in co-expression demonstrates expression of granzyme B in activated histiocytes.
Figure 2.7 MicroCT and radiographic images from the caudal vertebrae of 12-month-old mice. On the μCT images (A) wild-type (WT) mice have limited regions of bone loss at the metaphysis due to age-related osteoporosis. Tax+ mice have increased osteolysis due to histiocytic tumor infiltration at the metaphysis (arrows). Tax+/IFN-γ-/− mice have marked multifocal osteolysis secondary to tumor infiltration at the metaphyses. The regions of osteolysis have increased radiolucency (arrows). In addition, regions of metaphyseal osteosclerosis were seen by μCT as areas of increased radio-opacity compared to the metaphyses of wild-type and Tax+ mice (*). On the digital radiographs (B) there were multifocal regions of radiolucency in the metaphyses of Tax+ and Tax+/IFN-γ-/− mice compared to wild-type control mice. These areas of bone loss were due to histiocytic tumors that originated in tendon sheaths and invaded into bone. The vertebral cortices near the ‘cut-back’ zone the most common sites of bone invasion by the histiocytic tumors.
Figure 2.7
Figure 2.8 Percent bone volume in the caudal vertebral metaphyses of wild-type and Tax+ mice. The average percent bone (mean±SEM) in the metaphysis of Tax+ mice was significantly decreased compared to wild-type mice at sites of histiocytic tumor invasion (p<0.0001).
Figure 2.9 Plasma calcium concentration of Tax+ and wild-type mice.

Plasma calcium was measured in five, twelve-month-old mice from each genotype (mean±SD). Calcium concentrations were 12.5 mg/dL ± 0.6 in WT, 11.2 ±0.4 in Tax+ and 12.1 ±1.0 in Tax+/IFN-γ−/− mice.
Chapter 3: Osteolytic Sarcomas from Mice Expressing Human T Lymphotrophic Virus-1 Tax Viral Oncogene

Abstract

Human T Lymphotrophic virus-1 (HTLV-1) is the causative agent of adult T-cell leukemia/lymphoma (ATL), a hematopoietic malignancy with a high morbidity and frequent development of osteolytic metastases and humoral hypercalcemia of malignancy (HHM). The viral oncogene, Tax, encoded by the pX regulatory region of the HTLV-1 genome has been demonstrated to be both necessary and sufficient for neoplastic transformation; however, the function of Tax in development and progression of osteolytic metastases and HHM is not fully understood. In this study, we derived three cell lines from spontaneous tumors that formed on mice expressing HTLV-1 Tax under regulation of the granzyme B promoter and deficient in interferon-gamma. All three cell lines were used for in vitro gene expression assays, ex vivo osteolysis assays and in vivo xenograft models. Histopathology and flow cytometry demonstrated that two of these cell lines were monocyte/macrophage in origin. All three cell lines expressed HTLV-1 Tax, were immortalized in culture and formed tumors following injection into immunodeficient mice. Differences in expression of
PTHRP, IL-6, IL-1α and β, and G-CSF all correlated with differences in plasma calcium, tumor growth, metastasis and neutrophilic inflammation respectively. Based on these initial studies, these cell lines would be ideal for further examination of HTLV-1 Tax in osteolytic tumor formation, development of HHM and tumor-associated inflammation.

**Introduction**

Human T-Lymphotrophic virus-1 is a complex retrovirus and the causative agent of adult T-cell leukemia/lymphoma (ATL). ATL is a devastating disease with limited treatment options. Patients with HTLV-1-induced ATL frequently develop tumor-induced osteolysis and hypercalcemia with little associated osteoblastic activity.\(^1\), \(^2\) The formation of osteolytic metastases and hypercalcemia is a strong contributor to morbidity and mortality in ATL patients. Cellular transformation and formation of ATL following viral infection by HTLV-1 is attributed to expression of the viral oncoprotein, Tax. Tax is a regulatory protein located in the pX region of the viral genome. A number of studies have demonstrated the ability of HTLV-1 Tax alone to immortalize primary rodent cells.\(^3\), \(^4\)

Attempts to further understand the mechanism by which HTLV-1 Tax causes ATL and formation of osteolytic bone lesions has led to the development of several animal models that control the expression of Tax through specific promoters. Transgenic mouse models that express HTLV-1 Tax on the viral LTR
develop a number of lesions consisting of neurofibromatosis, muscle fiber degeneration, increased bone turnover, exocrinopathies, fibrosarcomas and rheumatoid arthritis-like lesions. Transgenic mice that express Tax under regulation of the CD4 promoter develop an inflammatory arthropathy. Mice that express HTLV-1 Tax under control of the human granzyme B promoter have been reported to develop hematopoetic malignancies and osteolytic lesions similar to those seen in ATL patients that increase in severity when the animals are crossed with interferon-γ knockout mice (IFN-γ−/−). Recent studies show expression of granzyme B occurring not only in T lymphocytes, but also in macrophages and further examination of mice expressing HTLV-1 Tax under regulation of the human granzyme B promoter in our lab demonstrated the histogenesis of spontaneous tumors in the transgenic mice to be histiocytic in origin. These animals provide an excellent platform to study bone-Tax interactions and further elucidate the cellular and molecular mechanisms of osteolytic tumors.

In this study, we derived three cell lines (T94, 501, Tom3) from tumors in Tax+/IFN-γ−/− mice. The histogenesis of these cells was confirmed using flow cytometry and further experiments were done to explore the osteolytic potential of the cells, the ability of these cells to grow in vivo, and differences in gene expression that further our understanding of the pathogenesis of HTLV-1 Tax-induced malignancies.
Materials and Methods

Cell Line Derivation

Three cell lines were derived from spontaneous tumors of C57BL6/SJL mice expressing HTLV-1 Tax under regulation of the granzyme B promoter (Tax⁺) and deficient in interferon gamma (IFN-γ⁻/⁻). The T94 cell line was derived from a mass on the tail of a 6-month-old female Tax⁺/IFN-γ⁻/⁻ mouse, the Tom3 cell line was derived from a mixed osteolytic/osteoblastic mass adjacent to the right radius and ulna of a 7-month-old male Tax⁺/IFN-γ⁻/⁻ mouse, and the 501 cell line was derived from an osteolytic mass on the right hind limb of a 6-month-old male Tax⁺/IFN-γ⁻/⁻ mouse. To establish cell lines from these tumors, mice were euthanized and tumors were aseptically dissected. Isolated neoplasms were minced on plates containing DMEM/F12 media (Invitrogen) with 25 μg/mL Plasmocin (InvivoGen, San Diego, CA) and 10% fetal bovine serum (FBS). Minced tumors were washed 3 times in media, plated at variable densities and incubated for 48 to 72 hours to allow release of individual cells from the minced tumor and adhesion to the flask. After 2 weeks in plasmocin-containing media, adherent cells were cultured in DMEM/F12 with 100 units penicillin and 100 μg streptomycin/mL and 10% FBS.

Initial engraftment of the T94 cell line in nude mice following subcutaneous injection resulted in small, slow-growing tumors, therefore rederivation was
performed by selecting the fastest growing subcutaneous tumor and performing the above steps to derive a new cell line selected for growth in vivo (T94S).

**Flow Cytometry**

Flow cytometry was performed on cultured cells. Cells were cultured to 70% confluence. Cells were trypsinized, washed and resuspended in FACS buffer (PBS with 2% FCS, 0.01% NaN₃). The cells were stained by incubation for 30 min at 4°C with a combination of the following fluorescent anti-mouse antibodies: anti-CD11b (Mac-1), anti-CD11c, MHCII, Ly6C and Ly6G (BD Biosciences). Cells were then washed in FACS buffer and analyzed by flow cytometry (C6 flow cytometer, Accuri Cytometers, Ann Arbor, MI)

**Calvarial Co-Culture Experiments**

Conditioned media was produced from each of the three cell lines for use in calvarial co-culture experiments. The media consisted of basal medium of DMEM/F12 supplemented with 0.1% RIA-grade bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO), 100 units/mL penicillin and 100 μg/mL streptomycin which had been cultured with 70% confluent cells for 72 hours. Following culture, media was harvested and centrifuged at 1000 RPM for 5 minutes to remove any latent cells or cell debris. This media was stored at -20°C prior to use in co-culture experiments.
Calvaria from 4-6-day-old BALB/c mouse pups were harvested following euthanasia by asphyxiation with carbon dioxide and subsequent decapitation. Two calvarial bone disks including frontal and parietal bones were collected from each mouse using a 3.5 mm diameter biopsy punch. Calvaria were washed once in phosphate-buffered saline (PBS) and then washed 3 times in culture media consisting of BGJb media (Invitrogen), 0.1% RIA-grade bovine serum albumin (BSA) (Sigma-Aldrich) and 100 μg/mL Normocin (Invivogen). Disks were then cultured for 16 hours at 37°C and 5% CO₂. Following overnight incubation, six disks were randomly distributed to each conditioned media group as well as a non-conditioned media control group.

Disks were cultured for 6 days in 50% conditioned media and 50% BGJb with 0.1% BSA and 100 μg/mL Normocin. On day 6, disks were collected, fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity using an acid phosphatase leukocyte kit 387A (Sigma-Aldrich). Following TRAP staining, calvaria were whole-mounted on glass slides using aqueous mounting medium and photographed. The photographed calvaria were analyzed for total bone area and resorptive perimeter using image analysis software, Image Pro Plus 6.0 (Media Cybernetics, Inc., Bethesda, MD). Media from the co-cultures were collected, centrifuged at 1000 RPM for 5 minutes and total calcium concentrations were determined using a Quanti-Chrom colorometric assay kit (BioAssay Systems, Hayward, CA) according to the manufacturer’s instructions.
Subcutaneous, intratibial and bone-adjacent inoculation of Tax-induced malignant cells in nude mice

Subcutaneous (N=3), intratibial (N=3) and bone-adjacent (N=2) injections of each of the Tax-induced malignant cell lines were performed in 6-week-old nu/nu mice (Harlan Laboratories, Inc., Indianapolis, IN) under general anesthesia with a 3% isofluorane-oxygen mixture. Specifically, mice receiving subcutaneous tumor cells were injected with $1 \times 10^6$ cells in 100 μL PBS into the subcutaneous tissue overlying the right scapula with a 25 ga needle; mice receiving intratibial tumor cells were injected with $2 \times 10^5$ cells in 20 μL PBS into the marrow cavity of the right tibia with a 27 ga needle and mice receiving bone adjacent tumor cells were injected with $5 \times 10^5$ cells in 50 μL PBS into the lateral surface of the right hindlimb adjacent to the mid-diaphysis of the tibia with a 25 ga needle. Mice were observed twice weekly for tumor growth evidenced by nodule formation or swelling at the sight of injection in any of the mice, or lameness in the intratibial and bone-adjacent injected mice. Mice were euthanized when the tumor size exceeded 2 cm in diameter, was ulcerated, or if persistent lameness was present in the injected leg. All animals were housed and treated in accordance with the University Laboratory Animal Resources guidelines, and experimental protocols were approved by the Institutional Laboratory Animal Care and Use Committee.
Histopathology

Following euthanasia of mice, whole blood was collected by percutaneous cardiac puncture, complete necropsies were performed on all mice and tissues were fixed in 10% neutral-buffered formalin for 48 hours at 4°C. Bones were decalcified for 10 days at 4°C in 10% EDTA (pH 7.4). Tissues were paraffin embedded, sectioned at 4 μm and stained with hematoxylin and eosin (H&E) for histopathological characterization.

Plasma Calcium

Plasma calcium concentrations were measured from the blood collected following euthanasia of the xenografted mice. Plasma calcium concentrations were measured using the QuantiChrom™ Colorimetric Calcium Assay Kit (BioAssay Systems, Hayward, CA).

Gene Expression Array

T94, 501 and Tom3 cells were cultured in triplicate to 70% confluence in 6-well culture plates. RNA was isolated from the cultured cells using the Absolutely RT-PCR RNA Miniprep Kit per manufacturer’s instructions (Agilent, Santa Clara, CA). RNA quality was assessed using the Agilent RNA 6000 Nano Assay. Following quality assessment, the purified RNA was then hybridized to the Affymetrix GeneChip Mouse 430 2.0 Genome Array (Affymetrix, Santa Clara, CA) at Ohio State University’s Comprehensive Cancer Center Microarray Shared
Resource. Correction for background expression and normalization were performed to avoid technical bias and gene expression level was summarized over the probeset using the RMA method.\textsuperscript{15}

**RNA extraction, reverse transcription, and real-time RT-PCR**

Total RNA from each cell line was extracted using the Absolutely RNA RT-PCR Miniprep Kit (Agilent). Approximately 0.5 $\mu$g of each RNA was reverse transcribed with SuperScript II Reverse Transcriptase and oligo(dT)$_{12-18}$ primer (Invitrogen). Real-time RT-PCR analysis was performed using QuantiTect SYBR Green PCR Kit (Qiagen) with the primers listed in Table 3.1. Real-time RT-PCR was performed on at least three replicate samples. Gene expression was normalized to 4 housekeeping genes by geometric averaging ($\beta$-actin, PP1a, UBC, and GAPDH).\textsuperscript{16}

**Statistical analysis**

Results are displayed with means and standard deviation as indicated. Data were analyzed using Student’s $t$-test or ANOVA and Bonferroni’s post hoc test. Normalized gene expression data (ΔCT) were analyzed using Student’s $t$-test and displayed as relative expression compared to the cell line with the lowest expression. Data with P values less than 0.05 were considered statistically significant. All statistical comparisons were performed with JMP Version 9 (SAS Institute Inc., Cary, NC).
Results

Cell Line Derivation and Characterization by Flow Cytometry

In culture, the T94 cells were moderately-sized and spindle-shaped with a single, central, oval-shaped nucleus. The 501 cells were comprised of a biphasic population with small numbers of large, elongate cells with smooth cell borders and smaller irregularly shaped cells with multiple, thin cytoplasmic projections from the surface. The Tom3 cells were moderately-sized with multiple, thin projections extending out from the cell surface and a single, central, round nucleus. Occasional large and multinuclear cells were present comprising less than 20% of the total cell population (Figure 3.1).

Surface markers for MHCII, CD11b, CD11c, Ly6G and Ly6C were used to label the three cell lines for flow cytometry. Approximately half of the T94 cells were positive for Ly6C, no other surface markers were positive in this cell line. Ly6C is non-specific and alone cannot definitively identify the cell line of origin. Cells that express Ly6G include monocyte/macrophages, granulocytes, endothelial cells, plasma cells, and thymocyte, NK-cell, and T-subsets. The T94 cells were adherent and spindle-shaped in culture which would more typical for macrophages or endothelial cells. The majority of Tom3 cells were positive for CD11b and Ly6C. These findings, along with the appearance of the cells in culture, would be consistent with an inflammatory macrophage. The 501 cells had a bimodal population, similar to what was seen in culture. The bimodal population consisted of CD11b$^{\text{low}}$ (23% of cells) and CD11b$^{\text{high}}$ (70% of cells) as
well as Ly6C\textsuperscript{low} expression. These findings were consistent with a macrophage/monocyte population, possibly resident monocytes (Figure 3.2).

**Osteolysis can be stimulated in vitro with conditioned media from 501 and Tom3 cells.**

Calvarial disks from neonatal mice measuring 3.5 mm in diameter were cultured for 6 days with a mixture of 50% BGJ\textsubscript{B} and 50% cell-conditioned media. After 6 days, the calvaria were TRAP stained and the percent lytic perimeter was measured. The control calvaria had 6.7% bone lysis (± 9.7). The T94 cell-conditioned media was not significantly different at 19.4% (± 21.7; p=0.38). The percent bone lysis with 501 and Tom3 cell conditioned media was significantly higher than the control (46.6% ± 23.9 and 62.6% ± 35.7) with p values of 0.01 and 0.001, respectively. Increased numbers of osteoclasts can be appreciated by the increase in fast garnet uptake resulting in red coloration to the calvaria and their osteoclasts (Figure 3.3).

Calcium levels were measured in the conditioned media before and after co-culture with calvaria. There was no significant difference between cell-conditioned media and the DMEM control (6.9 mg/dL ± 0.3). Following calvarial co-culture, the calcium level in control media was 8.0 mg/dL ± 0.6. The calcium level in the T94 media was significantly higher at 9.5 mg/dL ± 1.2 (p=0.003), 501 media was significantly higher at 10.8 mg/dL ± 0.9 (p<0.0001) and Tom3 media was significantly higher 11.0 mg/dL ±1.1 (p<0.0001) compared to the control.
Additionally, the calcium levels in the Tom3 and 501 cell-conditioned media were significantly higher than that of the T94 cell conditioned media with p values of 0.007 and 0.003, respectively (Figure 3.4).

**Derived cell lines engrafted in immunodeficient mice and induced neutrophilic infiltration, splenomegaly and hypercalcemia**

All cells injected in the nu/nu mice grew and all but one mouse (mouse 13, intratibial injection of 501 cells) met early removal criteria by 60 days post injection due to tumor size or lameness. The T94S cells were the most rapidly growing in all sites with an average survival of 19 days post injection (d.p.i.). The average survival of mice injected with 501 cells was 43 days and Tom3-injected mice had an average survival of 54 d.p.i. Two of the three mice injected intratibially with T94S cells had developed grossly visible lung metastasis at the time of euthanasia. A summary of the xenograft study is in Table 2.

Histologically, tumor cells had similar morphologic characteristics to the cells growing *in vitro*. The T94 cells at the site of injection were spindle-shaped and monomorphic with no inflammatory infiltrate. Metastatic lesions present in the lungs consisted of spindle-shaped cells with moderate neutrophilic infiltration. Tumors that formed at the site of 501 cell injection consisted of spindle-shaped cells with minimal to no neutrophilic infiltration. Several 501 cell tumors had a biphasic morphology. In the biphasic tumors, the central population of cells was large and round with large vesicular nuclei and the peripheral cells were spindle-
shaped with elongated nuclei. Interestingly, the central round-cell population was frequently accompanied by a marked neutrophilic infiltration that did not extend into the surrounding spindle-shaped tumor population. Tom3 cells formed tumors that consisted of large round cells with large vesicular nuclei and occasional bi- and multinucleated cells. There was marked neutrophilic infiltrate in all tumors formed by the Tom3 cells. The neutrophilic infiltrate frequently comprised greater than 50% of the total tumor volume. In addition, Tom3 tumor-bearing mice frequently had grossly apparent splenomegaly due to marked myelopoiesis (Figure 3.5).

Tumors from all cell lines injected intratibially extended from the marrow space through the cortex and into the surrounding soft tissue. Expansile growth within the soft tissues was frequently more severe than the tumor growth within the bone. All cell lines induced osteolysis when injected adjacent to bone or intratibially.

There was no significant difference in plasma calcium for mice injected in different locations with the same cell type. Mice injected with T94 cells had an average plasma calcium of 13.9 (± 0.9), 501 cell-injected mice had an average plasma calcium of 14.0 (± 0.6) and Tom3 injected mice had an average plasma calcium of 12.8 (± 0.5) mg/dL. A plasma calcium level over 13 mg/dL was used to make the diagnosis of hypercalcemia in the mice. The mice injected with 501 and T94 cells were considered to be hypercalcemic and had significantly higher plasma calcium levels than mice injected with Tom3 cells (Figure 3.6).
Metastatic T94 cells expressed the most IL-6 and PTHrP, and Tom3 cells with neutrophilia expressed the most CSF3, IL-1α and IL-1β.

Expression of HTLV-1 Tax was confirmed by real-time RT-PCR (Figure 3.7). Gene expression in the 3 cell lines was first examined using Affymetrix microarray. The results of the gene array revealed over 300 genes that had significantly different expression between the cell lines (data not shown). Real-time RT-PCR was performed to confirm the differences in expression for genes relating to osteolysis and the NFκB pathway. The genes selected for real-time RT-PCR were interleukin 6 (IL-6), receptor activator of nuclear factor-κB (RANK) and its ligand (RANKL), osteoprotegerin (OPG), parathyroid hormone-related protein (PTHrP), granulocyte-colony stimulating factor (G-CSF/CSF3), interleukin 1α and β (IL-1α and IL-1β). Significant differences were found in all genes and the differences in gene expression were similar to what was seen in the microarray (Figure 3.8). IL-6 and PTHrP were expressed at a significantly higher level in T94 cells compared to Tom3 and 501 cells. The marked increase in IL-6 expression in the T94 cells correlated well with the significantly faster tumor engraftment and growth in the T94 xenografts, since IL-6 is a potent growth stimulator in multiple tumors including prostate cancer, breast cancer and multiple myeloma.\textsuperscript{17-19} The increased expression of PTHrP in T94 cells correlated with earlier mortality in mice due to increased tumor growth rate. In addition to more rapid growth and progression, the expression of PTHrP by neoplastic cells has been associated with increased tumor metastasis.\textsuperscript{20} The T94
cell line was the only one of the three that produced grossly visible metastases in xenografted mice. OPG and RANKL expression were significantly higher in 501 cells compared to the T94 and 501 cells, whereas greater RANK was expressed in Tom3 and 501 cells compared to the T94 cells. Expression of CSF3, IL-1α and IL-1β were all significantly higher in Tom3 cells compared to the 501 and T94 cells with 501 cells expressing significantly greater mRNA levels than T94 cells. This finding was consistent with the marked neutrophilic infiltrate seen histologically in the Tom3 xenograft mice as well as the similar neutrophilic infiltrate seen in 501 xenografted mice.

**Discussion**

In this study, three cell lines were successfully derived, T94, 501 and Tom3. All three cell lines were immortalized in culture and formed tumors when injected into immunodeficient (nude) mice. Based on high expression of CD11b (Mac-1), the 501 and Tom3 cells were macrophage/monocyte in origin. The lack of MHCII in a monocyte/macrophage origin cell was unexpected; however, the antibody is haplotype specific and will not bind SJL mouse monocytes. Since these mice are a mix of C57BL/6 and SJL, it is likely the negative flow data was a result of the difference in haplotype. The T94 cells were poorly differentiated mesenchymal cells. All three cell lines induced osteolysis *in vivo* when injected
intratibially, T94 cells metastasize to the lungs following intratibial injection and
the T94 and 501 cells induced hypercalcemia regardless of the site of injection.

Xenograft mice injected with T94 and 501 cells frequently developed
hypercalcemia, regardless of the site of tumor cell injection. This finding is
consistent with humoral hypercalcemia of malignancy. Humoral hypercalcemia of
malignancy (HHM) is a paraneoplastic syndrome that is frequently encountered
in patients with ATL.\textsuperscript{21} PTHrP plays a central role in the pathogenesis of HHM in
patients with ATL because it is capable of both increasing osteoclastic bone
resorption and inhibiting the excretion of calcium by the kidneys.\textsuperscript{22} Initiation of the
NF-κB pathway by HTLV-1 Tax has been demonstrated to be a critical step in
cellular transformation and is frequently attributed to increased serum PTHrP and
subsequent development HHM in ATL patients.\textsuperscript{23} Previous studies in our
laboratory have demonstrated that the level of PTHrP expressed in ATL or
HTLV-1-infected cells was regulated by NF-κB.\textsuperscript{24} In these novel cell lines, the
level of PTHrP expression did not correlate to differences in Tax expression
within the cells suggesting that Tax initiated expression of the NF-κB pathway,
but continued expression was independent of Tax. However, PTHrP expression
in the cell lines did correlate well with the development of HHM. The level of
PTHrP expressed by the T94 and 501 cells was significantly greater than the
Tom3 cell line. The mice injected with T94 and 501 cells also developed HHM
and had significantly higher plasma calcium concentrations than mice with the
Tom3 cells.
Survivability and proliferation of neoplastic cells \textit{in vivo} directly correlated to the levels of PTHrP and IL-6 expressed in the different cell lines. The T94 cell line expressed significantly greater levels of both genes compared to the 501 and Tom3 cells. Mice injected with T94 cells had the shortest survival time, regardless of injection site and mice injected intratibially with T94 cells had multifocal lung metastases. The relationship between PTHrP expression and increased cellular proliferation has been demonstrated in multiple cancer cell types.\textsuperscript{20, 25, 26}

PTHrP cannot directly induce the differentiation of hematopoietic precursor cells to osteoclasts, therefore, the PTHrP in T94 and 501 cells did not correlate to the osteolysis and media calcium levels measured in the calvarial co-culture experiment.\textsuperscript{27} Additionally, differences in expression of RANK, RANKL and OPG did not correlate with the differences in osteolysis seen in the calvarial co-culture assay. In contrast, interleukin-1 induces osteoclast maturation and function even in the absence of osteoblasts and mesenchymal stem cells.\textsuperscript{28} The 501 and Tom3 cells expressed significantly higher levels of IL-1\textalpha\ and IL-1\textbeta than the T94 cells and had a significant increase in osteolysis and higher media calcium levels than the T94 cells and controls in the calvarial co-culture experiments.

Inflammation, specifically peripheral neutrophilia, neutrophilic infiltration into tumors and splenomegaly secondary to elevations in G-CSF (CSF3) and GM-CSF have been demonstrated in xenografts and transgenic mice as a direct result of HTLV-1 Tax expression.\textsuperscript{29-31} Tom3 cells had the highest level of CSF3
expression in vitro and subsequently had marked infiltration of xenograft tumors with non-degenerate neutrophils and severe splenomegaly characterized by marked granulopoiesis-myelopoiesis.

In this study, we derived three cell lines that express HTLV-1 Tax under regulation of the granzyme B promoter. These cell lines are immortal and formed tumors in immunodeficient mice. Through microarray and real-time PCR, we have demonstrated differences in gene expression between the three cell lines. As a result of these differences in gene expression, we have been able to support previous studies regarding the expression of PTHrP, IL-1, IL-6 and G-CSF as they relate to the formation of HHM, development of bone-invasive tumors, tumor proliferation and induction of neutrophilic inflammation and myelopoiesis in ATL. Based on these findings, these cell lines will serve as excellent models for further studies into the mechanism and potential treatments of HTLV-1 Tax-induced neoplasia, humoral hypercalcemia of malignancy and tumor-induced inflammation.

The formation and progression of ATL is a complex process that frequently takes years, even decades, to complete. Currently, the mechanisms involved in oncogenesis and disease progression, including the development of osteolytic bone metastases and hypercalcemia, is poorly understood. The viral oncogene, Tax, has been shown to be necessary and sufficient for neoplastic transformation. The development of transgenic mice expressing HTLV-1 Tax that develop consistent, osteolytic tumors was an instrumental first step in better understanding these processes.
understanding the role of HTLV-1 Tax in the formation and progression of osteolytic metastasis and humoral hypercalcemia of malignancy in ATL patients.
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<table>
<thead>
<tr>
<th>Gene</th>
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<td>mPP1A</td>
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**Table 3.1** Primer pairs used for real time RT-PCR
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<tr>
<th>No.</th>
<th>Experimental Procedure</th>
<th>Survival d.p.i.</th>
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Negative (-), Minimal (+), Mild (++) , Moderate (+++), Marked (++++), d.p.i.-days post injection

**Table 3.2** Summary of Xenograft Study Results
Figure 3.1. Photomicrograph of T94 (A), 501 (B) and Tom3 (C) cells. All cell lines were adherent to the culture flask. T94 cells were spindle-shaped, whereas Tom3 cells were stellate with multiple cytoplasmic projections. The 501 cells showed a mixture of both phenotypes; the stellate morphology was predominant.
Figure 3.2 Flow cytometry of T94, 501 and Tom3 cells. Flow cytometry was performed on all cells using antibodies for MHCII, CD11c, CD11b, Ly6C and Ly6G. T94 cells were positive only for Ly6C. The 501 cells were bimodal with more than half of the cells expressing CD11b and a small portion expressing low levels of Ly6C. Tom3 cells expressed CD11b and Ly6C. These data indicated that the 501 and Tom3 cells were macrophage/monocyte in origin.
Figure 3.2
Figure 3.3 Increased osteolytic bone resorption in calvaria cultured with 501 and Tom3 conditioned media. The percent eroded surface in 3.5 mm calvarial punches cultured in cell-conditioned media. The calvaria grown in 501 and Tom3 conditioned media had significantly more osteoclastic bone resorption than the calvaria cultured in control and T94 cell-conditioned media (p<0.05). Below are images of TRAP-stained calvaria following co-culture. The red periphery in the 501 and Tom3 calvaria is due to increased numbers of osteoclasts.
Figure 3.4 Calcium level in the media before and after calvarial co-culture.

There was no significant difference between the cell-conditioned media and control media before calvarial co-culture. After co-culture, the calcium concentration was significantly greater in calvaria cultured with cell-conditioned media compared to the controls. (*p<0.05, **p<0.0001)
Figure 3.5 Histopathology of xenografted mice. All three cell lines grew in immunodeficient (nude) mice. In the subcutis, the T94 (A) and 501 (B) cells were spindle-shaped with minimal to no neutrophilic infiltrate. The Tom3 cells (C) were large and round with vesicular nuclei and accompanied by an abundant neutrophilic infiltrate. The spleen of a mouse with a subcutaneous tumor of 501 cells (D) had no evidence of increased myelopoiesis. The inset shows a higher magnification of the red pulp with erythrocytes, lymphocytes, erythroid precursors and a megakaryocyte. The spleen of a mouse with a subcutaneous tumor of Tom3 cells (E) had marked expansion of the red pulp. The inset shows a higher magnification of the red pulp with large numbers of mature and immature granulocytes, larger myeloid precursors and a megakaryocyte. Lung metastasis was present only in mice injected intratibially with T94 cells (F). Unlike the subcutaneous tumor, the metastases had a moderate infiltration of neutrophils (inset). White bar=50µm, Black bar=100µm, H&E
Figure 3.5
Figure 3.6 Mice injected with all three cell lines developed hypercalcemia.

Mice injected with T94 and 501 cells, regardless of where the cells were injected, had significantly higher concentrations of plasma calcium than mice injected with Tom3 cells (p=0.01).

Figure 3.7 All three cell lines expressed HTLV-1 Tax. RT-PCR was used to demonstrate the presence of Tax mRNA in all three cell lines.
Figure 3.8 Differences in gene expression between the three cell lines correlated with increased growth rate, metastasis and inflammatory infiltrates. Real-time RT-PCR for murine IL-6, RANK, OPG, PTHrP, RANKL, CSF3, IL-1α and IL-1β. The symbol ‘*’ indicates p<0.05 and ‘**’ indicates p<0.0001 when compared to the lowest-expressing cell line, or as indicated.
Figure 3.8
Adult T-cell leukemia/lymphoma (ATL) is a devastating disease that occurs in approximately 4% of people infected with human T lymphotrophic virus type-1 (HTLV-1). ATL is an aggressive malignancy of CD4/CD25+ CD8- T lymphocytes. Patients who develop ATL have a short survival and frequently develop humoral hypercalcemia of malignancy (HHM) and osteolytic metastases that contribute significantly to morbidity and mortality. Development of ATL is attributed to expression of the viral oncogene, Tax. Tax is a transactivating protein and has been shown to be necessary and sufficient for oncogenesis in human and mouse tissues. The mechanism of oncogenesis and cellular immortalization is thought to be multifactorial, including trans-activation of CREB and NF-κB pathways which drive cellular proliferation as well as trans-repression of normal DNA repair mechanisms resulting in cellular mutagenesis. The function of Tax in the development of HHM and osteolytic metastasis, however, is not well understood.

Transgenic mice expressing HTLV-1 Tax under regulation of the granzyme B promoter consistently develop osteolytic tumors in tails and feet and tumors adjacent to the hyaline cartilage of the ear. In this dissertation, we characterized the cell type comprising the tumors formed in these mice. Through
gross and histologic examination, flow cytometry, immunohistochemistry and imaging, we determined that the tumors in these transgenic mice are histiocytic and originate from tendon sheaths, with fewer tumors forming from histiocytes in the endosteum, periosteum and perichondrium of bones.

Following characterization of the tumors in the Tax transgenic mice, we derived three cell lines for further study. The cell lines, T94, 501 and Tom3 were determined to be a poorly differentiated sarcoma and two macrophage/monocyte origin cell lines, respectively. All three cell lines were successfully immortalized in culture and formed tumors in immunodeficient (nude) mice following subcutaneous, intratibial and bone adjacent injection. Microarray and real-time RT-PCR demonstrated a number of genes with significantly different expression between the cell lines. These differences in gene expression correlated with differences seen in vitro and in vivo. Specifically, the development of HHM in mice injected with T94 or 501 cells correlated with significantly higher expression of PTHrP in these cell lines compared to the Tom3 cell line. Additionally, increased osteolysis in calvaria co-cultured with 501 or Tom3 conditioned media correlated with significantly higher expression of IL-1α and IL-1β in these cell lines. Finally, the development of marked splenomegaly due to increased splenic myelopoiesis and tumor infiltration with large numbers of non-degenerate neutrophils in mice injected with Tom3 cells correlated with increased expression of granulocyte-colony stimulating factor in this cell line compared to the T94 and 501 cells.
The future directions based on the results of this dissertation research include:

(1) Determine the effect of HTLV-1 Tax expression on cell survival and gene expression profiles in derived cell lines.

Tax is attributed to transactivation and trans-suppression of a number of genes in patients infected with HTLV-1. These changes in gene expression as a result of Tax expression in infected cells have been associated with increased proliferation, cell survival and accumulations of DNA damage/mutations that result in oncogenesis. Additionally, transactivation of the NF-κB pathway by Tax is associated with increased expression of PTHrP and subsequent development of HHM.

Inhibition of Tax expression could be performed through the introduction of siRNAs against HTLV-1 Tax into the three Tax-induced malignant cell lines. Examination of cellular proliferation, gene expression and osteolysis in vitro as well as tumor formation, metastasis and development of hypercalcemia in vivo would result in further understanding of the effect of continuous Tax expression on the expression of genes necessary for immortalization and formation of osteolysis and HHM.

(2) Direct examination of the effect of specific genes suspected to contribute to differences between Tax-induced malignant cell lines in vitro and in vivo.
The formation of HHM, lung metastasis and inflammation/splenomegaly that occurs in xenografted mice as well as increased osteolysis in calvarial co-cultures were attributed to differences in expression of PTHrP, IL-6, G-CSF and IL-1, respectively. To confirm the role that specific genes played in these phenotypic differences, induction of gene expression through transfection as well as inhibition of gene expression through siRNA could be used.

The effect of PTHrP on the development of HHM in mice injected with Tax-induced malignancies should be confirmed. Experiments in our lab have demonstrated the ability to upregulate gene expression through cellular transfection and inhibit gene expression through the use of siRNAs. These techniques could be utilized in all three cell lines to alter expression of PTHrP, IL-6, G-CSF and IL-1 to confirm their role in cellular growth, metastasis, osteolysis and inflammation in vitro and in vivo.

(3) Examination of gene expression within the bone microenvironment.

The microarrays and real-time RT-PCR in this dissertation were all performed on genes in culture. Although there were significant differences in gene expression between the cell lines that correlate with alterations in tumor phenotype and development of HHM, this study did not look specifically at differences in gene expression of a single cell line in tumors formed within the bone microenvironment and tumors not in contact with bone.
Initial studies to examine these differences through microarray were performed; however, because both the cells and tissue injected are mouse-origin, complete separation of tumor cells from surrounding stroma was not possible. To address this issue, cells could be transduced with GFP-luc. The expression of GFP would allow for selection of neoplastic cells by flow sorting. RNA from these cells could then be isolated and examined for differences in gene expression. The expression of firefly luciferase would allow for localization of small metastases \textit{in vivo} that would not been identified on routine gross post-mortem examination of xenografted mice.

Patients diagnosed with acute ATL currently have a poor prognosis with a short survival and limited treatment options. Understanding the role of Tax in the progression of ATL, formation of bone metastases and HHM will help further define Tax as a potential treatment target against this disease. Specifically, the potential use of monoclonal antibodies against proteins known to be transactivated by Tax following HTLV-1 expression as a target for use in ATL treatment. Concurrent research on the role HBZ, an anti-sense protein that is expressed later in the progression of HTLV-1 that functions to reduce the effect of Tax on NF-κB expression as well as the potential for new allogenic stem cell transplant protocols in conjunction are all potential treatment targets. ATL is a devastating disease, but novel therapeutic approaches based on the results of
multi-faceted mechanistic studies in the initiation and progression of cancer has the potential to offer effective treatment or prevention.
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