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Previously B lymphocytes have been reported to accumulate at the site of tumor development and to play a role in immune surveillance against metastatic tumors. Investigating this mechanism, we studied B lymphocyte production of extracellular matrix-degrading proteinases: matrix metalloproteinases (MMPs) and components of the urokinase plasminogen activator (uPA) system. Our studies include RT-PCR of CRL-1631 cDNA revealing mRNA for MMP-2, MMP-9, TIMP-1, TIMP-2 and uPAR. MMP-2 and MMP-9 activity was verified by gelatin zymography. TIMP-1, TIMP-2 and uPAR protein expression was confirmed by Western blot analyses. I also report, for the first time, MT-1 MMP gene and protein expression in B cells by RT-PCR and Western blot, respectively. CRL-1631 invasion through Matrigel model basement membrane was significantly inhibited by BB-94, confirming MMP involvement in this cell line's invasiveness. Therefore, B cells use multiple proteases in the degradation of the extracellular matrix, ECM, and this may be one factor responsible for their accumulation at the site of established tumors.

PRODUCTION OF EXTRACELLULAR MATRIX-

DEGRADING PROTEASES BY A RAT B CELL LINE,

CRL-1631

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PRODUCTION OF EXTRACELLULAR MATRIX-DEGRADING PROTEASES BY A RAT B CELL LINE, CRL-1631

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CHAPTER 1

INTRODUCTION

Historically, there have been two main functions attributed to B lymphocytes: antibody production and antigen presentation. Upon activation of its surface immunoglobulin receptor, the B cell differentiates into a plasma cell producing antibody molecules of the same specificity as the receptor (1,2). As an antigen-presenting cell, (APC), the activated B cell provides the two signals necessary for the priming of $CD4^+$T cells: the ligating signal through the T cell receptor, (TCR), via the MHC Class II/peptide complex and the co-stimulatory signal via CD28 ligation with the B-7 molecule (3).

Recently, however, in studies investigating the mechanism of how stress can affect tumor development, it has been observed that B lymphocytes may play a role in immune surveillance against metastatic tumors (4). A significant correlation was found between an increased B lymphocyte population in the lungs of Fisher 344 rats and decreased pulmonary metastases subsequent to tail vein injection of MADB106 mammary adenocarcinoma cells (4). One million MADB106 cells were administered to Fisher 344 rats via tail vein injection. Seven and eleven days later the animals were sacrificed and the number of lung metastases were measured and found to be ~10 metastases (mets) /cm^2 on day 7 and ~68 mets/cm^2 on day 11. Analysis of the lung lymphocytes also done seven and eleven days after tumor injection showed that in vehicle-injected control animals, the B lymphocyte percentage in the lungs was very low, usually less than 1% of the total lymphocytes. The B cell percentage after tumor cell injection, however, was elevated appreciably and significantly: 6-10% on days 7 and 11 after tumor cell challenge (4). Of particular interest is the fact that immunoneutralization of the B cell population resulted in a significant increase in the number of lung metastases. Anti- rat B cell antibody

(OX33 100μg IgG) was administered at various time points: simultaneously with tumor cells or 24 hours after tumor injection. Control animals only received tumor cells, without antibody to B cells. Again, the lung metastases were analyzed 7 days after injection. The control animals developed ~2 mets/cm^2; the animals with simultaneous antibody and tumor cell injections developed ~21 mets/cm^2; animals that received antibody 24 hours after tumor challenge developed ~2 mets/cm^2 (essentially no difference from control). Subsequent studies showed that even a 1-2 hr. delay of antibody administration after tumor cell injection decreased the ability of the anti-B cell antibody to increase lung metastases. These findings indicate that the anti-tumor influence of B lymphocytes occurs very soon after the tumor cells reach the lung (4). Similar findings were also found in the WAG rat using CC531 colon adenocarcinoma cells (4). The relationship between various lymphocyte populations prior to tumor challenge and subsequent tumor development was also investigated, using WAG rats and CC531 cells. It was determined that the baseline percentage of B lymphocytes in the peripheral blood prior to tumor challenge was positively correlated with the duration of survival and negatively correlated with the size of the primary tumor. It was also noted that the number of B lymphocytes decreased in the circulation after tumor cell injection (5). In summary, these findings, therefore, suggest the possibility that B lymphocytes participate in tumor immune surveillance.

It is realized that this participation may involve B cell activity very early after tumor cell injection within the peripheral circulation or it may occur within the lung. Based on the previous studies heretofore mentioned, I am proposing that the B cell tumor surveillance activity primarily occurs in the lung. Even though the exact mechanism involved in this B cell anti-tumor influence remains to be elucidated, I have focused my efforts on determining the mechanism used by B lymphocytes to position themselves within the tumor environment to effect such an influence.

There have been extensive studies conducted on other cell types of the immune system and their participation in tumor surveillance including cytolytic T cells, macrophages, and activated NK, A-NK, cells. It has been shown that adoptively transferred fluorescently labeled IL-2- activated NK cells accumulate within established metastases (6). These findings led to further studies showing that A-NK cells use BB-94 inhibitable matrix metalloproteinases, MMPs, to degrade extracellular matrices, thereby suggesting MMPs play a role in the accumulation of A-NK cells within cancer metastases (7). The production of MMP-2, MMP-9, MMP-11, MMP-13, MT1-MMP, MT2-MMP, and tissue inhibitors of matrix metalloproteinases, TIMP-1, and TIMP-2, was shown in IL-2 activated NK cells (8). Moreover, our recent studies have shown the expression of urokinase plasminogen activator, uPA , and urokinase plasminogen activator receptor, uPAR, on rat NK cells, again suggesting the possible involvement of these proteolytic enzymes in A-NK cell extracellular matrix degradation. (9) Indeed, the MMP and uPA/uPAR systems appear to work in concert in NK cell-mediated extracellular matrix degradation.

MY HYPOTHESIS is that B cells produce extracellular matrix-degrading proteases. If B lymphocytes produce such enzymatic activities, these proteases could participate in the binding to and degradation of extracellular matrix (ECM) components within the tumor stroma, thereby allowing the B lymphocytes to play a role in immune surveillance within the tumor microenvironment.

MMPs are a family of zinc-dependent endopeptidases collectively capable of degrading virtually all components of the ECM. The majority of MMPs are secreted enzymes, while only a few are membrane-associated. MMP activity is tightly controlled by activation of latent enzymes and tempered by inactivation as the result of MMP inhibitors, TIMPs, or tissue inhibitors of

MMPs (8). uPA is a serine protease that upon binding to its receptor, uPAR, cleaves plasminogen to plasmin which can either directly degrade various components of the ECM or indirectly degrade these entities by activating members of the MMP family.

Heretofore, there has been only minimal investigation into such proteases in B lymphocytes as well as into B lymphocyte participation in tumor immune surveillance. It has been shown that EBV-immortalized human B lymphocytes secrete the gelatinase, MMP-9, that correlates with the chemotactic migrating ability of these cells through model basement membrane-like ECM (10). IgG-positive plasma cells from diseased synovial and scleral tissue have been found to express MMP-3 and MMP-9 (11). In situ-hybridization has shown that lymphoblastic leukemia B cells constitutively express mRNA for MMP-2 and MMP-9 (12).While B lymphocytes have been implicated in the inhibition of tumor growth mediated by lymphotoxin, lymphoplasmacytic B cells have been found to infiltrate tumors, and B cells have also been shown to participate in the growth inhibition of pulmonary melanoma metastases achieved by a targeted antibody-lymphotoxin fusion protein (13,14,15) the role of degradative proteolytic enzymes in B cell extracellular matrix degradation remains only fragmentary. In this study, I examine not only the production of various members of the MMP family and the uPA system by a rat B lymphocyte cell line but also the possibility that these degradative proteases may contribute to B lymphocyte entry into the tumor environment where they may contribute to immune surveillance against metastatic tumors.

SIGNIFICANCE

There are several ramifications to the findings described herein delineating the production of extracellular matrix-degrading proteases by B lymphocytes. A better understanding of how B cells may influence tumor development was my primary purpose in initiating these studies. However, these studies will also provide insights in the B lymphocyte's role, not only in tumor challenge, but also in other diseases.

It is likely that this work can be incorporated into a future therapeutic model that consists of adoptively transferred B lymphocytes being used to penetrate the tumor stroma as outlined in this work. Their subsequent participation, either directly or indirectly through other immune mediators, can now be tested for their potential role in the establishment of an effective anti-tumor, anti-metastatic host defense.

CHAPTER 2

MATERIALS and METHODS

Reagents and Chemicals

Recombinant rat IL-4 was purchased from Sigma (St. Louis, MO), Antibody to MT1-MMP were obtained from Oncogene Research Products (Cambridge, MA) Antibodies to TIMP-1 and TIMP-2 were obtained from Calbiochem Corp. (La Jolla, CA). Antibody to uPAR was obtained from American Diagnostica, Inc. (Greenwich, CT) BB-94 was a generous gift of British Biotech (Oxford, UK).

Culture and Preparation of Cell Line

CRL-1631 cells, obtained from ATCC, are B cell lymphoblasts derived from rat plasmacytoma. The cells were cultured in Dulbecco's Modified Eagle Medium, Sigma Corp. (St. Louis. MO), with 4.5g/L glucose and 5% fetal bovine serum, FBS. The cells are grown at 37°C. with 10% CO_2. The cells are also cultured in serum-reduced medium, Opti-MEM 1, also in 10% CO_2 at 37° C.

RT-PCR

Total RNA was extracted from the CRL-1631 cells using RNeasy Column by Qiagen, (Chatsworth, CA). cDNA was subsequently synthesized using the RT-PCR kit by Stratagene (LaJolla, CA). PCR amplification of the CRL-1631 cDNA was then performed using the appropriate primers in the protocol set forth by Qiagen (Chatsworth, CA) using HotStar Taq DNA polymerase.

Agarose Electrophoresis

A 1% agarose gel in 5X Tris HCl/ Boric Acid/ EDTA Buffer, (TBE), was prepared with the addition of 2 µl Ethidium Bromide. The PCR product was run in the electrophoresis chamber in the presence of 10X loading buffer at 150V for 1 hr DNA bands were visualized by UV illumination. Band molecular weights were determined from standards using the AlphaImager (Alpha Innotech).

Gelatin Zymography

Gelatin (2mg/ml) was added to 10% SDS-PAGE gel. The serum-free conditioned media from CRL-1631 cells was loaded directly onto the gel. To maintain enzyme activity, the samples were neither boiled nor reduced prior to loading. After the gels were electrophoresed at 20 ma for 1.5 hrs, the gels were incubated in 2.5% Triton X-100 for 1 hr at room temperature. The gels were then transferred to 1% TX-100 and incubated for 18-24 hrs. after which the gels were stained in 0.1% Coomassie Blue, 50% MeOH and 7% Acetic Acid overnight and destained using 40% MeOH and 7% acetic acid.

Western Blot Analysis

Membrane fractions (cell lysates) or concentrated conditioned media (supernatants) of CRL 1631 cells were subjected to electrophoresis on 10% SDS-PAGE gel for 1.5 hrs at 30 mA. Proteins from the gel were then electro-blotted onto a PVDF membrane overnight at 30V in 4° C with 100V being applied for the last hour of the transfer. The membrane was then blocked for 1 hr with

1 % goat serum and 5% dry milk in 0.5% Phosphate Buffered Saline-Tween 20, (PBS-T). Monoclonal mouse anti-MT-1 MMP at 1:100 dilution, was used to detect the presence of CRL-1631 MT-1 MMP via overnight incubation at 4°C. Peroxidase labeled goat anti-mouse polyclonal secondary antibody at 1:50,000 dilution was applied for 1 hr at 25° C. The membrane was incubated for 3 min. with Luminol/Enhancer Solution and Peroxidase Super Signal Substrate from Pierce Chemical (Rockford, IL). The membrane was subsequently exposed on radiographic film for development of theband via chemiluminescence. Mouse monoclonal anti-TIMP-1 was used at 1:200 dilution and mouse monoclonal anti-TIMP-2 was diluted 1:100. The secondary antibodies and detection methods were the same as described previously. Rabbit anti-rat uPAR was diluted 1:50 and was probed with the peroxidase conjugated secondary antibody, goat anti-rabbit Ig. The same detection method was then employed.

Concentration of conditioned medium

After 3 days in culture, the B cells were placed in Opti-MEM reduced -serum medium for 24 hrs. The culture supernatants were collected, centrifuged to remove debris, and concentrated in Amicon Centriplus concentrators, Millipore Corp. (Bedsford, MA) up to 40 fold. Aliquots were frozen at -80° C. HT1080 control supernatants were previously prepared and stored in the same manner.

Preparation of whole lysates from CRL-1631 cells

Three day-old CRL-1631 cells were washed in 1X PBS, then lysed in an extraction buffer consisting of 0.1M Tris HCl, 5M NaCl, 0.5M EGTA, 0.5M EDTA, 10mM p-nitrophenyl p-guanidino-benzoate, (NPGB), 1000 u/ml aprotinin, and 1%Triton X-100. Lysates were cleared by centrifugation at 960 x g for 10 min. Lysates were then aliquoted and frozen at - 80° C.

Phosphatidylinositol Specific Phospholipase C Treatment (PtdIns-PLC)

Ptd-Ins-PLC treatment was performed as previously described (9) .

Briefly, 40 x 10^6 cells were washed in RPMI 1640, then incubated at 37° C for 1 hr in 1 ml RPMI with 1U/ml Ptd Ins-PLC, (Sigma, St.Louis, Missouri) 10mM NPGB, 1000u/ml aprotinin and 10μg/ml leupeptin. The cells were then spun down and the uPA/uPAR containing supernatant was run on SDS-PAGE with subsequent Western blot analysis.

Invasion Assay

The assay was performed in Biocoat Matrigel Invasion Chambers, Becton-Dickinson Laboratories (Bedford, MA) . The Matrigel was hydrated using 0.25 ml RPMI for 2 hrs at 37° C. A total of 100,000 CRL-1631 cells in 0.5ml of 0.2% BSA-Opti-MEM was placed in the top wells. Opti-MEM containing 0.2% BSA (0.75 ml) was placed in the bottom chambers. BB-94, 10 μM, was added to the top and bottom chambers of 6 of the 12 wells. IL-4 at 20 ng/ml was also added to the top and bottom chambers of 6 wells. The plates were then incubated at 37° C for 24 hrs. Top wells were removed and discarded. After 24 hrs incubation, the bottom chambers were labeled with 1μM calcein AM and incubated at 37°C for 45 min. The plate was then read on a Cytoflour II fluorescence plate-reader using an excitation wavelength of 485nm and an emission wavelength

of 530 nm. Standards with known concentrations of CRL-1631 cells were simultaneously run. The number of invading cells was calculated by plotting the fluorescence on the standard curve. All determinations were performed in triplicate.

CHAPTER 3
RESULTS

RT-PCR Analysis of cDNAs from CRL-1631 B cells

Total RNA was prepared from CRL-1631 cells and cDNA was synthesized using reverse transcriptase. The cDNA was then analyzed using known primers for rat MMPs, TIMPs, uPA and uPAR (Table I). Results in Figure 1A show that CRL-1631 cells express the mRNA for TIMP-1 and uPAR, Figure 1B shows the band representative of TIMP-2. Figure 2A indicates the gene transcript for MT-1 MMP and MMP-2 and MMP-9 is shown in Figure 2B. The gene transcript for uPA was not detected.

Gelatin Zymography Analysis of CRL-1631 Culture Supernatants

To assess the enzymatic activities of the MMPs found via RT-PCR, we performed SDS-PAGE gelatin zymography. Supernatants from CRL-1631 cells grown in serum-free medium were concentrated, as described in **Materials and Methods**, and analyzed by SDS-PAGE gelatin zymography, using HT 1080 cell supernatant as the positive control. Figure 3A shows 2 bands of lysis indicating the presence of two gelatinases: MMP-2 found at a molecular weight of 72 kDa and MMP-9 at a molecular weight of 92kDA, as confirmed in the control, HT 1080 cells. Use of the MMP inhibitor, BB-94, shown in Figure 3B ablated the gelatinase activity of these two enzymes, confirming that ,indeed, these bands are representative of MMPs.

Western Blot Analysis of MT1-MMP, TIMP-1, TIMP-2 and uPAR

After cells were treated with PtdIns-PLC as described in **Materials and Methods**, rabbit anti-rat uPAR was used and a band was detected at approximately 60 kDa representative of rat uPAR as seen in Figure 4A. The blot was subsequently stripped of anti-uPAR and re-probed using rabbit anti-rat uPA as the primary antibody. This resulted in the 60 kDa band, uPAR, being very diminished and the appearance of a well-defined uPA band at 55 kDa. Additional stripping and probing of the blot for non-specific rabbit IgG binding accounted for the presence of the higher molecular weight bands noted on the Western blot. Confirmation of MT1-MMP expression was obtained by using Western blot analysis from cell lysates, as described previously. Using mouse anti-MT1-MMP, a rat protein was recognized at a molecular weight of 60kDa, as shown in Figure 4B, which corresponds to the reported molecular weight reported for MT1-MMP. Figure 5A shows TIMP-1 was found to be expressed at 28kDa using mouse anti-TIMP-1. TIMP-2 in Figure 5B was found to be expressed at a MW of 27kDa when probed with mouse anti-TIMP-2. A similar higher than expected MW for TIMP-2 has also been reported for IL-2 activated mouse NK TIMP-2 by Kim et al (8). These two antibodies for TIMP-1 and TIMP-2 recognize the rat proteins as well as the mouse proteins.

Role of MMPs in Rat B Cell Invasion

To determine if MMPs play a role in B cell invasiveness, a Matrigel invasion assay was employed. CRL-1631 B lymphocytes were placed in a Matrigel invasion chamber containing an 8 μm pore-size filter coated with 100μg of Matrigel per cm^2. As shown in Figure 6, the invasion of the B cells through Matrigel in a 24 hr period was inhibited ~66% by 10μM BB-94. In addition, when IL-4 was used to increase the number of B cells with locomotor capability (16), this increase in migration was also inhibited by BB-94.

TABLE I
Primer Sequences Used in PCR of CRL-1631 cDNA

Enzyme	Forward Primers (5′—3′)	Reverse Primers (5′-3′)
MMP-2	GAGTTGGCAGTGAATACCT	GCCGTCCTTCTCAAGTGT
MMP-9	AGTTTGGTGTCGCGGAGCAC	CATGAGCGCTTCCGGCACTG
TIMP-1	CTGGCATCCTCTTGTTGCTA	AGGTATTGCCAGGTGCACAA
TIMP-2	AGACGTAGTGATCAGGGCCA	GTACCACGCGCAAGAACCAT
MT1-MMP	GTGCCCTATGCCTACATCCG	TTGGGTATCCGTCCATCACT
uPAR	ACAGAACGAGCGTGAAGGA	ATGAGGATAAGATGAGCAGG

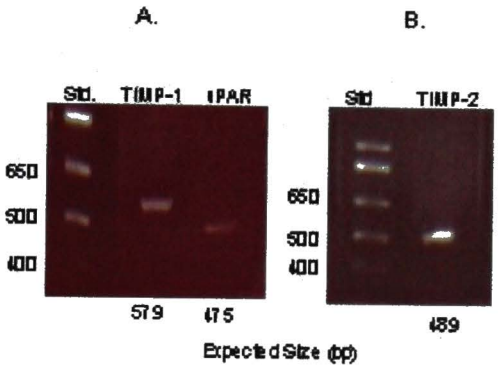


FIGURE 1. RT-PCR analysis of TIMPs and uPAR of CRL-1631 cells. Total RNA was isolated and cDNA was generated as previously described in Materials and Methods.RT-PCR analyses were performed using specific primers as outlined in Table I. Figure 1A, TIMP-1 at 579 bp and uPAR at 475 bp; Figure 1B, TIMP-2 at 498 bp,.

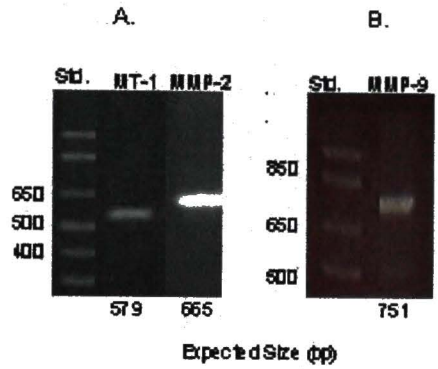


FIGURE 2. RT-PCR analysis of MMPs of CRL-1631 cells. Total RNA was isolated and cDNA was generated as previously described in Materials and Methods. RT-PCR analyses were performed using specific primers as outlined in Table I. Figure 2A, MT1-MMP at 579bp and MMP-2 at 665 bp; Figure 2B, MMP-9 at 751 bp.

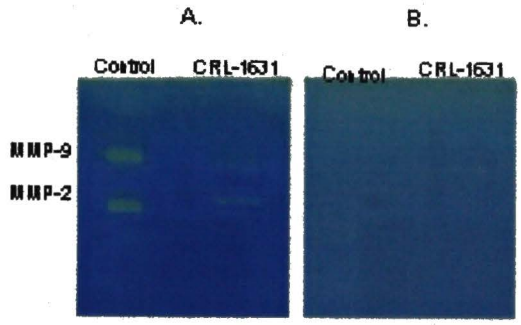


FIGURE 3. Gelatin zymography analysis of CRL-1631 and control, HT 1080, supernatants. Day 3 CRL-1631 cells were incubated in Opti-MEM for 24 hrs. Media supernatants were then filtered and concentrated as described in Materials and Methods. HT1080 supernatants were collected in a similar fashion. Figure 3A, the 92kDa and 72 kDa bands of gelatin lysis indicate MMP-9 and MMP-2 enzymatic activity respectively; Figure 3B, ablation of MMP activity in the presence of 10μM of the specific MMP-inhibitor, BB-94.

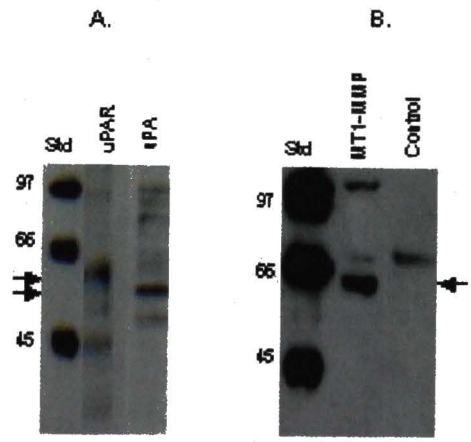


Figure 4. Western Blot Analysis of CRL-1631 uPAR and MT1-MMP. Figure 4A, uPA/uPAR supernatants were obtained by Phoshophatidylinositol-specific Phospholipase C treatment as described in Materials and Methods. SDS-PAGE and Western blots were run. The blots were blocked and incubated with rabbit polyclonal antibody to rat uPAR, specifically domain 1 of uPAR. Rat uPAR is denoted by a band at approximately 60 kDa that is consistent with the glycosylated protein. Stripping and re-probing of the blot using rabbit anti-rat uPA shows the band for uPA at ~55 kDa , confirming the 60kDa band on the original blot as uPAR. Figure 4B, cell lysates from CRL-1631 cells and control, HT 1080 cells, were prepared as described in Materials and Methods and run on SDS-PAGE gels with subsequent Western blot analysis. The blots were blocked and incubated with mouse monoclonal antibody to human MT1-MMP. The MT1-MMP protein is found at a MW of approximately 60 kDa .

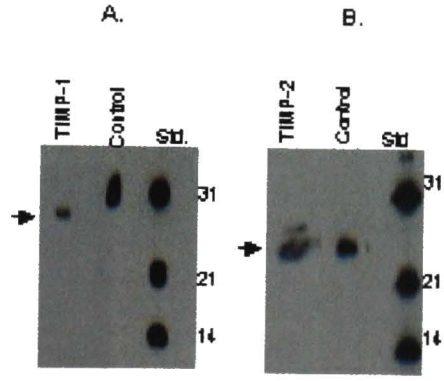


FIGURE 5. Western blot analyses of CRL-1631 TIMP-1 and TIMP-2. Media supernatants from CRL- 1631 cells and HT 1080 control cells were prepared as described previously in Materials and Methods. SDS-PAGE gels with subsequent Western blot analyses were performed. Figure 5A, blots were blocked and incubated with mouse monoclonal antibody to human TIMP-1, with known rat reactivity. TIMP-1 is indicated by the presence of a band at a MW of 28 kDa. Figure 5B, blots were blocked and incubated with mouse monoclonal antibody to human TIMP-2, with known rat reactivity. TIMP-2 is delineated by the presence of a band at MW of approximately 27 kDa.

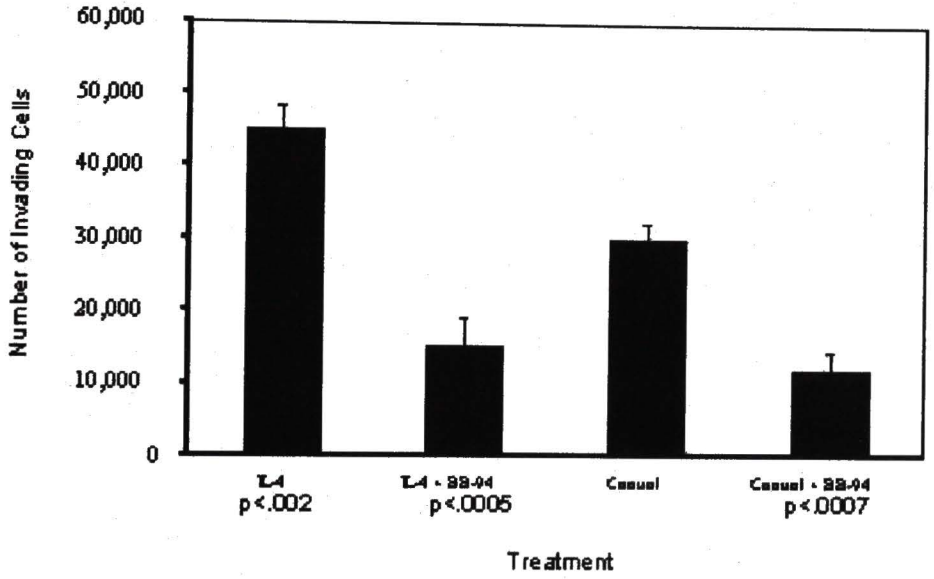


FIGURE 6. Inhibition of CRL-1631 Invasion through Matrigel model basement membrane using BB-94. 100,000 CRL-1631 cells in 0.5ml Opti-MEM containing 0.2% BSA were placed in the upper wells of Matrigel invasion chambers, with or without 10μM BB-94. IL-4 was used to stimulate cell motility. Results are expressed as cell numbers invaded through Matrigel. Each bar represents the average of at least triplicate determinations. (±SD).

CHAPTER 4

DISCUSSION

In this study I have examined the rat B cell line, CRL-1631, for the presence of various proteolytic enzymes known to be essential for the degradation of ECM/basement membrane. Herein, mRNA for MMP-2, MMP-9, MT1-MMP, TIMP-1, TIMP-2 and uPAR has been documented in these B lymphocytes.

MMP-2 and MMP-9 enzymatic activities were demonstrated by gelatin zymography of CRL-1631 cell supernatants. This activity was totally ablated when the conditioned medium was treated with the prototypical MMP inhibitor, BB-94, as also seen in the inhibition of MMPs of other cell types (8). Protein expression of MT-1 MMP has been verified by Western blot analysis as has the expression of TIMP-1, TIMP-2, and uPAR.

MT-1 MMP has been reported to be expressed in other immune-type cells: macrophages, T cells and NK cells. This, however, is the first report by Western blot, documenting the presence of MT1-MMP in B lymphocytes, specifically the rat B cell line, CRL-1631.

The presence of MT-1 MMP prompts one to postulate that there is probably cell surface binding of the B cell to ECM components, resulting in the proteolytic degradation of the basement membrane. It is known that MT-1 MMP, in conjunction with TIMP-2, activates latent MMP-2. The active MMP-2 is then capable of degrading collagen type IV, one of the primary constituents of the extracellular matrix. Indeed, it is felt that other MMP family members may also be converted to their active forms upon this binding to the extracellular matrix. This finding,

therefore, suggests an important potential role for membrane-associated ECM-degrading enzymes in B cell function.

In addition to the studies I have delineated previously, I also have unpublished data and findings included in this thesis appendix that further support my belief that B cells do, indeed, bind to ECM components and probably activate latent MMPs in the process. I have shown phenotypical changes in B cell morphology upon exposure to increasing concentrations of Matrigel model basement membrane. An increase in MMP-2, demonstrated on gelatin zymography, has been shown when these cells were grown on collagen IV-coated flasks. I have been unable to show direct B cell binding to tumor cells, specifically using CRL-1631 cells and CC531 colon adenocarcinoma cells. This observation, however, may imply a requisite preliminary process involving B cell adherence to and subsequent proteolytic degradation of the tumor stromal ECM, thus allowing the B lymphocyte to then influence the tumor microenvironment. Interestingly as well, this observation correlates with preliminary results obtained showing a decrease in the number of CC531 tumor cells when grown in the presence of CRL-1631 cells. The two cell types were physically separated by a 0.45μ transwell filter ,but with free exchange of media supernatants. It would, therefore, appear that these B lymphocytes may be capable of affecting these tumor cells when they are in the tumor environment.

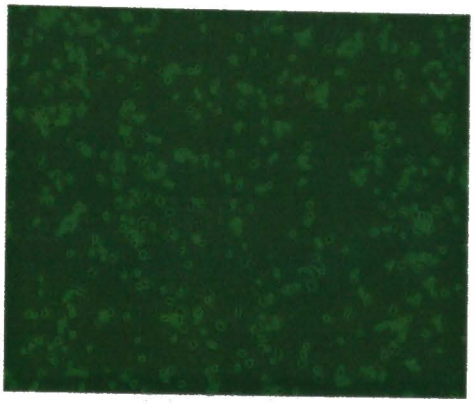
The detection of mRNA for uPAR as well as its protein expression in this cell line suggests not only another putative ECM degradative pathway for this B cell population, but also a possible cooperative protease cascade between the MMP family and the uPA system which is known to be operational in other invasive cell types including metastatic tumor cells and NK cells (9).

Further studies are warranted regarding the functional capabilities of these demonstrated proteolytic enzymes. As others have shown that MMPs definitively play a role in A-NK cell infiltration of cancer metastases and in migration through the basement membrane, I have attempted to examine these scenarios in this B cell population. I have investigated the ability of B cells to migrate through a model basement membrane and have demonstrated that MMPs play a role in this process. Additional related studies could continue, looking specifically for the cooperation of MMPs and uPAR. As mentioned previously, the potential role of these degradative enzymes in B cell/tumor binding and B cell/tumor stromal binding has also been under active investigation as well. It has previously been reported that TIMP-2 inhibits basic fibroblast growth factor induced human microvascular endothelial cell proliferation. Such anti-angiogenic potential using this cell line in association with rat lung microvessel endothelial cells can also be undertaken. It is known that B cells can produce various cytokines that may directly or indirectly, through other cells of the immune system, play a role in their immune surveillance capability. This immune modulatory scenario should also be investigated.

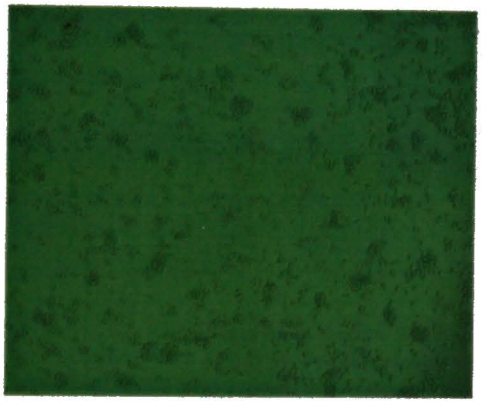
The correlation between an increased B lymphocyte population in the lungs of rats previously injected with MADB106 mammary adenocarcinoma cells or CC531 colon adenocarcinoma cells and decreased pulmonary metastases (4,5) is very intriguing and warrants additional investigation. In these studies, I have attempted to better understand the nature of this relationship between B cells and tumor cells. As tumor cell metastases is attributed to the invasion through and the degradation of extracellular matrices, I have postulated that matrix-degrading proteinases, including MMPs, may be produced by B lymphocytes and may participate in their effector role at the site of tumor challenge. Upon my test of this hypothesis, such enzymes are indeed produced by this cell type. I believe that these findings provide

important insights into the molecular mechanism of B cell function in immune surveillance against cancer metastases. Indeed, I believe that members of the MMP family and the uPA system found in B lymphocytes may be critical to the understanding of this B cell/tumor cell interaction and may constitute a key component in B lymphocyte participation in tumor immune surveillance. This B cell participation may lead to future anti-metastatic therapeutic modalities using adoptively transferred B lymphocytes, i.e..., as ECM-degrading vehicles to penetrate the surrounding tumor stroma. This could likely lead to the subsequent development of an unfavorable micro-environment for further tumor expansion, and thereby contribute to therapeutic efficacy against advanced tumors.

APPENDIX



Control

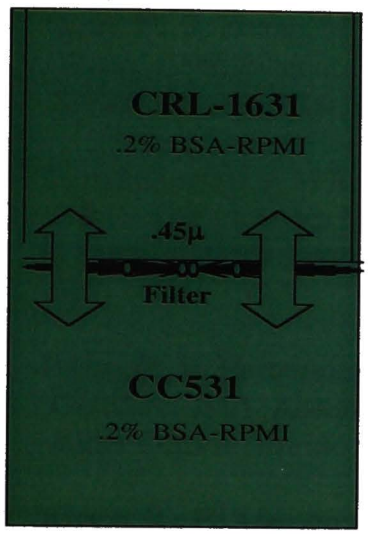


1mg/ml

5mg/ml

Figure 7. CRL-1631 Morphology with Increasing Matrigel Concentrations. 100,000 cells were plated on non-coated tissue flasks (control), or on Matrigel model basement membrane coated flasks (1mg/ml; 5mg/ml) and allowed to incubate at 37° C for 24 hrs. CRL-1631 cells exhibited increased clumping and "chaining" upon exposure to Matrigel, indicating probable binding to and invasion into the artificial basement membrane.

Figure 8. Gelatin Zymographic Analysis of CRL-1631 MMP-2 Production with Type IV Collagen Stimulation. Conditioned media supernatants from CRL-1631 cells grown on uncoated tissue flasks were compared to such supernatants from CRL-1631 cells grown on Collagen IV coated flasks. Equal amounts of protein were loaded for each sample. Collagen IV stimulation results in an increased production of MMP-2: this correlates with CRL-1631 binding to Collagen IV via MT1-MMP with its subsequent activation of MMP-2.



Control = cells + .2% BSA- RPMI

	24hrs	48hrs	72hrs
	Same as Control	Same as Control	Same as Control
	Same as Control	50% of Control	40% of Control

Figure 9. CRL-1631 / CC531 Incubation in Transwell Chamber.
CRL-1631 cells were placed in the top well of a transwell chamber and were separated from CC531 colon adenocarcinoma cells in the bottom chamber by a 0.45μ pore-size filter.Analyses of both cell types was conducted via MTT assay and compared to Control cells of each cell type at specified time points. Within the tumor microenvironment, CRL-1631 cells appear to exert an anti-tumor influence.

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