American Journal of Pathology, Vol. 152, No. 2, February 1998

Proteolytic Activity of Human Non-Hodgkin's Lymphomas

Anna E. Kossakowska,* Aleksander Hinek,† Dylan R. Edwards,‡ Megan S. Lim,*‡ Chun-Li Zhang,* Deanne R. Breitman,* Christopher Prusinkiewicz,* Andrea L. Stabbler,* Lawrence S. Urbanski,* and Stefan J. Urbanski*

From the Departments of Pathology and Pharmacology and Therapeutics,‡ University of Calgary, Alberta, and the Division of Cardiovascular Research, Hospital for Sick Children, Toronto, Ontario,† Canada*

This study was conducted to assess the net proteolytic activity of human non-Hodgkin's lymphomas (NHLs). We have compared the extracellular matrix (ECM)-degradative abilities of human NHLs, reactive lymphoid hyperplasias, and established lymphoid cell lines using Matrigel invasion and elastin degradation assays. The inhibition studies allowed identification of the classes of proteinases involved in ECM degradation. Our results indicate that lymphocytes and other leukocytes derived from both human NHLs and reactive lymphoid hyperplasias are capable of Matrigel penetration, but only cells derived from the high-grade human NHLs degrade elastin *in vitro*. Established lymphoid cell lines (both malignant and Epstein-Barr virus immortalized) do not produce MMP-9, do not penetrate the Matrigel, and do not degrade elastin. Moreover, in human NHLs, elastolytic activity is blocked by metalloproteinase inhibitors, while inhibitors of the other classes of proteolytic enzymes have only minor effects. This study identifies metalloproteinases as the most important class of proteinases involved in ECM degradation by NHLs. The previous studies suggest that, within this class, MMP-9 represents the key enzyme that plays a role in the biological aggressiveness of human NHLs. ***(Am J Pathol 1998, 152:565–576)***

Destruction of an extracellular matrix (ECM) accompanies many physiological and biological processes, including an inflammation and tumor invasion.[1,2] In the process of tumor invasion in carcinomas, malignant cells break through the natural barrier of basement membrane (BMs) and migrate within the ECM until they encounter blood or lymphatic vessels.[3,4] Vascular invasion and ECM destruction enables malignant epithelial cells to disseminate through the body. This is not the case in malignant lymphomas, as normal and malignant lymphocytes migrate freely from one lymphoid organ to another through the postcapillary venules lined by high endothelial cells.[5–8] Most non-Hodgkin's lymphomas (NHLs), especially low grade, grow initially in an expansile fashion, confined to the lymphoid organs.[8,9] Although low-grade NHLs often present initially as disseminated disease (clinical stage III or IV), destruction of the ECM of the extra lymphoid organs is not involved; rather, malignant cells spread directly from one lymphoid site to another. Only in advanced stages and in some cases of aggressive high-grade NHL does extensive ECM degradation occur, resembling the tumor invasion seen in carcinomas.[8,9]

Matrix metalloproteinases (MMPs) represent a family of proteolytic enzymes involved in the degradation of ECM components.[10–14] These enzymes have broad substrate specificities and, among other proteolytic enzymes, have been implicated in tumor invasion and metastasis.[4,11,12,14] We have previously demonstrated that one member of this family, MMP-9 (92-kd gelatinase B), is overexpressed by human high-grade NHLs and that this overproduction at the mRNA level is associated with worse survival.[15] This significant clinical information prompted us to investigate whether human NHL cells exhibit invasive behavior and ECM-degrading abilities *in vitro*.

We have studied a broad spectrum of proteinases that may play a role in the proteolysis that underlies the ability of human NHLs to degrade ECM.[1,11] As normal and malignant lymphocytes traverse the BMs of the terminal venules of the hematopoietic organs,[5,7,8] we have used Matrigel-coated filters to test the ability of the cells derived from different sources to penetrate this complex type of ECM barrier.[16–18] In addition, we have used purified human elastin (devoid of other ECM components) to test the lymphoid cells for their ability to produce proteolytic enzymes capable of proteolysis *in vitro*. Elastin is a substrate for MMP-9 as well as other MMPs, including MMP-2, MMP-7, and MMP-12.[10,19–21] This test also allows assessment of the contributions of other classes of proteinases that may participate in ECM degradation.

Supported by a grant from the Medical Research Council of Canada to A. E. Kossakowska (MT-12706). M. S. Lim is a Clinical Fellow supported by the Medical Research Council of Canada and AHFMR.

Accepted for publication November 6, 1997.

Address reprint requests to Dr. A. E. Kossakowska, Department of Histopathology, Foothills Hospital, 1403 29th Street N.W., Calgary, AB, Canada T2N 2T9. E-mail: anna.kossakowska@crha-health.ab.ca.

Our initial studies used total cellular populations derived from NHLs to mimic the *in vivo* situation as closely as possible. This approach was taken because malignant human tumors represent a heterogeneous cellular population composed of tumor cells, nonneoplastic inflammatory cells, and stromal cells.[9] The production and activation of proteinases important in tumor behavior appear to be dependent on the interactions of these various cellular components, which is not reflected in the analysis of pure lymphoma cell lines.[11] As controls, we have used cells derived from human hyperplastic tonsils and established lymphoid cell lines of B- and T-cell origin (derived from NHLs and normal lymphocytes that were Epstein-Barr virus (EBV) immortalized).

It has been shown previously that MMP-9 is produced by a wide variety of cells, including tumor cells, mononuclear phagocytes, and lymphocytes.[22–26] Mononuclear phagocytes and lymphocytes can be stimulated to increase MMP-9 production, and the increase in this enzyme expression has been associated with their increased ability to penetrate BMs.[16,26–28] Macrophages have been shown to degrade elastin as well, a function that was also correlated with the production of MMP-9.[21] Unstimulated lymphocytes, on the other hand, show very low levels of MMP-9.[16,28] In human NHLs, we have previously shown by *in situ* hybridization that MMP-9 transcripts are mainly present in large lymphoma cells and macrophages.[29] Polymorphonuclear leukocytes (PMNs) contain MMP-9 protein but no mRNA transcripts.[24] Our previous data linking MMP-9 production by human NHLs with survival indicated that this enzyme likely plays a pathogenetic role in the progression of these tumors, but its precise contributions to the cellular behavior were unknown.

We have demonstrated here that the ability of human NHL to degrade ECM components correlates with the activity of proteolytic enzymes, mainly metalloproteinases. The proteinase within this class that was found in our studies to be consistently active, as shown by gelatin zymography, was MMP-9. Immunocytochemical studies of invading cells from NHL cultures show that malignant lymphoma cells as well as nonneoplastic cells invade Matrigel and elastin membranes, suggesting the need for cellular interactions in this process. Furthermore, the production of MMP-9 decreased in short-term cultures of cells separated on the basis of their immunological profile, indicating that the presence of heterogeneous cell populations is necessary for the high expression levels of this proteinase.

Materials and Methods

Tissue Collection, Culture, and Analysis

Tissue was collected from NHLs and tonsils that were sent to the Department of Pathology, University of Calgary. Tissue was assessed by frozen section and divided sterilely into several portions. Adequate tissue was secured for diagnostic purposes, which in each case included histology, flow cytometric assessment of the cell surface markers and cell cycle parameters, and molecular analyses of immunoglobulin heavy chain and T-cell receptor-β gene rearrangements. All cases were tested for human immunodeficiency virus (HIV) and EBV by polymerase chain reaction (PCR). Only HIV- and EBV-negative cases were included in this study. The number of cases used was limited by tissue availability. Sixteen NHL cases and ten hyperplastic tonsils were analyzed by Northern blot and gelatin zymography. In 14 NHL cases and 10 hyperplastic tonsils there were enough cells for the Matrigel penetration assay. Eight NHL cases and three tonsils were tested in the elastin degradation assays. Single-cell suspensions prepared from freshly dissected specimens of NHLs and tonsils were washed and snap frozen. They were kept at −70°C until further analysis. A total of 10^8 cells were cultured for 7 days in RPMI medium containing 10% (v/v) fetal calf serum (FCS), L-glutamine, penicillin, and streptomycin. Aliquots of cells from tissue culture were collected every day and analyzed by flow cytometry for the percentage of lymphoid cells, PMNs, and macrophages using a panel of antibodies to cell-type-specific markers. Aliquots of these cells were used for Matrigel penetration and elastin degradation assays. Different cell types were isolated with the use of magnetic beads (Dynabeads, Dynal, New Hyde Park, NY) in 4 NHL cases and 10 tonsils. The separated cells were subsequently tested for MMP-9 production by gelatin zymography and reverse transcriptase (RT)-PCR. Portions of the original tumor were B-5 and formalin fixed, paraffin embedded, stained with hematoxylin and eosin (H&E), and antibodies detecting PMNs (CD15) and macrophages (CD68). The percentage of each cell type was assessed by flow cytometry and by counting the number of cells in 50 high-power (×400) fields of each tissue section examined.

Established cell lines of T- and B-cell origin (NC 37, Raji, Jurkat, and MOLT-4) were purchased from American Type Culture Collection (catalog items CCL 214, CCL 86, CRL 8163, and CRL 1582, respectively; Rockville, MD) and cultured in RPMI medium supplemented with 10% FCS, antibiotics and L-glutamine. The cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere.

Cell Separation

Cells were separated with the use of Dynabeads (Dynal) coated with monoclonal antibodies against T lymphocytes (anti-CD4 and anti-CD8, catalog items M-450 111.15 and 111.07), B lymphocytes (anti-CD19, catalog item M-450 111.03), and macrophages (anti-CD14, catalog item 111.11). Positive and negative selection was done according to standard protocols. Briefly, the cells were incubated with the appropriate amount of beads (the amount of beads depended on the tissue and the percentage of different cell types, which was established by flow cytometric analysis of the cells before isolation) and washed, and the cells that attached to the beads were isolated in the process of positive selection, whereas the remaining cells represented a population of cells depleted of one cell type (negative selection). After

selection, the cells were tested by flow cytometry for the purity of the cellular population.

RNA, Protein Extraction, Northern Blot, RT-PCR, Gelatin Zymography, Reverse Zymography, and Western Blot

These methods have been described previously.[29,30] Northern blots were hybridized with nick-translated, ^{32}P-labeled MMP-9 probe (Dr. G. Goldberg, Division of Dermatology, University School of Medicine, St. Louis, MO).[22] As a loading control, the blots were stained with methylene blue before hybridization, as previously described.[29]

RT-PCR was performed as described previously with the use of the primer-dropping method.[31] As a control, we have used glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used were as follows: MMP-9, 5′ primer was 5′CAACATCACCTATTGGATCC, and 3′ primer was 5′-CGGGTGTAGAGTCTCTCGCT; MMP-12, 5′ primer was 5′-CTGAACAGCTCTACAAGCCT, and 3′ primer was 5′-TCCAATGCCAGATCCAGGTC; GAPDH, 5′ primer was 5′-CGGAGTCAACGGATTTGGTCGTAT, and 3′ primer was 5′-AGCCTTCTCCATGGTGGTGAAGAC.

Gelatin zymography was performed as described previously.[29] Briefly, 100 mg of tissue was homogenized in 100 mmol/L PIPES buffer (pH 7.5) containing 1.5 mmol/L $MgCl_2$ and 5 mmol/L KCl. Samples to be analyzed for the presence of gelatin-degrading enzymes were electrophoresed in 0.1% sodium dodecyl sulfate (SDS), 10% polyacrylamide gels containing 1 mg/ml gelatin as a substrate. A powdered gelatin (Sigma Chemical Co., St. Louis, MO) was added to the water portion of the resolving gel and heated to 65°C until dissolved. The solution was allowed to cool, the remaining ingredients were added, and the gel was cast. After electrophoresis, the gel was washed in a solution of 2.5% Triton X-100, 50 mmol/L Tris chloride (pH 7.5), and 5 mmol/L $CaCl_2$ once for 15 and once for 45 minutes, rinsed in water three times, and incubated in 50 mmol/L Tris chloride (pH 7.5) and 5 mmol/L $CaCl_2$ for 18 to 24 hours. The gel was then stained with Coomassie blue. Most of the gel stains blue due to the presence of undigested gelatin. Clear bands against the background blue staining indicate the presence of gelatin-degrading enzymes in the sample. As a positive control we have used conditioned medium from the baby hamster kidney (BHK) cell line stably transfected with human MMP-9 in pNUT vector.

Samples to be analyzed for the presence of tissue inhibitors of MMP (TIMPs) activity were electrophoresed in 0.1% SDS, 12% polyacrylamide gels containing 1 mg/ml gelatin as above for zymography; conditioned medium (BHK gelatinase A) was added as a source of gelatin-degrading enzyme. Known standards of TIMP-1, -2, and -3 were loaded on each gel. Conditioned medium (CM) from BHK cells was used as a positive control for TIMP-1, -2, and -3, and matrix (M) from BHK cell cultures was used as an additional control for TIMP-3. Gels were electrophoresed as described above. After electrophoresis, the gel was washed, incubated, and stained as described for zymography. The majority of the gel does not stain intensely, due to the degradation of gelatin by BHK gelatinase A. Dark blue bands against a pale blue background represent TIMP activity.

For Western blotting, 10 μg of protein from each sample were boiled for 3 minutes in the presence of SDS gel-loading buffer and electrophoresed through a 8 to 10% SDS-polyacrylamide gel electrophoresis gel, according to the previously described methodology.[30] Aliquots of protein samples from tonsils 1 and 2 were also pretreated with dithiothreitol (DTT) in a concentration of 0.01 mol/L before gel electrophoresis. The gel was run at 150 V for 1 hour at room temperature. The proteins were transferred to nitrocellulose, blocked with 5% nonfat milk in Tris-buffered saline/Tween 20 (TBS-T) and incubated with 1:1000 diluted anti-MMP-9 and anti-TIMP-1 antibodies (Oncogene Science catalog items IM 1OL and IM 32L; Uniondale, NY) in TBS-T for 1 hour at room temperature. After washing, the blots were incubated with 1:5000 diluted goat anti-mouse antibody (Pierce catalog item 31430; Rockford, IL) in TBS for 1 hour at room temperature. After repeated washes, the membranes were incubated in an equal volume of detection reagent 1 and 2 (ECL Western blotting detection reagents from Amersham Life Science, Arlington Heights, IL) for 1 minute at room temperature and immediately exposed to x-ray film.

Matrigel Invasion Assay

Matrigel invasion assays were performed according to previously described methodology.[16,17] Invasion chambers were purchased from Collaborative Biomedical Products (Becton Dickinson catalog item 40480; San Jose, CA). Isolated cells were washed three times in serum-free RPMI medium and resuspended in serum-free RPMI medium, and 4×10^5 cells were placed in the upper compartment of the invasion chamber. The lower chamber was filled with 0.5 ml of serum-free RPMI. No chemoattractants were used. The chambers were incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2. Cells retrieved from the upper and lower chambers were counted and used for the preparation of cytospins, for flow cytometric analysis of cell surface markers, and for zymography. The cytospins were stained with H&E and cell-type-specific markers.

Elastin Degradation Assays

Three-dimensional scaffolds made of pure elastin membranes were prepared from fresh autopsy fragments of human aortas devoid of atherosclerosis as described previously.[32,33] The elastin membranes were cut into round pieces to fit snugly in the bottom of 24-well culture dishes, and then NHL and tonsil-derived cells suspended in RPMI medium containing antibiotics and 10% FCS were plated on the membrane top and cultured for 96 hours. The membranes were then rinsed, fixed in 10% formalin, and embedded in paraffin. Histological sections were prepared and immunostained with a panel of cell-

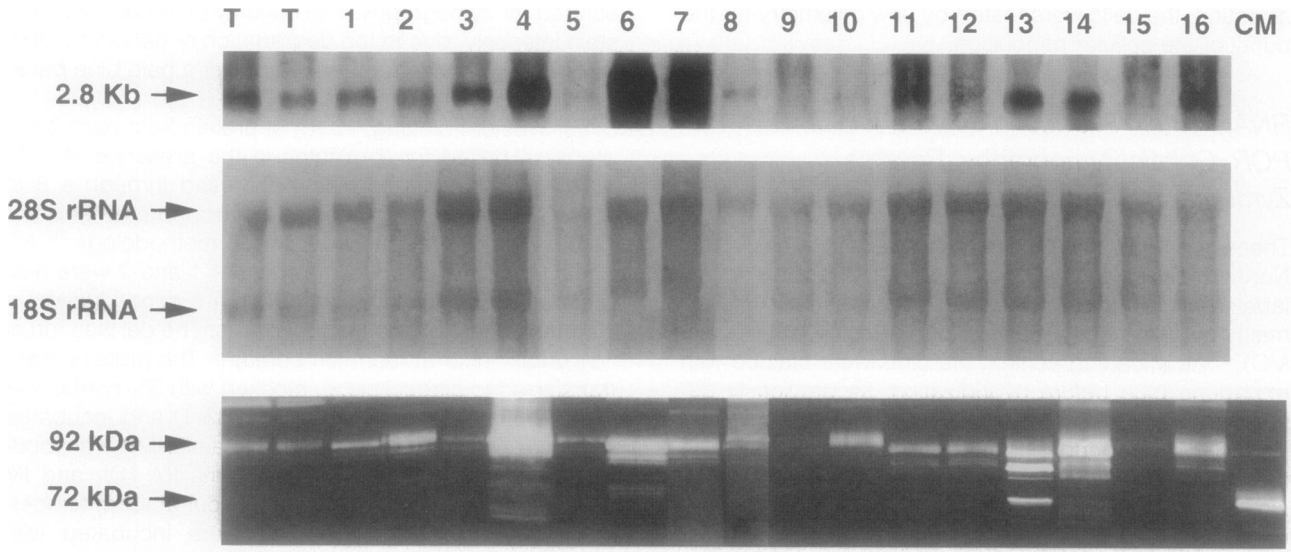

Figure 1. Expression of MMP-9 by human NHLs and hyperplastic tonsils. The **upper panel** shows Northern blots containing RNA extracted from suspensions of cells derived from tonsils (T) exhibiting follicular hyperplasia and human NHLs (case descriptions in Table 1). The **middle panel** shows the results of the methylene blue staining of the Northern blots before hybridization and represents the loading control. The **lower panel** shows gelatin zymography of protein extracts from the same cases as shown in the **upper panels** . MMP-9 (indicated as 92 kd) was identified by molecular weight and by comparison with a standard from culture medium (CM) from baby hamster kidney (BHK) cells stably transfected with an MMP-9 expression construct (P. Beaudry and D. Edwards, unpublished). The higher molecular weight bands represent MMP-9 dimers, and the lower molecular weight bands correspond to activated forms of the enzyme. MMP-2 (72-kd gelatinase A) is indicated as 72 kd.

type-specific antibodies to identify cells that attached to or penetrated into the membranes.

Single-cell suspensions from NHLs and tonsils were plated in 24-well culture dishes (5×10^5 cells per well) and maintained until confluency (48 hours) in RPMI medium containing 10% FCS, L-glutamine, penicillin, and streptomycin. The medium was then changed for serum-free RPMI (1 ml per well), and elastolytic activity of enzymes released from the cultured cells over an 18-hour period was assayed in triplicate, measuring degradation of ^{3}H-labeled insoluble elastin substrate added to each well (100 μl per well) as described previously.[34,35] Background was established by measuring the elastin degradation in wells that contained labeled elastin but no cells. To assess the type of elastolytic enzymes secreted by cultured cells, parallel cultures were treated with class-specific proteinase inhibitors before the administration of ^{3}H-labeled elastin. These included *O*-phenanthroline (0.01 mol/L), EDTA (0.002 mol/L), leupeptin (0.002 mol/L), and phenylmethylsulfonyl fluoride (0.002 mol/L). At the end of the incubation, media were collected and centrifuged (8000 $\times$ *g* for 4 minutes), and 100-μl aliquots of supernatant containing the solubilized degradation products were mixed with 4 ml of scintillation fluid and counted in triplicate in a liquid scintillation counter. Results of three separate experiments (originally expressed in cpm per well) were combined and finally expressed as percentage inhibition of the elastolytic activity.

In Situ *Hybridization*

In situ hybridization was performed as described by us previously.[29] A fragment of MMP-9 from the 5′ end of the cDNA to a *Bam*HI site at nucleotide 390[22] was subcloned into pBluescript KS−, and sense and antisense probes were made from *Not*I- and *Eco*RV-restricted templates, with T3 and T7 RNA polymerases, respectively. These probes were labeled by the incorporation of ^{35}S-labeled rUTP using Riboprobe Gemini kit (Promega, Madison, WI). Parallel slides were hybridized with antisense and sense probes using identical conditions. Hybridization with sense RNA probes provided negative controls.

Results

The results of Northern blot analysis, RT-PCR, and gelatin zymography are shown in Figures 1 and 2. The 16 cases of human NHL analyzed for MMP-9 expression are identified in Table 1. In addition, we have analyzed 10 cases of hyperplastic tonsils and four established lymphoid cell

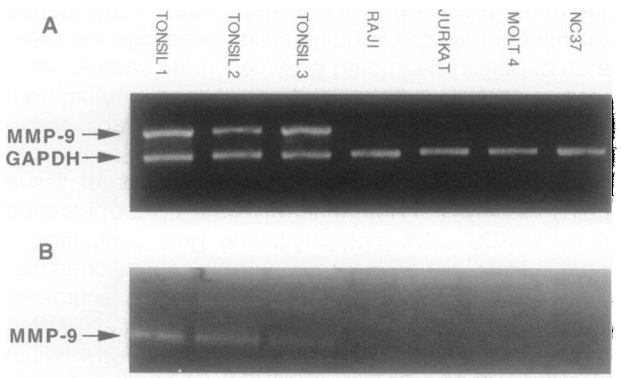

Figure 2. RT-PCR analysis of MMP-9 mRNA transcripts (**A**) and gelatin zymography (**B**) of proteins derived from hyperplastic tonsils and established lymphoid cell lines. Three separate cases of tonsil hyperplasia are labeled as tonsil 1, 2, and 3, and lymphoid cell lines include Raji, Jurkat, MOLT4, and NC37. PCR amplification of MMP-9 cDNA was performed for 30 cycles and of GAPDH for 20 cycles.

Table 1. Characteristics of the NHLs

Case	Diagnosis, WF	Diagnosis, REAL	Cellular lineage
1	Follicular, predominantly large cell	Follicular center lymphoma, follicular, grade III	B-cell
2	Follicular, predominantly large cell	Follicular center lymphoma, follicular, grade III	B-cell
3	Diffuse, small cleaved cell	Mantle cell lymphoma	B-cell
4	Large cell immunoblastic	Diffuse large B-cell lymphoma	B-cell
5	Follicular, predominantly small cleaved cell	Follicular center lymphoma, follicular, grade I	B-cell
6	Large cell immunoblastic	Anaplastic large-cell lymphoma	T-cell
7	Large cell immunoblastic	Diffuse large-B-cell lymphoma	B-cell
8	Diffuse, mixed large and small cell	Peripheral T-cell lymphoma	T-cell
9	Large cell immunoblastic	Diffuse large-B-cell lymphoma	B-cell
10	Large cell immunoblastic	Diffuse large-B-cell lymphoma	B-cell
11	Diffuse, large cell	Diffuse large-B-cell lymphoma	B-cell
12	Diffuse, large cell	Diffuse large-B-cell lymphoma	B-cell
13	Large cell immunoblastic	Diffuse large-B-cell lymphoma	B-cell
14	Diffuse, large cell	Diffuse large-B-cell lymphoma	B-cell
15	Diffuse, small cleaved cell	Mantle cell lymphoma	B-cell
16	Diffuse, large cell	Diffuse large-B-cell lymphoma	B-cell

WF, Working Formulation; REAL, Revised European-American Classification of Lymphoid Neoplasms. Cellular lineage—cellular lineage was defined in all cases by immunocytochemistry, flow cytometric analysis of cell surface markers, and Southern blot analysis of immunoglobulin heavy chain and T-cell receptor-β gene rearrangements.

lines (two of B-cell and two of T-cell origin, one derived from Burkitt's lymphoma). The immunophenotypes of the NHLs and tonsils are shown in Table 2. All of the NHLs and tonsils showed MMP-9 expression at protein or RNA levels (Figure 1). In NHL cases 9 and 15, mRNA was undetectable by Northern blot analysis, and in NHL cases 5 and 10, very low mRNA levels were detected (Figure 1). The tonsils studied showed both MMP-9 mRNA transcripts and proteins, and the expression of both was similar in all cases. In NHLs, MMP-9 expression was variable, and the results of zymography and Northern blot did not always correspond (Figure 1). In four of the NHL cases in addition to MMP-9, MMP-2 (72-kd gelatinase A) protein was also detected by gelatin zymography (cases 4, 6, 13, and 14). None of the lymphoid cell lines examined showed expression of MMP-9 at the mRNA or protein level as studied by RT-PCR and gelatin zymography, respectively (Figure 2). The highest MMP-9 expression levels were observed in some of the large-cell NHLs (cases 4, 6, 7, 11, 13, 14, and 16). Case 6 represents an anaplastic large-cell lymphoma of T-cell origin, whereas the other cases were of B-cell origin. Admixtures of nonneoplastic T cells did not appear to influence the levels of MMP-9 expression. The percentages of macrophages, PMNs, and natural killer cells were small in all cases studied (Table 2).

Table 2. Immumophenotypes of the NHLs and Tonsils

Case	$CD3^+$ $CD4^+$	$CD3^+$ $CD8^+$	$CD19^+$	$CD15^+$	$CD14^+$	$CD16^+$ $CD56^+$	$CD68^+$
1	7%	3%	91%	0%	ND	1%	4%
2	21%	5%	76%	1%	ND	ND	2%
3	4%	3%	84%	0%	ND	0%	1%
4	4%	4%	86%	0%	ND	ND	3%
5	15%	3%	80%	0%	ND	0%	0%
6	56%	8%	36%	1%	0%	1%	4%
7	1%	2%	94%	1%	ND	2%	2%
8	70%	16%	14%	1%	ND	1%	2%
9	1%	1%	99%	1%	3%	ND	ND
10	23%	20%	60%	ND	ND	ND	ND
11	4%	1%	93%	ND	ND	ND	ND
12	16%	1%	80%	ND	ND	ND	ND
13	2%	2%	95%	ND	ND	0%	ND
14	18%	12%	68%	3%	2%	1%	ND
15	2%	3%	92%	1%	1%	ND	ND
16	7%	4%	87%	1%	1%	0%	ND
T1	37%	12%	53%	1%	1%	0%	1%
T2	32%	11%	30%	1%	1%	0%	0%
T3	49%	10%	45%	1%	1%	0%	1%
T4	32%	9%	66%	0%	0%	1%	0%
T5	30%	8%	55%	1%	0%	0%	ND
T6	43%	12%	55%	2%	5%	2%	ND
T7	32%	9%	66%	0%	0%	1%	ND
T8	32%	5%	61%	1%	0%	1%	ND
T9	23%	5%	70%	1%	0%	1%	ND
T10	42%	8%	50%	1%	0%	1%	ND

T, tonsil; ND, not done.

Table 3. Invasion of Lymphoid Cells from Different Sources

% of Invasive cells	Source of cells
5.0 ± 0.5%	Tonsils*
4.4 ± 0.7%	NHLs†
0.0 ± 0.0%	Raji
0.0 ± 0.0%	NC37
0.04 ± 0.005%	Jurkat
0.005 ± 0.001%	MOLT-4

Each sample was tested by three assays and the cell lines were tested on three separate occasions during exponential growth. Raji is a Burkitt's lymphoma cell line, NC-37 is a B-cell line, and Jurkat and MOLT-4 are T-cell lines.
*Represents an average of 10 tonsils.
†Average of 14 cases.

Cells derived from all of the studied tonsils, cell lines, and 14 of the NHL cases (cases 1 to 10 and 13 to 16 in Table 1) were tested for their ability to penetrate Matrigel-coated filters. The cells from the lower chambers were collected, counted, and assessed morphologically. The average percentage of cells derived from different sources migrating through Matrigel is shown in Table 3. Only a small percentage of cells derived from NHLs and tonsils showed the ability to cross the Matrigel-coated filters, and Raji and NC37 B-cell lines did not penetrate Matrigel (Table 3). Occasional Jurkat and MOLT-4 T cells were recovered from the lower chambers (Table 3). In two NHL cases (cases 1 and 8 from Table 1), enough cells were retrieved from the upper and lower chambers to perform flow cytometric analysis of the cell surface markers. These cells were also analyzed by gelatin zymography. A schematic representation of the invasion chamber, as well as the results of immunophenotyping and gelatin zymography for NHL cases 1 and 8, are shown in Figure 3. Immunophenotyping confirmed our morphological observation that all of the cell types populating human NHLs are capable of Matrigel penetration, and the percentage of different cell types in the upper and lower chambers was similar. Gelatin zymography showed that the migrating cells produced MMP-9 protein. Cells derived from tonsils have a similar ability to penetrate Matrigel, and immunophenotyping of the cells derived from four tonsils showed that cells of T- and B-cell lineage as well as macrophages and PMNs are capable of crossing this barrier (Table 4). Thus, Matrigel does not appear to represent a physical barrier that discriminates between the penetrating abilities of leukocytes and NHL cells. Cells from four NHL cases (cases 4 to 7) were cultured on pure elastin membranes, and cells derived from cases 4, 6, and 7 were able to penetrate the elastin networks and destroy the superficial elastin layers. Case 7 is shown in Figure 4A. Immunostaining showed that cells of B- and T-cell lineage as well as macrophages penetrated the elastin membranes (data not shown). Cells derived from three hyperplastic tonsils grew on top of the elastin membranes, retaining their viability, but they did not penetrate the membranes (Figure 4B). Cells from

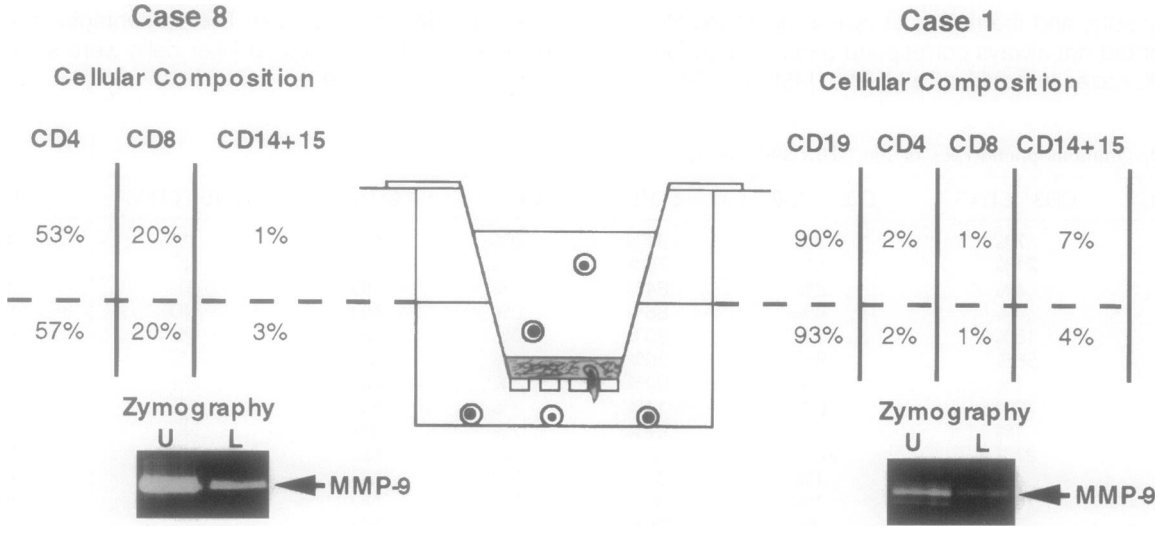

Figure 3. Schematic representation of Matrigel invasion assay. The cells collected from the lower and upper chambers were subjected to flow cytometric analysis, and the results from NHL cases 8 and 1 (according to Table 1) are shown on both sides of the drawing. Protein extracts from the cells from the lower and upper chambers were tested by gelatin zymography, and the results for both cases are shown underneath the flow cytometry results. U, upper chamber; L, lower chamber.

Table 4. Immunophenotypes of Tonsillar Cells Migrating through the Matrigel

Case	$CD4^+$	$CD8^+$	$CD19^+$	$CD15^+$	$CD14^+$	$CD16^+$ $CD56^+$
T1	36%	8%	54%	1%	1%	0%
T2	51%	10%	37%	2%	2%	0%
T3	55%	14%	27%	1%	1%	1%
T4	34%	9%	58%	1%	1%	1%

T, tonsil.

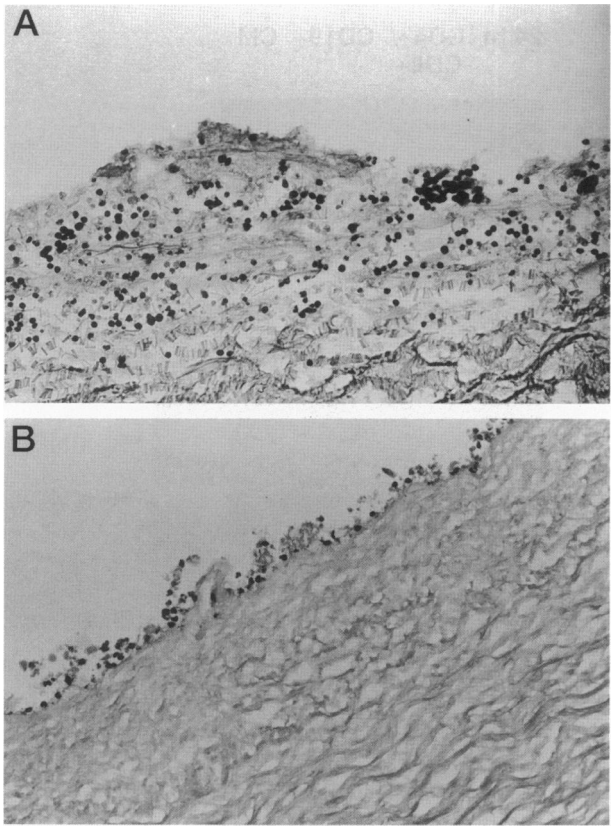

Figure 4. Elastin degradation by human NHL. Cells originating from human NHL (**A**; case 7 according to Table 1), but not from a human hyperplastic tonsil (**B**; tonsil 1), plated on top of the elastin membranes penetrate into (up to 0.5 mm) and degrade this three-dimensional scaffold made of insoluble elastin. Magnification, ×200.

tonsils 1, 2, and 3, from eight NHL cases (cases 1 to 8 from Table 1), and from lymphoid cell lines were used for the *in vitro* elastolytic activity assay. The elastolytic activity was measured by the degradation of radiolabeled elastin, and the results are shown in Figure 5. Tonsils showed no significant elastolytic activity. Among the NHL cases, the highest levels of the elastin degradation were seen in cases 4, 6, and 7. These were all large-cell NHLs, which showed high MMP-9 levels (Figure 1). Elastolytic activity was partially inhibited by inhibitors of metalloproteinases (*O*-phenanthroline and EDTA) and in some cases by inhibitors of serine and cysteine proteinases (phenylmethylsulfonyl fluoride and leupeptin; Table 5). Established lymphoid cell lines showed no elastolytic activity (data not shown).

To determine which cells are responsible for MMP-9 production in human NHLs, *in situ* hybridization studies were performed on large-cell NHL cases (cases 4, 6, and 7). It was demonstrated that MMP-9 mRNA is produced by lymphoma cells as well as macrophages (Figure 6, A and B). In the next step, different cell types were separated with the use of magnetic beads (Dynabeads) coated with monoclonal antibodies against T (anti-CD4 and anti-CD8) and B (anti-CD19) lymphocytes. Cells after 24 hours of culture, even before their separation into individual cell types, showed much lower levels of MMP-9 protein than homogenates of the entire tissue (Figure 7A).

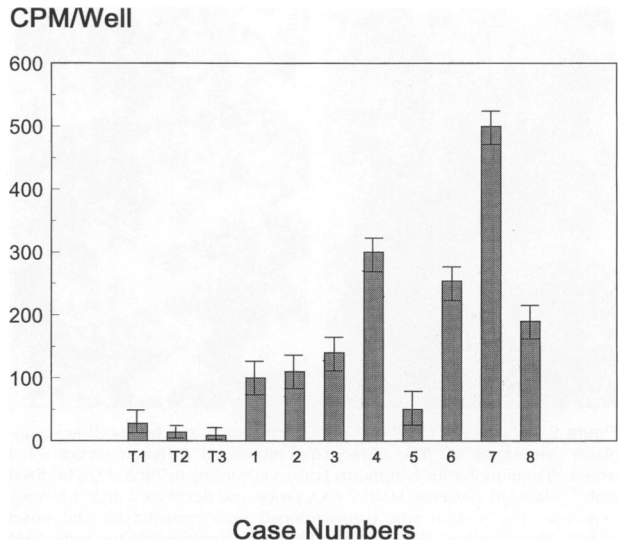

Figure 5. Elastolytic activity of human NHLs and tonsils. Only cells derived from human NHLs are capable of efficient degradation of insoluble elastin in an *in vitro* assay with ^{3}H-labeled elastin substrate. Elastolytic activity is expressed in cpm released above the background obtained from wells with labeled elastin that did not contain cells. The results represent the means of triplicate or quadruplicate assays from each case. NHL case number (according to Table 1) is indicated underneath each result. Tonsils are labeled as T1, T2, and T3.

This is in keeping with the fact that MMP-9 is a secreted proteinase present largely in the extracellular space. Changes in MMP-9 expression were also noted at the mRNA level with higher expression in the extracts from the entire tissue than in isolated cells after 24 hours of culture (Figure 7B). Fibroblasts cultured from NHLs showed MMP-2 protein expression and no MMP-9 protein or mRNA transcripts. Four NHL cases (cases 13 to 16 in Table 1) were used for cell separation, and each cell type was analyzed by gelatin zymography. These results are shown in Figure 8, A (case 13), B (case 14), C (case 15), and D (case 16)). Cells separated from NHLs showed higher levels of MMP-9 in the entire population of cells cultured for 24 to 48 hours than in isolated T and B lymphocytes (Figure 8, A–D). MMP-9 expression in isolated T and B lymphocytes was variable in different NHL cases. In case 13 (Figure 8A), the highest MMP-9 expression was noted in the isolated T cells ($CD8^{+}$ and $CD4^{+}$ mixture), although the tumor was of B-cell phenotype. In addition, all cells derived from this case expressed MMP-2. In case 14, the separated B lymphocytes

Table 5. Percentage of Elastolytic Activity Inhibition

Case	O-Phenathroline/EDTA	Leupeptin	PMSF
1	10%		
2	15%		
3	36%	21%	21%
4	67%	20%	18%
5			
6	44%	24%	
7	20%	30%	10%
8	14%		

Inhibition of less that 10% was considered insignificant. PMSF, phenymethylsulfonyl fluoride.

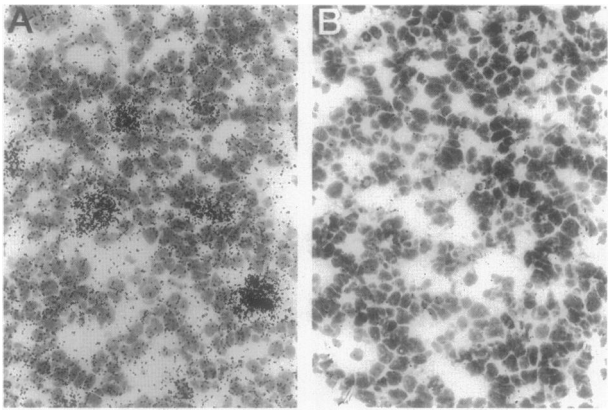

Figure 6. Localization of MMP-9 mRNA transcripts in a large-cell immunoblastic lymphoma. **A:** The photograph represents a frozen section of a large-cell immunoblastic lymphoma (case 4 according to Table 1) hybridized with ^{35}S-labeled antisense MMP-9 RNA probe and developed after a 3-week exposure. The section was counterstained with hematoxylin and eosin (H&E). Magnification, ×200. **B:** The photograph represents the same case hybridized under the same conditions with ^{35}S-labeled sense MMP-9 RNA probe and developed after a 3-week exposure. Magnification, ×200.

(CD19$^+$ cells) showed the highest MMP-9 expression, and this tumor was of B-cell origin (Figure 8B). Cells isolated from this case also expressed MMP-2. In cases 15 and 16, the expression of MMP-9 in isolated cells was low, and no MMP-2 activity was detected (Figure 8, C and D). Cells separated from 10 human tonsils were studied by gelatin zymography and RT-PCR. As all tonsils showed similar results of gelatin zymography and RT-PCR, representative results are shown in Figure 9, A and B. The MMP-9 protein and mRNA levels were low in the cells isolated from the tonsils, and the highest expression was observed in CD4$^+$ and CD8$^+$ T cells. MMP-2 protein was detected only in the fibroblasts cultured from the tonsils. Activated forms of MMP-9 were seen in the protein extracts from cells isolated from NHLs and tonsils (Figures 1, 8, and 9). Isolated B and T lymphocytes from NHL case 14 and from three tonsils were able to penetrate the Matrigel-coated filters, but not enough cells were available to perform the elastolytic activity assays.

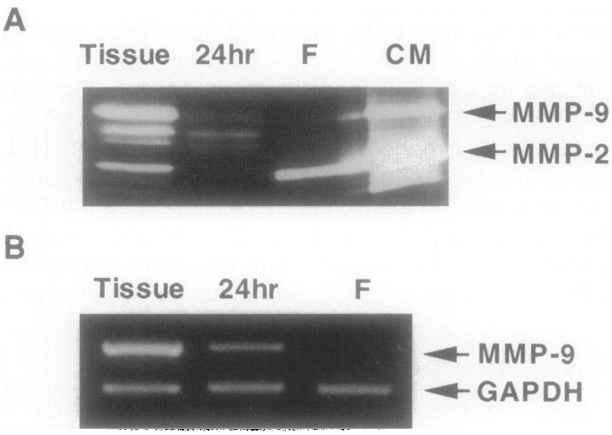

Figure 7. Gelatin zymography (**A**) and RT-PCR (**B**) analysis of proteins and RNA derived from tissue and cells separated from human NHL. **A:** Proteins derived from NHL tissue (labeled as tissue) show a higher activity of MMP-9 on the gelatin zymography than the extracts from the cultured cells (labeled as 24hr, the entire cell population at 24 hours of culture). MMP-2 activity is present in the protein extracts from the entire snap-frozen tissue and from the fibroblasts (F) cultured from the same NHL case (after 7 days of culture). Bands between MMP-9 and MMP-2 represent activated forms of MMP-9. **B:** Lower expression of MMP-9 in cells at 24 hours of culture is also noted at the mRNA level as tested by RT-PCR. Fibroblasts (F) show no detectable MMP-9 transcript. PCR amplification of MMP-9 cDNA was performed for 30 cycles and of GAPDH for 20 cycles.

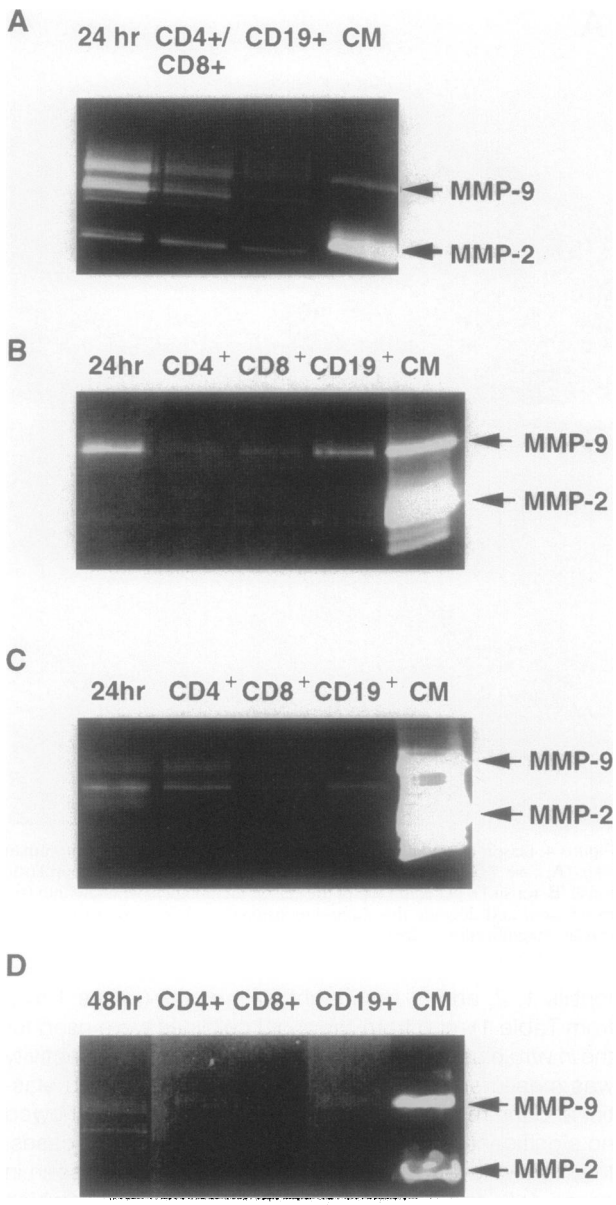

Figure 8. Gelatin zymography of proteins derived from cells isolated from NHLs. In NHL cases 13 (**A**), 14 (**B**), 15 (**C**), and 16 (**D**), the activity of MMP-9 tested by gelatin zymography decreased after 24 to 48 hours of culture (24 hr and 48 hr) as compared with the entire cell population at 0 hours (Figure 1) and is lower in the isolated T and B lymphocytes (CD4$^+$, CD8$^+$, CD19$^+$ cells isolated through the process of positive selection). MMP-2 activity is noted in the entire cell populations from NHL cases 13 and 14, and in B (CD19$^+$) and T lymphocytes (CD4$^+$/CD8$^+$) isolated from these cases. Bands between MMP-9 and MMP-2 represent activated forms of MMP-9. CM, conditioned medium from BHK cell line transfected with human MMP-9 was used as positive control.

The identity of MMP-9 in NHL cases 13 to 16 and tonsils 1 and 2 was further confirmed by the Western blot analysis (Figure 10A). No significant differences in the MMP-9 expression were noted in the Western blot anal-

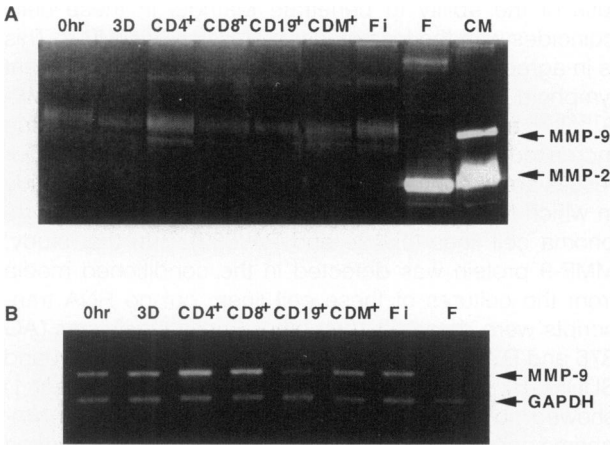

Figure 9. Gelatin zymography (**A**) and RT-PCR (**B**) analysis of proteins and RNA from cells separated from a human hyperplastic tonsil. Experimental conditions were the same as in Figure 8. 0 hr, cells separated from tissue at the time of collection; 3D, cells collected from culture on the third day; $CD4^+$, $CD8^+$, positive selection for T lymphocytes; $CD19^+$, positive selection for B lymphocytes; CDM^+, mixture of CD4, CD8, and CD19-coated beads, positive selection for T and B lymphocytes; Fi, cells centrifuged through Ficoll-Hypaque gradient depleting the cellular population of PMNs; F, fibroblasts after 7 days of tissue culture. Bands between MMP-9 and MMP-2, seen on gelatin zymography, represent activated forms of MMP-9 (**A**). PCR amplification of MMP-9 cDNA was performed for 30 cycles and of GAPDH for 20 cycles (**B**).

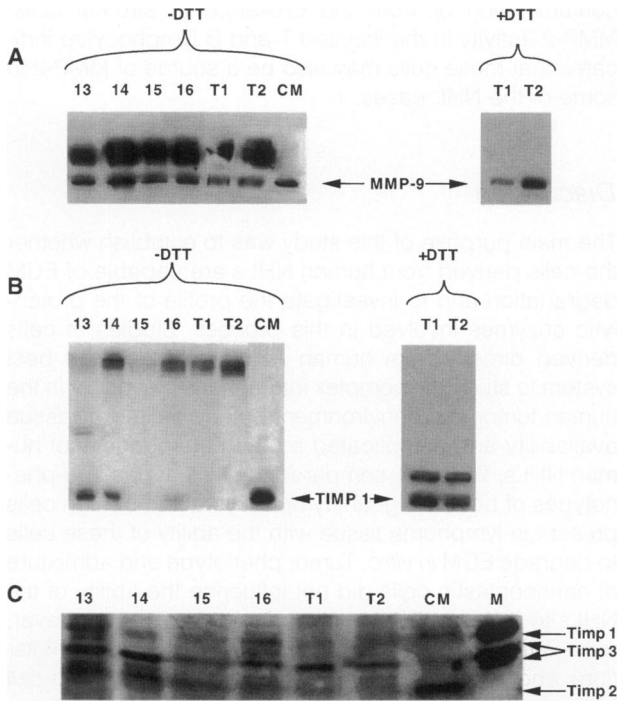

Figure 10. Western blot analysis (**A** and **B**) and reverse zymography (**C**) of proteins derived from NHLs and tonsils. The Western blots were probed with anti-MMP-9 antibodies (Ab-2, Oncogene Science catalog item IM 1OL; **A**) and with anti-TIMP-1 antibodies (Oncogene Science catalog item IM 32L; **B**). MMP-9 (92-kd gelatinase) position was determined by comparison with molecular weight standards and a positive control. CM, conditioned medium from BHK cells transfected with MMP-9. The high molecular weight bands (approximately 200 kd) represent MMP-9 dimers and possibly MMP-9/TIMP complexes. Treatment of protein extracts from tonsils (T1 and T2) with DTT (+DTT) caused a disappearance of the high molecular weight bands (**A**). Free TIMP-1 (indicated by **arrows**) is observed in NHL cases 13, 14, and 16 (case numbers according to Table 1). High molecular weight bands are seen in all cases, and these bands disappear after treatment with DTT (+DTT). In the protein extracts from tonsils (T1 and T2) treated with DTT, two bands are observed. The lower molecular weight band represents free TIMP-1, and the higher molecular weight band most likely represents as yet uncharacterized complexes (**B**). Reverse zymography analysis of proteins derived from NHLs (case numbers 13 to 16 according to Table 1 and tonsils T1 and T2) shows the presence of TIMP-1, -2, and -3 (indicated by **arrows**). The identity of the TIMPs was established by their molecular weight and comparison with the positive controls. CM, conditioned medium from BHK cells; MT, extracellular matrix from BHK cells used as positive control for TIMP-3. The upper band in the MT control represents the glycosylated form of TIMP-3 (**C**). The NHL case number (according to Table 1) is indicated on top. Tonsils are labeled as T1 and T2.

ysis, whereas more pronounced differences were seen on gelatin zymography (Figures 1 and 10A). In addition, high molecular weight bands were detected by Western blot (Figure 10A). These bands may represent MMP-9 dimers, but on gelatin zymography the enzymatic activity in this area was much weaker than the signal seen on the Western blot (Figures 1 and 10A). In addition, the same high molecular weight bands were seen on the Western blot probed with anti-TIMP-1 antibodies (Figure 10B), indicating that they may represent MMP-9/TIMP-1 complexes. This hypothesis is further supported by the fact that the high molecular weight bands disappeared after boiling and treatment of the protein samples from tonsils with DTT, which breaks the complexes (Figure 10, A and B). After such treatment, only free MMP-9 protein was detected (Figure 10A). TIMP levels were analyzed in NHL cases 13 to 16 and tonsils 1 and 2 by reverse zymography and Western blot using anti-TIMP-1 antibodies (Figure 10, B and C). Reverse zymography showed the presence of three TIMPs: TIMP-1, -2, and -3 (Figure 10C). In most cases, the highest activity was seen in the region of TIMP-3, and TIMP-1 activity appeared very low (Figure 10C). No clear TIMP-2 band was seen in tonsil 2 (Figure 10C). TIMP-1 levels varied on Western blot, and free TIMP-1 was demonstrated in NHL cases 13, 14, and 16 (Figure 10B). In addition, higher molecular weight bands were observed, which appear to represent TIMP-1/MMP-9 complexes (Figure 10B). These complexes were present in the area corresponding to the MMP-9 dimers and disappeared after DTT treatment (Figure 10B). Additional lower molecular weight complexes were seen in NHL case 13 and after boiling and DDT treatment of the protein samples from tonsils (Figures 10B). The precise nature of these complexes is uncertain, and they were not present in the blots probed with anti-MMP-9 antibodies (Figure 10A).

No other MMPs (including macrophage metalloelastase, MMP-12)[36] mRNA transcripts were detected in the samples examined in this study by Northern blot. We have analyzed NHL cases 3 and 10 to 12 and four tonsils by RT-PCR, and this analysis showed MMP-12 transcripts in all of these samples (data not shown). Levels of MMP-12 transcripts in NHLs and tonsils were similar. MMP-2 activity on gelatin zymography was seen in the cells isolated from four NHL cases (Figure 1), in the extract of the entire, snap-frozen NHL tissue (Figure 7), in the fibroblasts cultured from NHLs and tonsils (Figures 7 and 9), and in T and B lymphocytes from cases 13 and 14 (Figure 8, A and B). In the case of fresh tissue, the presence of active MMP-2 protein may result from blood

contamination or from the presence of stromal cells. MMP-2 activity in the isolated T and B lymphocytes indicates that these cells may also be a source of MMP-2 in some of the NHL cases.

Discussion

The main purpose of this study was to establish whether the cells derived from human NHLs are capable of ECM degradation and to investigate the profile of the proteolytic enzymes involved in this process. Studies of cells derived directly from human NHLs represent the best system to study the complex interactions that occur in the human tumor microenvironment but are limited by tissue availability and complicated by the heterogeneity of human NHLs. We have compared the NHL types and phenotypes of both malignant lymphocytes and benign cells present in lymphoma tissue with the ability of these cells to degrade ECM *in vitro*. Tumor phenotype and admixture of nonneoplastic cells did not influence the ability of the NHLs to degrade ECM *in vitro*. This ability was, however, associated with a high grade of NHL (Working Formulation) and large-cell morphology. High-grade large-cell NHLs are known to overexpress MMP-9, and we have shown previously that high mRNA levels of this proteinase correlate with a poor clinical outcome of human high-grade NHL.[15,29] These findings suggest that increased production of proteolytic enzymes may play a role in the pathophysiology of these neoplasms. The identification of the gelatinases mRNAs is, however, not synonymous with the presence of their enzymatic activity, as mRNA codes for the protein, which at first is present in the inactive latent form.[10] The enzymatic activity of gelatinases is dependent on the activation of their latent forms.[10,13]

We have used *in vitro* invasion and elastin degradation assays to study the ability of cells derived from human NHLs and hyperplastic tonsils (nonneoplastic tissue of cellular composition similar to NHL) to penetrate Matrigel-coated filters and to degrade ECM components. Matrigel represents reconstituted BM, the natural barrier that prevents cells from migrating freely from one site to another. Elastin, a resilient ECM component, is a substrate for degradation by several proteolytic enzymes, including members of the MMP family (MMP-9, MMP-2, MMP-7, and MMP-12).[10,19–21,36] Its degradation was used here by us as a simple model for measuring the abilities of cells to attack and penetrate a natural physiological barrier. Although elastin does not represent the protein, which has to be physiologically degraded in the process of a tumor invasion, it does provide a convenient substrate to test the activity of proteinases *in vitro*.

We have shown that cells derived from NHLs and tonsils are capable of Matrigel penetration and that these cells produce MMP-9 protein. Neoplastic and nonneoplastic lymphoid cells of T- and B-cell origin as well as macrophages and PMNs were found to penetrate Matrigel. In contrast, analysis of the established cell lines, which represent pure populations of lymphoid cells, showed that these cells do not penetrate Matrigel. The loss of the ability to penetrate Matrigel in these cells coincides with the loss of the expression of MMP-9. This is in agreement with previous studies demonstrating that lymphoid cells *in vitro* can be induced to produce MMP-9[16,26,28] and that MMP-9 production correlates with the increased cellular invasion of monoblastoid cells.[27] Our results are in contrast with the results of a previous study in which MMP-9 was demonstrated in two Burkitt's lymphoma cell lines (JiJyoe and PA 682).[26] In that study, MMP-9 protein was detected in the conditioned media from the cultures of these cell lines, but no RNA transcripts were detected. Two other Burkitt's cell lines (AG 876 and DW 6), follicular center cell lines (SUDHL-4 and SUDHL-6), and T-cell lines (MOLT-4, Jurkat, and Peer 1) showed no MMP-9 production.[26] Raji, the Burkitt's lymphoma cell line used in our present study, was not tested in this previous report.

Significant elastin degradation was observed in our study only in NHL cases and not in tonsils or established lymphoid cell lines. The highest elastolytic activity was displayed by cells derived from large-cell NHL cases, and these cells were capable of penetrating an elastin matrix *in vitro*, whereas cells derived from hyperplastic tonsils showed no such ability. Our observations and previously published data suggest that malignant lymphocytes may use different mechanisms to migrate out of the endothelium-lined channels.[5,7] As has been stated before by others,[8] normal lymphocytes are destined to migrate, and they move from one lymphoid organ to another through the terminal venules of lymphoid organs.[5,7,8] Lymphocytes and macrophages that participate in the immunological response use MMPs to cross the BMs.[37] Low-grade NHLs are usually confined to lymphoid organs and, although they frequently present clinically as high stage (III and IV), they do not initially destroy tissue boundaries. In this respect, low-grade NHLs populate lymphoid organs in a manner similar to normal lymphocytes. They are unable to destroy ECM to the point that would result in an invasive growth pattern. High-grade tumors, however, behave as invasive neoplasms and show metastatic behavior similar to that of carcinomas.[9] These tumors cause extensive permeation of many organs with destruction of their anatomical boundaries. In part, this behavior is dependent on cellular interactions between neoplastic, nonneoplastic cells, and stroma. The importance of cellular interactions is demonstrated in our gelatin zymography assays of the separated cells, where we saw that gelatinolytic activity decreased in cells isolated from tissue and separated on the basis of their surface markers.

We have shown that both benign and malignant cells were able to invade the Matrigel and elastin membranes, but the separation of different cells populating lymphomas resulted in a decrease in gelatinolytic activity of the different cell fractions when compared with cultured unseparated fresh tumors. The MMP-9 levels were higher in the samples of the entire tissue than in the isolated cells, which could be partially attributed to the fact that this gelatinase is a secreted proteinase. This difference was, however, also noted at the mRNA level, and the isolated cells showed lower expression of MMP-9 mRNA than the

extract from intact NHL tissue. This observation suggests that tumor microenvironment and cellular interactions play an important role in the control of MMP-9 expression. It has been shown that contact with ECM components stimulated MMP-9 production by macrophages.[38] This analysis and our previous studies by *in situ* hybridization indicate that this gelatinase is produced by nontumorous histiocytes populating lymphomas as well as the malignant lymphocytes.[29]

We have shown in the present study that human malignant NHLs are capable of extracellular matrix degradation due to the function of proteinases. The proteolytic activity was in most cases partially inhibited by the metalloproteinase inhibitors and in some cases by cysteine and serine proteinase inhibitors. From these inhibition studies, it appears that the metalloproteinases represent the main class of proteinases responsible for this enzymatic degradation but in some cases may be accompanied by proteolytic enzymes from other classes. Our data indicate indirectly that, among the members of the MMP family, MMP-9 is likely to be the critical proteinase involved. This is in agreement with our previous observations that overexpression of MMP-9 (as analyzed by Northern blot) correlated with aggressive biological behavior of NHLs.[15] The precise role of MMP-9 in ECM degradation by human NHLs still needs to be, however, established by studies using specific inhibitors (eg, anti-MMP-9 monoclonal antibodies). In this as well as in our previous studies, other MMPs were detected only in isolated NHL cases.[29] Low levels of MMP-12 transcripts were detected by RT-PCR, but MMP-12 RNA expression was similar in hyperplastic tonsils and in NHLs.[10,36] It was shown by others that some of the lymphoid cell lines and tonsillar lymphocytes produce MMP-9 but no MMP-2.[26] In our study, MMP-2 was detected in the cells derived from four NHL cases and was present in both B and T lymphocytes isolated from two of these cases. Our present and previous findings indicate, therefore, that other proteinases may contribute to the proteolysis in the individual NHL cases.[29]

It is possible that TIMPs play a role in controlling the proteolytic activity of MMPs *in vivo*. In our previous studies, we have demonstrated that TIMP-1 mRNA levels correlate with MMP-9 transcripts in most cases of human NHL, although *in situ* hybridization showed TIMP-1 and MMP-9 to be expressed by different cell types.[29,39] There were, however, a number of instances when MMP-9 and TIMP-1 RNA levels were not concordant.[15,29] Other TIMPs (eg, TIMP-2 and TIMP-3) may play a physiological role in the control of MMP proteolytic action in NHL.[40,41] It has been shown that T and B lymphocytes show different TIMP expression patterns.[26] TIMP-1 was shown to be expressed mainly by B cells, and TIMP-2 expression was restricted to T cells.[26] In the present study we have demonstrated that TIMP-1, -2, and -3 are produced by cells derived from human NHLs and tonsils. Active TIMP-1 levels, tested by reverse zymography, were low. Western blot analysis demonstrated that TIMP-1 was present in complexes with MMP-9. This latter finding explains the discrepancies between Western blot, zymography, and reverse zymography. Active forms seen in gelatin zymography and reverse zymography represent only a fraction of these proteins, as the complexes will not be active in these enzymatic assays. Different types of complexes between MMP-9 and TIMP-1 have been described,[42] but the precise composition of some of the complexes seen on the Western blot is uncertain at present. Analysis of these complexes is beyond the scope of this study.

Our present study demonstrates that metalloproteinases, likely in combination with other enzymes, play an important role in the matrix-degrading and invasive characteristics of cells derived from human NHLs. Such an ability presumably allows the lymphoid tumors to destroy their surrounding ECM and spread through the body in a fashion similar to that of carcinomas.[9,11,43,44] This mode of spread is different from that of normal and reactive lymphocytes (which are unable to degrade or penetrate elastin) and is likely dependent on the cellular interactions that occur in malignant lymphoid tissues, as it is not observed in pure established cultures of benign or malignant lymphocytes. It appears that, at a certain stage of differentiation, a subset of high-grade human malignant lymphoma cells must acquire the ability to destroy ECM in a manner similar to that of carcinoma cells. Such an ability may be responsible for varying biological behavior of different NHL types. In addition, inhibition of certain proteolytic enzymes (eg, MMP-9) may offer future therapeutic potential for human NHLs.

Acknowledgments

We wish to acknowledge Ms. Nona Dolphin, Ms. Tammy Andrews, and Ms. Susan Watson for secretarial assistance. These investigations were approved by Medical Ethics and Biohazard Committees at the University of Calgary.

References

1. Alexander CM, Werb Z: Extracellular matrix degradation. Cell Biology of Extracellular Matrix. Edited by Hay ED. New York, Plenum Press, 1991, pp 255–302
2. Stetler-Stevenson WG: Dynamics of matrix turnover during pathological remodeling of the extracellular matrix. Am J Pathol 1996, 148: 1345–1350
3. Pauli BU, Knudson W: Tumor invasion: a consequence of destructive and compositional matrix alterations. Hum Pathol 1988, 19:628–639
4. Kohn E, Liotta L: Molecular insights into cancer invasion: strategies for prevention and intervention. Cancer Res 1995, 55:1856–1862
5. Stauder R, Hamader S, Fasching B, Kemmler G, Thaler J, Huber H: Adhesion to high endothelial venules: a model for dissemination mechanisms in non-Hodgkin's lymphoma. Blood 1993, 82:262–267
6. Cottier H, Turk J, Sobin L: A proposal for a standardized system of reporting human lymph node morphology in relation to immunological function. Bull WHO 1972, 47:375–417
7. Duijvestijn A, Hamann A: Mechanisms and regulation of lymphocyte migration. Immunol Today 1989, 10:23–28
8. Müller-Hermelink HK, Lennert K: Lymphocytes Are Destined to Migrate Malignant Lymphomas Other Than Hodgkin's Disease. Edited by Lennert K, Mohri N, Stein H, Kaiserling E, Müller-Hermelink HK. New York, Springer-Verlag, 1978, pp 6–9
9. Cotran RS, Kumar V, Robbins SL: Malignant lymphomas. Robbins Pathologic Basis of Disease. Edited by Cotran RS, Kumar V, Robbins SL. Toronto, WB Saunders, 1989, pp 708–717

10. Matrisian LM: The matrix-degrading metalloproteinases. BioEssays 1992, 14:455–463
11. Mignatti P, Rifkin DB: Biology and biochemistry of proteinases in tumor invasion. Physiol Rev 1993, 73:161–195
12. Ray J, Stetler-Stevenson W: The role of matrix metalloproteases and their inhibitors in tumour invasion, metastasis and angiogenesis. Eur Respir J 1994, 7:2062–2072
13. Woessner JF Jr: Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J 1991, 5:2145–2154
14. Stetler-Stevenson WG, Hewitt R, Corcoran M: Matrix metalloproteinases and tumor invasion: from correlation and causality to the clinic. Cancer Biol 1996, 7:147–154
15. Kossakowska AE, Urbanski SJ, Huchcroft SA, Edwards DR: Relationship between the clinical aggressiveness of large cell immunoblastic lymphomas and expression of 92 kd gelatinase (type IV collagenase) and tissue inhibitor of metalloproteinase-1 (TIMP-1) RNAs. Oncol Res 1992, 4:233–240
16. Leppert D, Waubant E, Galardy R, Bunnett NW, Hauser SL: T cell gelatinases mediate basement membrane transmigration in vitro. J Immunol 1995, 154:4379–4389
17. Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kozlowski JM, McEwam RN: A rapid in vitro assay for quantitating the invasive potential of tumor cells. Cancer Res 1987, 47:3239–3245
18. Kleinman HK, McGarvey ML, Hassell JR, Star VL, Cannon FB, Laurie GW, Martin GR: Basement membrane complexes with biological activity. Biochemistry 1986, 25:312–318
19. Mecham RP, Heuser JE: The elastic fiber. Cell Biology of Extracellular Matrix. Edited by Hay ED. New York, Plenum Press, 1991, pp 79–109
20. Senior RM, Griffin GL, Fliszar CJ, Shapiro SD, Goldgerg GI, Welgus HG: Human 92- and 72-kilodalton type IV collagenases are elastases. J Biol Chem 1991, 266:7870–7875
21. Katsuda S, Okada Y, Okada Y, Imai K, Nakanishi I: Matrix metalloproteinase-9 (92-kd gelatinase/type IV collagenase equals gelatinase B) can degrade arterial elastin. Am J Pathol 1994, 145:1208–1218
22. Wilhelm SM, Collier IE, Marmer BL, Eisen AZ, Grant GA, Goldberg GI: SV40-transformed human lung fibroblasts secrete a 92-kd type IV collagenase which is identical to that secreted by normal human macrophages. J Biol Chem 1989, 264:17213–17221
23. Sato H, Kida Y, Mai M, Endo Y, Sasaki T, Tanaka S, Seiki M: Expression of genes encoding type IV collagen-degrading metalloproteinases and tissue inhibitors of metalloproteinases in various human tumor cells. Oncogene 1992, 7:77–83
24. Mainardi CL, Pourmotabbed TF, Hasty KA: Inflammatory phagocytes and connective tissue degrading metalloproteinases. Am J Med Sci 1991, 302:171–175
25. Ståhl-Bäckdahl M, Sudenbeck BD, Eisen AZ, Welgus HG, Parks WCJ: Expression of 92 kd type IV collagenase by eosinophils associated with basal cell carcinoma. J Invest Dermatol 1992, 99:497–503
26. Stetler-Stevenson M, Mansoor A, Lim M, Fukushima P, Kehrl J, Marti G, Ptaszynski K, Wang J, Stetler-Stevenson WG: Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in reactive and neoplastic lymphoid cells. Blood 1997, 89:1708–1715
27. Watanabe H, Nakanishi I, Yamashita K, Hayakawa T, Okada Y: Matrix metalloproteinase-9 (92 kd gelatinase/type IV collagenase) from U937 monoblastoid cells: correlation with cellular invasion. Cell Sci 1993, 104:991–999
28. Zhou H, Bernhard EJ, Fox FE, Billings PC: Induction of metalloproteinase activity in human T-lymphocytes. Biochem Biophys Acta 1993, 11:174–178
29. Kossakowska AE, Urbanski SJ, Watson A, Hayden LJ, Edwards DR: Patterns of expression of metalloproteinases and their inhibitors in human malignant lymphomas. Oncol Res 1993, 5:19–28
30. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual, ed 2. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1989
31. Wong H, Anderson WD, Cheng T, Riabowol KT: Monitoring mRNA expression by polymerase chain reaction: the "primer-dropping" method. Anal Biochem 1994, 223:251–258
32. Hinek A, Rabinovitch M: The ductus arteriosus migratory smooth muscle cell phenotype processes tropoelastin to a 52-kd product associated with impaired assembly of elastic laminae. J Biol Chem 1993, 268:1405–1413
33. Prosser IW, Whitehouse LA, Parks WC, Ståhle-Bäckdahl M, Hinek A, Park PW, Mecham RP: Polyclonal antibodies to tropoelastin and the specific detection and measurement of tropoelastin in vitro. Connect Tissue Res 1991, 25:265–279
34. Leake DS, Hornebeck W, Brecheimier D, Robert L, Peters TJ: Properties and subcellular localization of elastase-like activities of arterial smooth muscle cells in culture. Biochem Biophys Acta 1983, 761: 41–47
35. Banda MJ, Werb Z: Mouse macrophage elastase. Purification and characterization as metalloproteinase. Biochem J 1981, 193:589–605
36. Belaaouaj A, Shipley JM, Kobayashi DK, Zimonjic DB, Popescu N, Silverman GA, Shapiro SD: Human macrophage metalloelastase: genomic organization, chromosomal location, gene linkage, and tissue-specific expression. J Biol Chem 1995, 270:14568–14575
37. Goetzl EJ, Banda MJ, Leppert D: Matrix metalloproteinases in immunity. J Immunol 1996, 156:1–4
38. Khan KMF, Falcone DJ: Role of laminin in matrix induction of macrophage urokinase-type plasminogen activator and 92-kd metalloproteinase expression. J Biol Chem 1997, 272:8270–8275
39. Kossakowska AE, Urbanski SJ, Edwards DR: Tissue inhibitor of metalloproteinases-1 (TIMP-1) RNA is expressed at elevated levels in malignant non-Hodgkin's lymphomas. Blood 1991, 77:2475–2481
40. Stetler-Stevenson WG, Krutzsch HC, Liotta LA: TIMP-2: identification and characterization of a new member of the metalloproteinase inhibitor family. Matrix Suppl 1992, 1:299–306
41. Leco KJ, Khokha R, Pavloff N, Hawkes SP, Edwards DR: Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues. J Biol Chem 1994, 269:9352–9360
42. Kolkenbrock H, Hecker-Kia A, Orgel D, Kinawi A, Ulbrich N: Progelatinase B forms from human neutrophils: complex formation of monomer/lipocalin with TIMP-1. Biol Chem 1996, 377:529–533
43. Urbanski SJ, Edwards DR, Gelfand GAJ, Hinek A, Lim M, Kossakowska AE: Proteinases in pulmonary neoplasia. Lab Invest 1995, 72:155A
44. Urbanski SJ, Hinek A, Lim M, Kossakowska AE: Expression of gelatinases A and B mRNA does not correlate with their enzymatic activity in human sporadic colorectal neoplasia. Lab Invest 1995, 72:70A