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Matrix Metalloproteinases Produced by Rat IL-2-Activated NK Cells

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We have previously documented that adoptively transferred IL-2-activated NK (A-NK) cells can accumulate within cancer metastases. Electron microscopic studies of pulmonary metastases have revealed that adoptively transferred A-NK cells that accumulate within metastases bind to endothelial cells and are able to traverse basement membranes. We have now extended these morphologic studies. We report that rat A-NK cells produce two matrix metalloproteinases: MMP-2 and MMP-9, as determined by SDS-PAGE gelatin zymography. These activities are inhibited following incubation with BB-94 (batimastat), a specific inhibitor of matrix metalloproteinases but not with 3,4-dichloroscoumarin, an inhibitor of neutral serine proteases. The identity of MMP-2 was confirmed by Western blots using a polyclonal Ab against human MMP-2, whereas reverse transcriptase-PCR analysis of mRNA extracts of A-NK cells has confirmed the presence of MMP-9. In addition, we report for the first time that A-NK cells can migrate through a model basement membrane-like extracellular matrix. Moreover, the ability of A-NK cells to migrate through this model basement membrane was partially inhibited by BB-94; however, BB-94 has no effect on A-NK cell-mediated cytotoxicity, suggesting that matrix metalloproteinases do not contribute to cytolytic function of A-NK cells. In sum, our studies show that A-NK cells employ BB-94-inhibitable matrix metalloproteinases to degrade extracellular matrices. This suggests that matrix metalloproteinases may play a role in the accumulation of A-NK cells within cancer metastases. The Journal of Immunology, 1998, 160: 4248–4253.

In recent years, considerable interest has focused on IL-2-activated NK (A-NK) cells for their potential role in the therapy of established cancer metastases (1–4). This interest is related in part to our finding that fluorescence-labeled, adoptively transferred A-NK cells selectively accumulate within established pulmonary and hepatic tumor metastases in a time-, dose-, and IL-2-dependent manner (5–12). It appears that the microenvironment of tumor metastases, including tumor cells, microvascular endothelial cells, and subendothelial extracellular matrices, influences the physiologic function of A-NK cells (13). Electron microscopic studies have revealed that within 16 h after injection, A-NK cells were able to migrate out of tumor vessels and establish direct contact with tumor cells and microvascular endothelial cells; in some cases, intravital interactions between A-NK cells and tumors have revealed that the formation of contacts between the two cell types is preceded by extensive cytokinetic actions by the A-NK cells through microvessel walls, including the basal laminae and through the extracellular space (6, 14). In some structural studies of tumor metastases following adoptive transfer, A-NK cells have been found in situ to penetrate through the subendothelial extracellular matrix (6). Recent studies have ultrastructurally analyzed the interactions of A-NK cells and tumor cells in an extracellular matrix environment. These studies have suggested that A-NK cell cytotoxic capacity might be dependent on the activation of the effector cells to a locomotive state as a consequence of their interaction with extracellular matrices (14). The microenvironment encountered by A-NK cells within tumor metastases might influence their cytolytic function, since effector cell–tumor cell contact takes place only following the triggering of the effector cell to an activated locomotive state, probably involving a proteolytic activity required for invasiveness into tissue (14). In addition, it has been suggested that NK tumor cell lines have the ability to degrade extracellular matrices (15, 16). While adhesion molecules have been implicated in the ability of lymphocytes and NK cells to selectively adhere to endothelial cells (17, 18), we hypothesized that A-NK cell-associated matrix metalloproteinases (MMPs) might exist. Moreover, such enzymes might also contribute to basement membrane degradation and thereby contribute to the capacity of adoptively transferred A-NK cells to accumulate within cancer metastases.

The MMPs are a family of enzymes that consists of at least 14 members. Of these, 10 are soluble secreted enzymes, while the other 4 are membrane bound (19, 20). Although this family of enzymes shares a number of properties in common, they differ in their substrate specificities and their distribution within various cell types. The most widespread MMPs are MMP-2 (gelatinase A) and MMP-9 (gelatinase B). These enzymes have been reported in numerous normal and transformed cells, including lymphocytes. Both MMP-2 and MMP-9 have been reported to cleave type IV collagen, which provides the scaffold for the assembly of basement membranes (21).

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3 Abbreviations used in this paper: A-NK, IL-2-activated NK cells; MMP, matrix metalloproteinase.

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Based on our studies of neutral serine proteases of NK and A-NK cells (22–25) and our studies of both neutral serine proteases and MMPs of invasive, metastatic tumor cells (26–30) we hypothesized that A-NK cells, like metastatic tumor cells, might also employ MMPs in the degradation of basement membranes in situ that could contribute to the accumulation of A-NK cells within tumor metastases. This would be of importance since while A-NK cells have been reported to produce neutral serine proteases, with the capacity to degrade glycoprotein components of basement membranes, the issue of whether MMPs are produced by A-NK cells has never been reported. Recent studies have shown that T cells produce both MMP-2 and MMP-9 and that these enzymes contribute to the ability of T cells to migrate through model basement membranes (31) and endothelial monolayers (32). Herein we report that A-NK cells produce MMP-2 and MMP-9. Moreover, BB-94, a broad spectrum inhibitor of MMPs, inhibits the capacity of A-NK cells to invade through artificial basement membrane-like extracellular matrices. We therefore conclude that A-NK cells produce MMPs with the capacity to degrade type IV collagen in the basement membrane. Moreover, by virtue of this degradative capacity, these MMPs may play an important role in contributing to the ability of A-NK cells to accumulate within established cancer metastases following their adoptive transfer.

Materials and Methods

Animals

Male Fischer 344 rats were obtained from Taconic Farms (Germantown, NY) and housed in a specific pathogen-free facility.

Reagents and chemicals

Tissue culture medium and FBS were purchased from Life Technologies (Grand Island, NY). Recombinant IL-2 was a generous gift of Chiron (Emeryville, CA). All other chemical reagents were of the highest available commercial purity.

Preparation A-NK cells

A splenocyte single-cell suspension was prepared. Erythrocytes were removed by treatment with NH4Cl and the resulting mononuclear cell preparation was passed through a nylon wool column to remove B cells and monocytes (33). The resulting mononuclear cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 2 mM glutamine, 5 × 10−3 M 2-ME, 1% MEM nonessential amino acids, and 6000 IU/ml recombinant human IL-2. Nonadherent cells were removed after 48 h and the adherent cells were cultured in the conditioned medium for another 3 days. In agreement with the literature, rat A-NK cells prepared by this method are >97% NK cells as determined by staining with the 3.2.3 mAb, which recognizes the 97% NK cells

Tumor inoculation, injection of A-NK cells, and electron microscopy preparation

Wag rats were inoculated subcapsularly with 107 CC531 cells (a colon carcinoma that metastasizes to the liver) (36). After 10 days, 40 × 106 Wag rat A-NK cells were injected via the hepatic artery. After 24 h, the liver tumors were removed, fixed in 2.5% glutaraldehyde, postfixed in 0.5% OsO4, dehydrated, and embedded in Agar 100. Ultrathin sections were cut and examined using a Zeiss 902 CEM.

Purification of MMPs from A-NK conditioned medium

After 5 days in culture, A-NK cells were placed in Opti-MEM (Life Technologies) supplemented with 6000 IU/ml recombinant human IL-2 and 100 ng/ml of PMA (Sigma, St. Louis, MO) for an additional 3 days. PMA was added to enhance the production of MMPs (37, 38). MMPs were purified from conditioned media as previously described (39). Briefly, culture supernatants were collected, centrifuged to remove debris, and ammonium sulfate added to 60% saturation. The resulting precipitate was resuspended in collagenase buffer (0.2 M NaCl, 0.05 M Tris-HCl, 0.01 M CaCl2, pH 7.4) and dialyzed against two changes of the same buffer. The ammonium sulfate fraction was then applied to a gelatin-agarose (Sigma) affinity column equilibrated with 50 mM Tris- HCl, 0.5 M NaCl, 5 mM CaCl2, 0.02% Brij 35, pH 7.6, and incubated overnight with end-over-end rotation at 4°C. The column was washed in the same buffer and eluted with equilibration buffer containing 5% DMSO and 10% glycerol. The eluate was concentrated in Amicon (Beverly, MA) Centricon preparators, dialyzed against collagenase buffer, concentrated again on Amicon Microcon concentrators, and aliquoted frozen at −80°C. MMP-2 and MMP-9 were also isolated from media supernatants of HT1080 cells (American Type Culture Collection, Rockville, MD), a human fibrosarcoma cell line that has been documented to produce both MMP-2 and MMP-9 (39).

Gelatin zymography

Samples were subjected to SDS-PAGE zymography with modifications (40). The nonreduced samples were applied to a 10% slab gel containing 2 mg/ml of gelatin. After electrophoresis, the gel was washed at room temperature for 1 h in washing buffer (50 mM Tris- HCl, pH 7.5, 5 mM CaCl2, 1 μM ZnCl2, 2.5% Triton X-100) and then incubated overnight at room temperature in the same buffer containing only 1% Triton X-100. For inhibition studies, gel slices were incubated in the presence of 10 μM 3,4-dichloroisocoumarin or 10 μM Batimastat (BB-94) (a generous gift of British Biotech, Oxford, U.K.) during the wash and incubation steps. The gel was stained with a solution of 0.25% Coomassie brilliant blue R-250 and destained in 7% acetic acid and 10% methanol.

Reverse transcriptase (RT)-PCR

Total RNA was isolated from HT1080 or A-NK cells, using RNeasy columns (Qiagen, Chatsworth, CA). cDNA synthesis was performed using the Superscript II Preamplification System from Life Technologies. For each reaction, 2 μg of total RNA were reverse transcribed using random hexamer according to the protocol supplied by Life Technologies in the presence of 40 U of RNase inhibitor. The cDNA was amplified using 100 pmol each of the forward MMP-9 primer (5′-AGATCTCAACCTTGGAG3′) and reverse MMP-9 primer (5′-GCCCTTGAGATGGAATGTTG3′). These primers were a generous gift of Dr. Andrea Larson. Samples were also reverse transcribed and amplified using primers specific for actin (Clontech Laboratories, Palo Alto, CA), to verify that the RNA was intact and could be reverse transcribed and amplified. We also performed reactions with no RNA to verify that samples were not contaminated with DNA. Samples were amplified for 30 cycles in a DNA thermal cycler using the following conditions: 94°C for 1 min, 52°C for 2 min, and 72°C for 3 min.

Southern blot analysis of PCR products

PCR products were analyzed on 1% agarose gels. Gels were alkaline-denatured andblotted onto nylon membranes (Boehringer Mannheim, Indianapolis, IN) overnight with 10× SSC, or were transferred using a vacuum blot apparatus ( Hoefer Scientific Instruments, San Francisco, CA). DNA was UV-cross-linked onto the membranes and blots were hybridized to the appropriate probe, washed, and incubated with anti-digoxigenin Ab conjugated to alkaline phosphatase, as described in the Genius Non-Radioactive Labeling Kit (Boehringer Mannheim). For labeling, cDNA products were isolated in low melting point agarose and random primed with digoxigenin-dUTP. The cDNA used for detection of rat MMP-9 were a kind gift of Dr. Andrea Larson (Magee-Women’s Research Institute, Pittsburgh, PA). For detection, Lumi-Phos (Boehringer Mannheim) was pipetted onto membranes in plastic sheet protectors, and then blots were exposed to x-ray film.

Western blot analysis

HT1080 and Rat A-NK-purified media supernatants were separated on 10% SDS polyacrylamide gels under reducing conditions and then transferred to a nitrocellulose membrane using an LKB Multiphor II semidry blotting apparatus. The membranes were blocked overnight at room temperature in T-PBS, pH 7.5 (0.1% Tween-20) with 5% BSA and 5% sheep serum. After washing, the blot was incubated with peroxidase-coupled sheep anti-human MMP-2 (The Binding Site, San Diego, CA) at a 1:1000 dilution for 1 h at room temperature. The blots were washed three times in T-PBS and the bands were detected using SuperSignal CL-HRP Substrate System (Pierce Chemical, Rockford, IL). The resulting chemiluminescence was recorded on ECL Hyperfilm (Amersham, Arlington Heights, IL).

Invasion assay

The assay was performed in Bicocut Matrigel Invasion Chambers (Becton Dickinson Labware, Bedford, MA). The Matrigel was prepared by hydrating for 1 h in Opti-MEM. A total of 500,000 cells in 0.2 ml were loaded...
into the top well and 1.0 ml of Opti-MEM was added to the bottom chamber. The plates were incubated at 37°C for 24 h. After incubation, the plates were centrifuged at 1200 rpm for 5 min to pellet any invading cells. The top chambers were removed and the bottom chambers were labeled with 1 mM calcein AM and incubated at 37°C for 45 min. The plate was read on a CytoFluor II fluorescent plate reader (PerSeptive Biosystems, Framingham, MA) using an excitation wavelength of 485 nm and emission wavelength of 530 nm. Standards with a known number of A-NK cells were run simultaneously. The number of invading cells was calculated by plotting the fluorescence on the standard curve. All determinations were performed in triplicate.

Results

Identification of MMPs of rat A-NK cells

Samples of concentrated A-NK supernatants were subjected SDS-PAGE gelatin zymography. These samples indicated the presence of gelatinolytic activity. To more accurately determine the number of gelatinolytic activities in these supernatants, these samples were purified by gelatin agarose affinity chromatography. These samples were analyzed by SDS-PAGE gelatin zymography in conjunction with authentic MMPs purified from HT1080 supernatants. The results, shown in Figure 1, indicate that A-NK cells produce two gelatin-cleaving enzymes that correspond to the 72-kDa MMP-2 and the 92-kDa MMP-9 in HT1080 cells. In this preparation of gelatinases from HT1080 cells, MMP-2 yields two active bands corresponding to the 72-kDa proenzyme and the 66-kDa active form. The presence of the processed forms of these enzymes varied between preparations; however, the identity of both forms of MMP-2 was confirmed by Ab staining (see Fig. 3).

To confirm that these enzymes were metalloproteinases, gelatin zymograms were incubated in the presence or absence of 10 mM protease inhibitors during the overnight incubation and the renaturation steps. Incubation with 3,4-dichloroisocoumarin, a general inhibitor of serine proteases (41), produced no change in the pattern of bands observed in the zymograms of samples from either HT1080 or A-NK cells (Fig. 2C); however, incubation with BB-94, a selective inhibitor of MMPs (42), resulted in the complete ablation of enzymatic activity in all bands (Fig. 2B). Similar results were obtained with o-phenanthroline, a general inhibitor of metalloenzymes (data not shown).

Although the m.w.s of these gelatinolytic activities corresponded to the known m.w.s of the enzymes isolated from HT1080 supernatants, we wished to confirm their identity using Abs raised against MMP-2 and MMP-9. Western blots using mouse mAbs raised against human MMP-2 and MMP-9, although able to identify the bands from the HT1080 human fibrosarcoma, were unable to detect the rat A-NK enzymes. However, a polyclonal Ab raised against human MMP-2 was able to recognize both rat A-NK and HT1080 MMP-2 (Fig. 3).

Analysis of rat A-NK mRNA for MMP transcripts

Since we were able to conclusively identify only MMP-2 by Western blotting, an analysis of rat A-NK mRNA was undertaken using RT-PCR to identify MMP-9 transcripts using MMP-9-specific primers. RNA was isolated from HT1080 cells and from rat A-NK cells that had been treated with 100 ng/ml of PMA. As shown in Figure 4, MMP-9 transcripts were readily detectable in HT1080 cells that were used as a positive control, as well as in rat A-NK cells that had been treated with PMA. MMP-9 transcripts were also detected in mRNA isolated from A-NK cells that had not been treated with PMA (data not shown).

Role of MMPs in rat A-NK cell invasion

A Matrigel invasion assay was used to determine whether these MMPs influenced the ability of rat A-NK cells to invade through
a basement membrane. Rat A-NK cells were placed in a Matrigel invasion chamber that consists of an 8-μm pore size filter coated with 100 μg of Matrigel protein per cm². As shown in Figure 5, the invasion of rat A-NK cells through Matrigel in a 24-h period was inhibited by over 50% by BB-94 with a p value of 0.0003; however, no significant inhibition of rat A-NK invasion was observed when cells were treated with inhibitors of serine proteases, chymostatin, and benzamidine. The chemoattractant FMLP also caused no significant change in the migration of A-NK cells through Matrigel. In addition, BB-94 does not cause a functional impairment of A-NK cells or affect viability over the course of the assay. When BB-94 (10 μM) was included in cytotoxicity assays, no effect on lytic activity against either YAC-1 or P815 targets was observed (data not shown).

In vivo invasion of rat A-NK cells through extracellular matrix
A-NK cells were injected into animals that had previously been inoculated with the CC531 colon carcinoma. These cells, when injected into the liver capsule, grow in a multilobular fashion. Islets of tumor cells are surrounded by a fibrous tissue capsule. An electron microscopic examination of these tumors showed A-NK cells that appeared to be in the process of dissolving some of the matrix surrounding the tumor (Fig. 6). The A-NK cell in this image can be distinguished from surrounding tumor cells by numerous dense core granules, characteristic in the literature for A-NK cells (i.e., large granular lymphocytes), present in the cytoplasm making it distinct from both the CC531 tumor cells and the normal liver tissue (6).

Discussion
Adaptively transferred A-NK have been shown to accumulate within tumor metastases (5, 6). We have previously documented that A-NK cells can leave the microvasculature and migrate into the tumor interstitium to subsequently establish localized contact with tumor cells. To achieve this localization, it is clear that A-NK cells have to traverse the capillary subendothelial basement membrane. This process and subsequent movement of A-NK cells through the tumor interstitium would likely require degradation of the components of these extracellular matrices. As is the case for tumor cell invasion through, and degradation of, extracellular matrices, we hypothesized that matrix-degrading proteases might be produced by A-NK cells, including MMPs. In this report, we have demonstrated that A-NK cells produce at least two MMPs (MMP-2 and MMP-9). Moreover, we have shown that inhibition of these proteases by the MMP inhibitor BB-94 leads to an inhibition of the migration of A-NK cells through Matrigel, a model, artificial basement membrane-like extracellular matrix.

MMPs have been reported in T cells. These include MMP-2 (31, 43), MMP-9 (31, 38, 43–46), MMP-3 (43), and MMP-10 (47). In addition, the production of these T lymphocyte-derived enzymes has been reported to be influenced by IL-2 (31, 44, 46, 48) and other cytokines (48). These MMPs have also been implicated in the ability of T cells to traverse model basement membranes (31, 46) and have been implicated in the process of inflammation.

Before the studies reported herein, only two reports of MMPs in NK cells have appeared in the literature (15, 48). Human NK cells were found to produce barely detectable levels of MMP-2 (48). The rat RNK-16 cell line, however, was found to produce MMP-1 and MMP-3, but not MMP-2 or MMP-9 (15). These findings are in contrast to results that we report herein that indicate that rat A-NK cells produce MMP-2 and MMP-9. We did not see any components in the m.w. range reported for MMP-1 or MMP-3. These discrepancies may be due to one or more differences in the cells used in each study. Our results were obtained with A-NK cells. IL-2 has been found to up-regulate the production of MMPs in T cells and may perform a similar function in NK cells. More important, however, may be the nature of the RNK-16 cell line used by others. This cell line, although an accepted model for aspects of NK cell function, was derived from a rat large granular lymphocyte leukemia. Since RNK-16 cells are tumor cells, it is entirely possible that they may express an array of proteases representative of both migratory effector cells as well as those expressed by tumor cells that might independently contribute to protease-mediated cellular degradation of extracellular matrices. Therefore, it is unclear whether the RNK-16 cell line serves as an optimal model for examining the production of effector cell proteolytic enzymes, including MMPs.
Although we have noted that BB-94 at 10 µM is very effective at inhibiting the enzymatic action of MMP-2 and MMP-9 in vitro, this same concentration caused only a 50% decrease in A-NK cell invasion through a model basement membrane. It is therefore likely that other proteases are also involved in this process. Indeed, in the case of invasive tumor cells, we and others have documented a role for degradation of glycoprotein components of the extracellular matrix by neutral, serine proteases, e.g., plasmin, distinct from and additive to matrix degradation by MMPs (30). Indeed it is of interest that plasmin, generated by the activation of plasminogen by uPA (urokinase-type plasminogen activator) produced by T lymphocytes, can cleave the extracellular matrix component tenascin C into an adhesive substrate for T lymphocyte adhesiveness (49). Incubation with general serine protease inhibitors produced no effect on A-NK cell invasion through Matrigel. It could be that other MMPs that are not as susceptible to BB-94, specialized membrane-associated MMPs, or other mechanistic classes (cysteine or aspartic acid) are involved in this process. These possibilities are currently under investigation. In addition, we have previously investigated neutral, serine proteases produced by A-NK cells for their role in cell-mediated cytotoxicity. Nevertheless, it does not appear that A-NK cell MMP-2 and MMP-9 contribute to cytotoxicity.

Our studies support the view that immune effector cells involved in lysis of malignant cells (NK cells and CD8+ T cells) secrete MMPs that are utilized to traverse basement membranes. This function would allow these cells to leave the vasculature, migrate to tumor sites, and have the opportunity to interact with and lead to the destruction of malignant cells that they encounter. These results also demonstrate that an MMP inhibitor, already in clinical trials for metastatic tumor cell MMPs but spare those of A-NK cells. In light of these studies, it is therefore important to assess the effects of MMP inhibitors being considered for clinical development not only on tumor growth and spread but also on the ability of effector cells of the immune system to localize within tumor deposits.

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References


