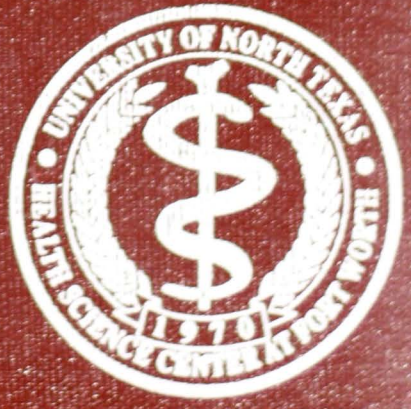
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Adoptively transferred natural killer (NK) cells can infiltrate tumors and directly kill malignantly transformed cells without prior sensitization. This makes NK cells ideal for cancer immunotherapy, not only for their tumoricidal capacities, but also as drug carriers. This dissertation investigates the role of NK cell urokinase plasminogen activator (uPA) system in NK cell invasion through extracellular matrices (ECMs), its cooperation with matrix metalloproteinases (MMPs), the mechanism by which interleukin-2 (IL-2) upregulates NK cell uPA/uPAR, and the *in vivo* antitumor therapeutic potential of NK cells as drug delivery vehicles.

uPA and its receptor uPAR were detected in human and rat NK cells using RT-PCR, casein plasminogen zymography, western blots, and fluorescence microscopy. *In vitro* invasion assays showed a role for the uPA system in NK cell invasion, alone and in cooperation with MMPs: this was achieved by using selective plasmin inhibitors in combination with selective MMP inhibitors in ECM/Matrigel invasion assays. uPA's regulation by the ECM proteins collagen type IV, laminin, and fibronectin was investigated and results show a downregulation of NK cell uPA mRNA by these proteins. IL-2, however, a potent NK cell stimulator, increases both uPA and uPAR, coinciding with an increase in NK cell invasion. This IL-2 upregulation has transcriptional and posttranscriptional components, the latter mediated by uPA and uPAR destabilizing

mRNA binding proteins. The use of NK cells as drug delivery vehicles was illustrated by *in vivo* studies which demonstrated that NK cells linked to doxorubicin were more efficacious in prolonging the survival of tumor bearing mice than either treatment alone.

The work presented in this dissertation has substantial impact on the field of adoptive immunotherapy for cancer treatment. Engineering NK cells to transiently express high amounts of uPA and/or uPAR may increase the invasive capacities of NK cells, resulting in greater infiltration of tumors by ex-vivo activated NK cells. Moreover, this enhanced infiltration may allow a greater delivery of anticancer drugs to established metastatic tumors. This can potentially lead to more efficacious and possibly curative NK mediated adoptive immunotherapy, thereby constituting a novel means to overcome current limitations to NK cell-mediated adoptive therapy of cancer metastases.

THE UROKINASE PLASMINOGEN ACTIVATOR SYSTEM
IN NK CELLS: ITS ROLE IN INVASION
AND ITS REGULATION BY IL-2

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DISSERTATION

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COMMONLY USED ABBREVIATIONS

A-NK:	Activated NK Cells
ECM:	Extracellular Matrix
IL-2:	Interleukin-2
IFN-γ:	Interferon-γ
LAK:	Lymphokine Activated Killers
MHC:	Major Histocompatibility Complex
MMP:	Matrix Metalloproteinase
mRNABP:	mRNA Binding Protein
NK Cells:	Natural Killer Cells
PAI:	Plasminogen Activator Inhibitor
RTPCR:	Reverse transcriptase polymerase chain reaction
uPA:	Urokinase plasminogen activator
UPAR:	Urokinase plasminogen activator receptor
VN:	Vitronectin

CHAPTER I

INTRODUCTION TO THE STUDY

Cancer and the Immune System

The immune system plays a major role in the prevention of cancer prior to tumor formation by eliminating cells which have undergone malignant transformation (a process known as immuno-surveillance), and following tumor establishment by preventing and controlling metastases (1,2). Individuals with immunodeficiency disorders such as Wiskott-Aldrich Syndrome, ataxia telengiectasia, X-linked agamma globolinemia, X-linked lymphoproliferative syndrome, and Chediak-Higashi Syndrome appear to have higher incidences of some cancers compared to immunocompetent individuals (3,4). Furthermore, the prevalence of AIDS related neoplasms such as Kaposi's sarcoma (5,6), and the emergence of cancers associated with immunosuppression following organ transplantation have emphasized the role of the immune system in the protection against cancer (7-11). Various immune cells have been shown to play a role in the immune response against cancer such as monocytes/macrophages (12-15), dendritic cells (16,17), T-lymphocytes (18-20), and natural killer (NK) cells (21,22). In light of the numerous reports which link immunodeficiency with cancer, and in view of the high incidence of cancer in

immunodeficient individuals, it is reasonable to hypothesize that certain malignancies may develop primarily as a result of the transformed cells escaping recognition by the immune system and/or evading the cytotoxic mechanisms of immune cells.

Adoptive Cellular Immunotherapy

The use of immune cells in the treatment of established tumors has shown promise in the fight against cancer. One form of cancer therapy employing the immune system is known as adoptive cellular immunotherapy. In adoptive cellular immunotherapy, the tumor-bearing host's immune cells are collected following stimulation of the host with immunostimulants such as interleukin-2 (IL-2), interferon-γ (IFN-γ), or lipopolysaccharides (LPS). The type of immunostimulant used is determined by the type of cells to be used in the immunotherapy (23). Following the expansion of the cells *in vitro*, the cells are then readministered to the tumor-bearing host. Adoptive immunotherapy using activated immune cells has proven to be an effective weapon in the treatment of some forms of cancer (24,25). Many cells have been used in this form of immunotherapy: T-cells (26-28), dendritic cells (29,30), NK cells (31,32), and monocytes/macrophages (23,33,34) are frequently used as part of immunotherapy regimens either alone, or in conjunction with anticancer drugs. Advances in pharmaceuticals have enabled effector cells to be used in adoptive immunotherapy not only for their killing abilities, but also as drug carriers (35). Moreover, improving effector cell anti-tumor functions by gene transfection has increased the potential for the

use of immune cells in cancer therapy, and has shown promise in the treatment of some cancers (36-38).

Lymphokine Activated Killer (LAK) Cells and NK Cells

Adoptive immunotherapy for the treatment of cancer using lymphokine-activated killer cells has shown promise in the treatment of various malignancies (24,25). LAK cells were discovered in the 1980's while investigators were searching for lymphocytes with the capacity to lyse malignantly transformed cells (39). LAK cells are peripheral blood lymphocytes that attain tumor lytic abilities following their stimulation with high doses of IL-2 (40,41). The use of these cells to treat both human and animal tumors has in some cases yielded successful therapeutic results (24,25,42). Since their discovery, extensive research has been undertaken to identify the exact morphology and phenotype of LAK cells. Studies of human, rat, and mouse have yielded strong evidence that LAK cells are composed primarily of lymphokine-activated (e.g. IL-2 activated) NK cells (43,44).

NK cells are large granular lymphocytes that make up 3-15% of peripheral blood lymphocytes, 5% of spleen cells, and approximately 25% of liver lymphocytes (45-47). NK cell precursors have been detected in the bone marrow and the thymus, however, they are almost completely absent in the lymphatic vessels and lymph nodes (48,49). Characteristic NK cell surface markers include the IgG Fc-receptor IIIA (CD16), neural cell adhesion molecule (NCAM, CD56), and NK1.1. NK cells lack CD3 which is found

on T-cells in association with the T-cell receptor (TCR) (50). One of the most distinguishing features of NK cells is their ability to lyse target cells without prior sensitization (50). The spontaneous cytotoxicity of NK cells makes them ideal immune cells functioning in the first line defense against pathogens as part of the innate immune system, in contrast to T-cells which must be exposed to specific antigens for their activation, rendering a latent period of 7-10 days before T-cells develop their initial or primary reactivity (51). NK cells have been shown to play a major role in anti-viral immunity (52,53) and in the prevention of neoplasm formation following malignant transformation (22,54,55)

A hallmark of NK cell-mediated cytotoxicity is the lack of NK cell dependency on major histocompatibility complex antigens for the activation of NK cell cytolytic machinery (50). For this reason, lysis of target cells by NK cells is often referred to as non-MHC restricted cytotoxicity, in contrast to T-lymphocytes which must be activated by MHC class antigens for effective killing of target cells. Moreover, the sensitivity of target cells to killing by NK cells appears to be inversely related to target cell expression of MHC glycoproteins (56,57). The mechanism of MHC inhibition of NK cell cytolytic machinery has been extensively investigated over the last decade. Briefly, NK cells express two types of MHC recognizing receptors: killer inhibitory receptors (KIR) which belong to the immunoglobulin (Ig) superfamily (58), and a number of other receptors which belong to the C-type lectin family such as the human CD94, and the murine Ly-49 (59). MHC downregulation and/or alterations in MHC structure by foreign antigens following viral transfection or malignant transformation prevents NK killer inhibitory

signal transmission and increases NK cell cytotoxic machinery which in turn causes target cell lysis (60).

On the other hand, NK cell-mediated cytotoxicity can be activated by NK receptors expressed on the cell surface. Common NK cell activating receptors include CD16, NKR-P1, NK-TR1, and 2B4 (59,61-64). With the exception of CD16, these receptors bind to ligands expressed on the surface of target cells. CD16 is the Fc receptor for IgG which mediates the phenomenon known as antibody-dependent-cell-mediated cytotoxicity (ADCC) (65,66) whereby an antigen bound antibody activates the cytotoxicity of NK cells following its binding to CD16. The binding and attachment of NK cells to target cells also appears to be a prerequisite for the triggering of NK cell cytotoxicity (59,61). This suggests an important role for NK expressed adhesion molecules such as α and β-integrins in NK cell cytotoxicity (67,68). Finally, some of the KIRs may trigger rather than inhibit NK cell cytolysis possibly due to differences in their transmembrane and intracytoplasmic domains (69). Studies have shown that both CD94 and Ly-49D are involved in the activation of NK cell cytotoxicity (70,71).

Target cell cytolysis is mediated by a number of cytokines and cytotoxic compounds which are released by NK cells following their activation. Tumor necrosis factor (TNF-α) is one of the major cytolytic molecules released by NK cells which induces apoptosis in target cells (72,73). NK cell granules have also been shown to play an important role in the cytotoxicity mediated by NK cells. Two major components of NK granules are perforin, and granzymes. Perforin causes cell death by forming pores in target cell membranes leading to osmotic lysis of cells (74); in conjunction with

granzymes such as granzymes A and B, perforin has been shown to induce apoptosis in target cells (75-81). NK cell induction of apoptosis in target cells has also been shown to be mediated by the Fas pathway and caspases (47,82-85). Furthermore, NK cells have been shown to secrete cytokines as they engage and kill target cells. IFN-γ, one of the major cytokines involved in cell-mediated cytotoxicity, has been shown to be secreted by NK cells. IFN-γ can directly cause target cell death, or it can activate and enhance the cytotoxicity of other cells of the immune system such as T cells, macrophages, and other NK cells (86-88). Indirect tumor killing can also be achieved by NK-endothelial cell interactions. The interaction of NK cell with endothelial cell within the tumor vasculature can potentially cause damage to the tumor blood supply ultimately resulting in ischemic tumor cell death (89,90).

NK Cell Adoptive Immunotherapy

Adoptive immunotherapy using NK cells has shown promise for the treatment of some cancers, with the most dramatic responses seen in melanoma and renal cell carcinoma patients (24,25,42,91,92). Following their activation with IL-2, prior to their administration into the tumor bearing host, NK cells become adherent to plastic and their antitumor cytotoxicity increases (93). Adherent, activated NK (A-NK) cells, also known as A-LAK (adherent-lymphokine activated killer) cells, are responsible for most of the anti-tumor effects exhibited by LAK cells (43,94). Following their adoptive transfer, A-NK cells have been shown to localize within tumors, selectively accumulate within the

malignant lesions, and establish cell to cell contact with their target cells (95-97). Ribeiro et al. demonstrated that following their tissue infiltration, NK cells were retained within the tumor tissue for longer times in comparison with other cell types (89).

In addition to their direct anti-tumor effects, A-NK cells have the potential to serve as carriers for anticancer agents primarily because of their capacity to specifically infiltrate tumors. In order to reduce the toxic effects of anti-cancer drugs, and to increase the concentration of the drugs within the milieu of the tumor, red blood cells (RBC) and liposomes have both been used as vehicles for anticancer drug delivery (98-102). However, because of their lack of tumor specificity, RBCs and liposomes in some cases get trapped in organs such as the liver, therefore limiting their therapeutic potential (103). In contrast, the selective infiltration of tumors by A-NK cells (96,97) makes A-NK cells ideal candidates for drug delivery. Zanovello et al. have shown that A-NK cells loaded with ricin, a plant toxin, showed significant inhibition of metastases in a lung cancer model (103). To further take advantage of the ability of A-NK cells to selectively accumulate within tumor tissue, gene transfection of NK cells has shown promise in cancer immunotherapy. Nagashima et al. demonstrated enhanced anti-tumor efficacy using A-NK cells transfected with the IL-2 gene following their administration to tumor bearing host (104).

Proteases Used in Leukocyte and NK Cell ECM Degradation and Invasion

In order for leukocytes, including NK cells, to reach target tumors, they must degrade and penetrate through a complex barrier of extracellular matrix (ECM) proteins. Leukocytes have been shown to produce a variety of proteases with differing specificities for ECM proteins. Some of the well characterized proteases include the serine proteases (i.e. urokinase plasminogen activator (uPA), plasmin, cathepsin G), matrix metalloproteinases (MMPs) (i.e. interstitial collagenase (MMP-1), gelatinases (MMP-2 and 9)), cysteine proteinases (i.e. cathepsins S, L, B, H), and aspartic proteinases (i.e. cathepsin D). These proteases differ in their structures, substrate specificities, active sites, and the pH required for optimal activity (105). Because ECMs can have a large number of protein components such as laminin, collagen, fibronectin, and vitronectin, the numerous proteases which are used by leukocytes enables them to degrade and move through a variety of ECMs in many different tissue types. The mechanism by which leukocytes employ the uPA system and the MMPs in their invasion and migration into pathologic tissue has been well characterized for neutrophils and monocytes (106-114). Our laboratory has investigated and clearly demonstrated the role MMPs play in NK cell invasion (115-117); however little is known about the uPA system in NK cells and its role in NK cell invasion.

The work presented in this dissertation primarily focuses on the following four topics: 1) identifying components of the uPA system in NK cells, 2) examining the role the uPA system plays in NK cell invasion through ECM, 3) exploring the

cooperation of the MMPs and the uPA system in the ECM degradation by NK cells, and 4) investigating the mechanism of regulation of uPA and uPAR by IL-2 in NK cells.

The Urokinase Plasminogen Activator (uPA) System

I. uPA

The uPA system consists of the serine proteases uPA and plasmin, the uPA receptor (uPAR), and plasminogen activator inhibitors (PAI-1 and PAI-2). uPA is a 54 kD, 411 amino acid, highly specific serine protease that is primarily responsible for the activation of plasminogen to plasmin. uPA is secreted as an inactive single chain proenzyme (sc-uPA), which is converted into an active two-chain enzyme (tc-uPA) following the cleavage of Lys158-Ile159 bond. Tc-uPA is made up of the A-chain and the B-chain which are held together by a disulfide bond (Cys148-Cys279). The A-chain contains the amino terminal end of uPA, known as the amino terminal fragment (ATF), and the kringle domain; the B-chain contains the serine protease domains of uPA composed of the typical serine protease catalytic triad His, Ser, Asp. The ATF contains the growth factor domain (GFD) of uPA which is composed of amino acids 1-48 which is responsible for the binding of uPA to its high affinity receptor uPAR. The 33 kD active low molecular weight uPA (LMW-uPA) is formed by cleavage of either Glu143-Leu144 by MMPs (118) or Lys135-Lys136 by uPA itself or plasmin (119), which releases the

ATF. In contrast to other similar neutral serine proteases e.g. trypsin, which has the capacity to cleave hundreds of substrates, uPA is highly selective and has a very restricted repertoire of substrates. The most efficient substrate for uPA is plasminogen which is cleaved by uPA at Arg561-Val562 bond; moreover, uPA has the capacity to attack single Arg-X or Lys-X bonds of fibronectin, hepatocyte growth factor/scatter factor (HGF), diphtheria toxin, uPA, and uPAR, although much less efficiently than plasminogen (120).

In addition to its role as a proteolytic enzyme, uPA has been shown to contain mitogenic and chemotactic properties. Rabbani et al. demonstrated that the GFD of uPA, which is necessary for the binding of uPA to uPAR, exhibits growth promoting effects on the human SaOS-2 osteosarcoma cells, and that the fucosylation at Thr18 within this domain is essential for this mitogenic effect (121). Moreover, De Petro et al. reported uPA mitogenic activity in normal human fibroblasts that was dependent on uPA-uPAR binding (122). There have also been many reports demonstrating the role of uPA as a chemotactic agent (123-126). This chemotactic effect may be dependent on uPAR's domain 1-domain 2 linking epitope $S_{88}R_{89}S_{90}R_{91}Y_{92}$,which may interact with cellular chemotactic receptors following conformational changes caused by uPA binding to uPAR, or following the proteolytic cleavage of uPAR or its soluble form suPAR by proteases like chymotrypsin (126,127).

II. Plasminogen/Plasmin

Plasmin, like uPA, is a two-chain serine protease that catalyzes the cleavage of Lys-X and Arg-X bonds. The inactive proenzyme plasminogen, which is a 791 amino acid single chain polypeptide, is cleaved by plasminogen activators at Arg561-Val562 to form the active two-chain form of the enzyme plasmin. Unlike uPA, plasmin has a much broader substrate specificity having the capacity to degrade many ECM proteins such a fibronectin (FN), vitronectin (VN), fibrin, and collagen type IV (128,129). Plasmin also has the ability to regulate other ECM proteases such as the MMPs by activating the zymogen forms of the MMPs such as proMMP-1 and proMMP-3 (130). Plasmin was also shown to have the ability to degrade MMP inhibitors such as TIMP-2, and thereby increase the activity of MMPs (130). The interactions of the uPA system with the MMPs is examined in detail in **chapter III.**

Within the vasculature, plasmin plays a major role in clot lysis because of its ability to degrade fibrin. In addition, plasmin appears to be centrally involved in other physiological and pathological processes. Extravascularly, where approximately 40% of plasminogen is found (131,132), plasmin plays a major role in physiological processes where cell movement is essential such as leukocyte invasion in inflammation (105,133), mammary cell involution after lactation (134), breakdown of the follicular wall for ovulation (135), trophoblast invasion into the endometrium during embryogenesis (136), and keratinocyte accumulation after wound healing (137). Furthermore, plasmin has been shown to play a role in pathological processes where increases in cell movement are

responsible for the detrimental effects of disease. In the case of cancer, plasmin has been shown to play a major role tumor invasion and metastases (120,130,138-140) and in tumor angiogenesis (141). In processes involving cell movement, plasmin exerts its role primarily by its capacity to degrade ECM components. An important role for plasmin in these latter processes is supported by the ability of cell-bound plasmin to directly degrade ECM proteins (142) such as proteoglycans, laminin, fibronectin, and type IV collagen (120,129,130).

III. uPAR

The receptor for uPA, uPAR, is a heavily glycosylated 3 domain extracellular receptor protein that is linked to the cell surface by a glycosyl phospatidylinositol (GPI) anchor. The K_d value for uPA/uPAR interactions has been reported to range between 0.1-4 μM, varying with the type of the cells and the assay conditions (143). Although domain-I of uPAR was shown to be crucial in the binding of uPA to uPAR (144), domains II and III of uPAR have also been shown to play a major role for efficient and high affinity uPA-uPAR binding (145-147) and for uPAR-vitronectin (VN) binding (148). uPAR has been shown to play a major role in cell invasion and migration through the ECM. In the proteolytic pathways involving uPA and plasminogen, Ellis et al. showed that the K_m for plasminogen activation by receptor bound uPA decreased 40 fold from 25μM in solution (in the absence of cell bound uPA) to 0.67μM with receptor bound uPA (149)--this is below the normal plasma concentration of plasminogen

implying a high physiological significance of this reaction. The increase in plasmin generation following the binding of uPA to uPAR is believed to be a result of uPA-uPAR complexes which result in catalytically favorable interactions of uPA with plasminogen; however, there has been contradicting evidence regarding the direct effects of uPAR on uPA's proteolytic properties (150,151). Furthermore, uPAR plays a major role in localizing uPA and therefore the proteolytic activities during migration and invasion to focal cell contacts and to polarized extensions at the leading edge of migrating cells known as invadopodia (110,152-156). uPAR has also been shown to play a role in the downregulation of the uPA system by its capacity to internalize uPA-PAI complexes in conjunction with the α_2 macroglobulin receptor/LDL receptor related protein (LRP/α_2-MR) (157-159); following their internalization, uPA-PAI-1 complexes are degraded and uPAR is recycled back to the cell surface (160). Although uPA-PAI-1 complexes have been shown to directly bind LRP/α_2-MR (157,161,162), Li et al. showed that uPA-PAI-1 internalization is enhanced following the binding of uPA-PAI-1 complexes to uPAR (163). The internalization of uPA-PAI-1 has also been shown to occur independently from the LRP/α_2-MR via interactions with the 275-kD cation-independent mannose 6-phosphate (Man-6-P)/insulin-like growth factor-II (IGF-II) receptor (CIMPR) (164,165). In contrast to the uPAR interactions with LRP/α_2-MR which leads to the recycling of uPAR, the uPAR-CIMPR interactions directs uPAR to lysosomes which may lead to the degradation of uPAR (164). In addition, uPA has been implicated in regulating its own activity by its capacity to cleave the ligand binding domain of uPAR, a process which

appears to be very important in controlling and limiting uPA activation and uPA initiated proteolytic cascades in the absence of PAIs (166).

Aside from its role in uPA/plasminogen mediated proteolysis, uPAR plays a major role in various aspects of cellular migration and invasion such as cellular adhesion and signaling needed for effective cell movement. Regarding adhesion, uPAR has been shown to interact and bind to VN, an ECM component which usually circulates in the plasma and is immobilized in ECM associated with cancer, angiogenesis, atherosclerosis, areas undergoing wound healing, and inflamed tissues, (120,167-172). While in the plasma VN is believed to circulate in a monomeric "native" conformation (n-VN). In the ECM VN is found in a multimeric "extended" form, and this latter form of VN is believed to be involved in the association with uPAR (173-175). The association of uPAR and VN appears to be mediated via domains II and III of uPAR, enhanced by uPA, and inhibited by PAI-1 (148,173,176-178). Since integrins have also been shown to associate with VN (178,179), the uPAR-VN interactions may cause a clustering of uPAR and the integrins at VN rich ECM areas thereby enhancing uPAR-integrin mediated signaling (173). Regarding proteolysis, VN associated with either the ECM or the cell surface has been shown to bind soluble uPAR (suPAR)-uPA complexes and possibly concentrate the uPA proteolytic pathways to VN rich areas of the cell or the ECM (180). Because of PAI-1's capacity to compete with uPAR and integrins for the binding of VN (173,178,181,182), in a VN rich ECM the balance between cell adhesion and cell detachment leading to cell movement is significantly governed by the interactions of PAI-1 and VN.

Since uPAR is an extracellular receptor with no intracellular domains, it must cooperate with other cellular proteins to carry out its signaling. uPAR has been show to be associated with various subunits of integrins including α_v, α_3, α_5, α_6, β_1, β_2, and β_3 (120,183-186). These associations with the integrins allows uPAR to play a major role in secondary signaling during the migration of cells through the ECM (176,187). uPAR mediated signaling occurs through a variety of second messenger pathways such as tyrosine phosphorylation of focal adhesion kinase (FAK) and mitogen activated protein kinase (MAPK) (188,189), phosphorylation of cytokeratins (125) and nonreceptor tyrosine kinases of the *src* family (184), activation of p56/p59^{hck} kinase (126), induction of the early response genes *fos, jun, and myc* (190,191), activation of *ras*/extracellular signal regulated kinases 1 and 2 (ERK1 and ERK2) (192,193), de novo synthesis of diacylglycerol (194), cAMP formation (195), and through calcium mediated pathways (196). These second messenger pathways have been shown to promote and regulate cellular movement through the ECM.

uPA System in Tumor Cell Invasion

The role of the uPA system in the cellular invasion and migration has been well documented especially in the areas of cancer and cancer metastasis (120,197-200). uPA/uPAR/PAI have been shown to be significant prognostic markers of relapse in human tumors (201), indicating a strong correlation between the uPA system and tumor invasiveness in a variety of cancers including bladder cancer (202), stomach cancer

(203), ovarian cancer (204), pleural cancer (205), pancreatic cancer (206), lung cancer (207), breast cancer (208), liver cancer (209), thyroid cancer (210), monocytic and mylogenous leukemias (211,212), chondrosarcoma (213), and neuroblastoma (214). Because of this major role in tumor invasion, components of the uPA system have been a central target in cancer therapy (199). Numerous uPA inhibitors have shown promise in anti-tumor therapy. Amiloride, which is better known as a diuretic, was shown to have uPA inhibitory effects and has demonstrated the potential to be an antitumor agent (215-217). The peptide Pyr-Leu-Arg-CHO, a leupeptin analogue inhibitor of both uPA and plasmin, was able to inhibit the *in vitro* invasion of human fibrosarcoma cells (218). uPAR has also been a target for antimetastatic therapy (120,219). A fusion protein composed of the EGF portion of uPA and the Fc portion of human IgG was shown to be a high affinity antagonist of uPAR and was able to inhibit neovascularization and growth of mouse B16 melanoma tumor (220). Mishima et al. have shown that limiting the role of the uPA system by using a uPAR binding peptide, known as Ångstrom 6 (Å6), derived from non-receptor binding region of uPA in conjunction with cisplatin was effective in inhibiting the angiogenesis and growth of a glioblastoma tumor model *in vivo* (221). In another study, Å6 was also able to inhibit breast cancer cell invasion and endothelial cell migration, thereby inhibiting tumor progression and angiogenesis (222).

uPA System in Leukocytes

The uPA system has been shown to play a major role in the migration of neutrophils (223,224), T cells (225,226), macrophages (111,227), and dendritic cells (228). Gyetko et al. reported a role for uPAR in the migration of macrophages independent of uPAR's role in proteolytic pathways (229). Similarly, Resnati et al. found an increase in the p56/p59hck Src-family tyrosine kinase activity following uPAR-mediated chemotaxis in monocytes (126). Furthermore, Gyetko et al. demonstrated that mice deficient in uPAR ($uPAR^{-/-}$) have profoundly diminished neutrophil recruitment to the lung in response to *Pseudomonas aeruginosa* pneumonia compared with wild-type mice ($uPAR^{+/+}$) (224). Moreover, Waltz et al. reported a dramatic decrease in neutrophil chemotaxis in uPAR deficient mice due to a deficiency in uPAR mediated chemotactic signaling, independent of a deficiency in the proteolytic activities of uPA (230). May et al. showed that $uPAR^{-/-}$ mice have an abrogated β2 integrin dependent adhesion of leukocytes to endothelial cells with a concomitant reduction of leukocyte infiltration compared with $uPAR^{+/+}$ mice (231). Regarding uPA, Shapiro et al. showed that uPA deficient mice ($uPA^{-/-}$) are predisposed to *Staphylococcal botryomycosis* infection, pleuritis, and effacement of lymphoid follicles in the spleen and the lymph nodes (232). Furthermore, in response to *Cryptococcus neoformans* infection, $uPA^{-/-}$ mice showed a decrease in $CD4^+$ cell recruitment, an increase in pulmonary CFU, and a significant decrease in survival compared to wild-type ($uPA^{+/+}$) mice (227).

Il-2 Regulation of uPA and uPAR

IL-2 has been shown to be the most potent stimulator of NK cells. Unlike IFN-γ which primarily increases NK cell cytotoxicity, IL-2 has been shown to increase both the cytotoxicity and the proliferation of NK cells (233-237). Furthermore, NK cells have been shown to express both the intermediate and high affinity IL-2 receptors which are responsible for the cytotoxic and proliferative effects of IL-2 stimulated NK cells (234,235,238-241). Manyak et al. reported an increase in chymotrypsin-like and trypsin-like proteases following the activation of NK cells with IL-2, and related the increase in these proteases to the enhancement of NK cell-mediated cytotoxicity (242). Nykjaer et al. showed an increase in T-cell expression of uPAR following activation with IL-2 (143).

Because it is the most frequently used activator of NK cells prior to NK adoptive immunotherapy, understanding the effects of IL-2 on uPA and uPAR coupled with its effects on NK invasion and tumor infiltration, is a major point addressed in this work. One of the major problems with IL-2 activated NK cell therapy has been the toxicity encountered in this form of immunotherapy, which is not related to the cellular dose, but to the dose of IL-2 (143). Therefore, understanding the effects of IL-2 on NK cells and the mechanisms that lead to the enhanced NK cell properties following their activation with IL-2 is essential for optimizing the efficacy of IL-2 activated NK cell immunotherapy while reducing the toxicity caused by IL-2 activation. In addition to our

investigations of the effects of IL-2 on NK cell uPA and uPAR, we also examined the mechanisms by which IL-2 regulates uPA and uPAR.

There has been extensive work done on the mechanism of regulation of uPA, uPAR and PAI-1 (205,243-246). The regulation of these components of the uPA system has been shown to be primarily controlled through posttranscriptional pathways involving mRNA binding proteins (mRNABPs). In the case of uPAR, a 50 kD protein was detected that bound to a 51 nucleotide fragment of the uPAR mRNA coding region. This interaction caused decreased stability of the uPAR mRNA and was coupled with a decrease in uPAR expression (205,244,245). Similarly, uPA has been shown to be regulated posttranscriptionally by a 30 kD uPA mRNABP which is believed to decrease the stability of the uPA mRNA. Unlike the uPAR mRNABP-uPAR mRNA interactions, the uPA mRNABP associates with a 66 nucleotide region of the 3'-untranlsted region of uPA mRNA (243). In addition to the aforementioned studies which showed primarily a posttranscriptional regulation of uPA and uPAR, Maity et al. demonstrated that hypoxia mediated uPAR induction was controlled both transcriptionally and posttranscriptionally (247). In **chapter IV**, we investigate the effects of IL-2 on NK cell uPA and uPAR, and we examine the mechanisms responsible for the noted results.

Although there has been extensive work done on the uPA system in neutrophils, monocytes, T-cells, and dendritic cells, there has been very little work done on the uPA system in NK cells (248-250). Certainly, to date, there have been no reports linking the uPA system to NK cell invasiveness, particularly through ECM. The invasion into

tumors by adoptively transferred NK cells may strongly rely on the protein composition of the ECM, the proteases produced by the NK cells, and ECM protease inhibitors within the tumor microenvironment (105). Although some ECM proteins can provide a substrate for the migration of NK cells stimulating their approximation to tumor cells (251-254), other ECM proteins and protease inhibitors within the tumor microenvironment may form barriers hindering NK cell infiltration of tumors (255). Regardless of the mechanism of killing by NK cells, whether it entails direct NK-tumor cell or NK-endothelial cell contact followed by the induction of apoptosis or necrosis, or whether it involves the activation of other immune cells within the tumor tissue by NK cell released cytokines, the infiltration of tumors by NK cells appears to be crucial for effective tumor therapy. Similarly, the documented potential of NK cells to be used as vehicles for drug delivery also relies on the successful infiltration of tumors by NK cells. In view of the studies reviewed above, it is likely that understanding the role of the uPA system in NK cell invasion of tumors may be of key importance in deriving strategies to enhance the therapeutic efficacy of NK cells following their adoptive immunotherapy for treatment of cancer metastases.

Specific Aims

1. Identify uPA and uPAR on NK cells and investigate the role of the uPA system in NK cell invasion.
2. Demonstrate the cooperation between uPA and MMPs in NK cell invasion, and examine the effects of various ECM components on NK cell uPA.
3. Investigate the effects and the mechanisms by which IL-2 regulates NK cell uPA and uPAR.
4. Determine the antitumor efficacy of NK cells as drug delivery vehicles, and consider how insights into the uPA system might lead to improvements in drug delivery.

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CHAPTER II

uPA and uPAR Contribute to NK Cell Invasion Through the Extracellular Matrix

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ABSTRACT

Background: The urokinase plasminogen activator (uPA) system has been implicated in cellular invasiveness of tumor cells and immune cells. Herein we provide evidence for the production by natural killer (NK) cells of both uPA and its receptor (uPAR). Materials and Methods: Western blot analysis, RTPCR, casein/plasminogen zymography, and fluorescence microscopy were employed to detect uPA and uPAR on NK cells. NK cell invasiveness was examined using Matrigel invasion assays. Results: NK cell uPA appeared at its characteristic molecular weights, is enzymatically active in casein/plasminogen zymography, and is recognized by monoclonal antibodies. uPAR was detected by RTPCR and fluorescence microscopy. Matrigel invasion assays demonstrated an active role of uPA in NK cell invasion. Conclusion: The uPA system contributes to extracellular matrix (ECM) degradation by NK cells, which may be essential for NK cell accumulation into metastases, and may be prerequisite for their killing of tumor cells following NK cell adoptive transfer.

INTRODUCTION

Natural killer (NK) cells constitute a subgroup of lymphocytes that is distinct from T and B cells. They are spontaneously cytotoxic large granular lymphocytes (LGL) that are involved in natural immunity against virally infected cells and transformed cells without prior sensitization (1,2). NK cells play an important role in controlling various cancers and preventing them from metastasizing (3). Lymphokine activated killer (LAK) cells, which are primarily comprised of activated NK cells (4,5), have shown promise in adoptive immunotherapy protocols to treat various malignancies (6-8). These cells in some studies were capable of an 80-95% numerical reduction of metastases (9).

Following their activation with IL-2, NK cells become adherent to plastic. These adherent, lymphokine-activated killer cells, originally known as A-LAK cells and now termed activated NK (A-NK) cells, are highly cytotoxic against various tumor targets (10). Their adherence to plastic facilitates the isolation and expansion of pure populations of cells with an NK phenotype (5). Interest in the clinical potential of adoptively transferred effector cells has been heightened due to the therapeutic efficacy noted with LAK cells in the treatment of various murine and human tumors (6,11-14).

Basse et al showed that adoptively transferred A-NK cells were clearly localized within murine B16 melanoma lung metastases in a time, IL-2, and dose dependent manner (9) ; furthermore, A-NK cells not only accumulate within established tumor metastases, but also establish cell to cell contact with tumor cells (15). Direct contact between tumor cells and LAK cells *in vitro* can determine the level of tumor-cell lysis

(16,17). It has been shown that the activation of NK cell cytotoxicity depends on the binding of NK cell receptors to their appropriate ligands on tumor cells (18); this close contact determines whether the NK cell lytic machinery is activated or inhibited. The mechanism of tumor cell lysis *in vivo* may also depend on close contact between A-NK cells and tumor cells (15,19). This implies that *in vivo* the number of A-NK cells that reach the tumor site and establish contact with tumor cells might be critical in determining the success of A-NK cell-mediated cancer immunotherapy.

Previous electron microscopy studies of tumor tissue have shown that A-NK cells have the capacity to bind tumor cells and penetrate the basement membrane/extracellular matrix (ECM) as they approach their target cells within cancer metastases (15,20), suggesting an active role for basement membrane degrading proteases in tumor infiltration by A-NK cells. The uPA system is composed of the serine protease urokinase plasminogen activator (uPA), its receptor (uPAR), plasmin, and plasminogen activator inhibitor (PAI). The uPA system plays a role in ECM degradation both directly, by its capacity to degrade glycoprotein components of the basement membrane/ECM (e.g. laminin), and indirectly, by activating matrix metalloproteinases (MMPs) which subsequently degrade type IV collagen (21). uPA has been shown to be crucial in tissue remodeling for invasion, migration, and adhesion in various cell types (22,23). It plays an important role in the migration of the oocyte during the time of ovulation (24), in embryogenesis (25), and in tumor cell invasion and metastasis (26-28). After uPA binds its receptor on the surface of cells, it is effectively converted from an inactive single chain form to an active two-chain protease. Two-chain active uPA then converts inactive

single chain plasminogen to plasmin, the active two-chain form. Plasmin is able to degrade ECM components, as well as activate other proteases such as matrix metalloproteinases, which also play an important role particularly in the degradation of the collagen components of the ECM (21,29-31). It is well documented that these processes enable various types of tumor cells to migrate through the basement membrane/ECM to reach their target sites.

Several reports have indicated the presence of uPA in NK cells (31,32). We have reported the detection of several cell associated and extracellular molecular weight forms of plasminogen activator in NK cells, including atypical 100 kD, 78 kD, 72 kD, 45 kD, 28 kD, and 26 kD forms (31). Carpen et al were also able to detect NK cell uPA primarily in subcellular compartments (32). Herein we confirm and extend these observations, and provide evidence which shows for the first time that uPA and uPAR play an important role in the invasion and migration of NK cells through the ECM. Our data therefore suggests that uPA/uPAR in NK cells may contribute to the *in vivo* ability of adoptively transferred A-NK cells to traverse basement membrane and accumulate in established cancer metastases. This in turn may optimally activate NK cells *in vivo*, which may be prerequisite for tumor cell cytolysis.

MATERIALS AND METHODS

Cells and cell culture. U937 (human monocytic cell line), YT (human non-ATL leukemic cell line, a kind gift from Dr. Porunellor Mathew), and RNK-16 (F344 rat leukemic large granular lymphocytic (LGL) cell line) were cultured in RPMI 1640 with 10% FBS, 55 _M 2-mercaptoethanol, 100 U/ml penicillin, 100 _g/ml streptomycin sulfate, 2 mM glutamine, 0.1 mM MEM nonessential amino acids, and 1mM sodium pyruvate. All cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air at a density of approximately 10^6 cells/ml.

Invasion assay chemoattractant. 3T3 murine fibroblast cell line was grown in DMEM with 10% FBS, 100 U/ml penicillin, 100 _g/ml streptomycin sulfate, 2 mM glutamine. At 80-90% confluency, the 3T3 complete media was removed, the cells were washed 3X with RPMI-1640, and were re-incubated for 24 hours in RPMI-1640 with 0.1% BSA, 100 U/ml penicillin, 100 _g/ml streptomycin sulfate. Following the incubation, the media was removed, centrifuged to remove cell debris, and was filtered though a 0.22 _m filter. Stock 3T3 conditioned media was used as a chemoattractant in the invasion assays.

Cell homogenization. Prior to lysing, the cells were harvested and washed three times in PBS. Cells were then incubated in lysis buffer (10mM Tris HCl pH 7.4, 1% Triton X-100, 0.5% IGEPAL CA-630 (nonionic detergent), 150 mM NaCl, 20 mM NaF, 1mM EGTA, 1mM EDTA) for 15 minutes on ice, with repeated vortexing. Cells homogenates

were then centrifuged at 500 x g for 10 minutes to separate nuclei. The pellet was discarded and the supernatant was removed and stored at -20 °C until later use.

Phosphatidylinositol specific phospholipase C (PtdIns-PLC) Treatment. PtdIns-PLC treatments were performed as described by Ragno et al (33). Briefly, cells were harvested and washed 3 times in RPMI 1640. Cells were then re-suspended at 40 x 10^6 cells/ml in 0.5% BSA-RPMI-1640, and PtdIns-PLC form Sigma (St. Louis, MO) was then added at 1 U/ml. After incubating the cells at 37 °C for 45 minutes, the cells were centrifuged and the uPA/uPAR enriched supernatants were analyzed by Western blot and zymography.

Zymographic analyses. Visualization of protease activity was performed as previously described by Roche et al (34). Briefly, 0.2% w/v casein Hammarsten was added to 10% SDS-PAGE gels with or without 2 mg/ml lys-plasminogen. Sample proteases, phospholipase C extracts, and cell homogenates were then loaded onto the gels. To retain enzymatic activity, the samples were not boiled or reduced prior to loading. Following electrophoresis, the gels were incubated in 2% Triton X-100 in H_2O for about 2 hours at room temperature. The gels were then incubated in 0.1M glycine-NaOH buffer pH 8.3 for 4-6 hours at 37°C and then were stained with 0.025% Coomasie blue G-250, 40% methanol, 7% acetic acid, for 6 hours. The gels were destained for 6 hours in 40% methanol, 7% acetic acid.

Western Blotting. Phospholipase C extracts were subjected to electrophoresis on 10% SDS-polyacrylamide gels under non-reducing conditions. Gels were then electrobloted onto PVDF membranes, which were then blocked for 1 hour with 1% BSA, 2% goat serum in PBS-T (0.5% Tween-20 in PBS). Mouse anti-human uPA monoclonal antibody from Oncogene Research Products (Cambridge, Massachusetts, USA) was used at a final concentration of 2 μg/ml. Goat anti-mouse peroxidase conjugate (Pierce Chemical, Rockford, IL) was used as a secondary antibody for uPA detection. Bands were detected using SuperSignal CL-HRP Substrate System (Pierce Chemical, Rockford, IL). The resulting chemiluminescence was recorded on Hyperfilm ECL (Amersham, Buckinghamshire, England).

Reverse transcriptase (RT)-PCR. Total RNA was isolated from YT and RNK-16 cells using RNeasy columns (Qiagen, Chatsworth, CA). cDNA synthesis was performed using the RT-PCR kit from Stratagene (La Jolla, CA). For each cDNA synthesis, total RNA from NK cells was reverse transcribed using random hexamer or oligo $(dT)_{16}$ primer in a volume of 50 μl each, according to the protocol supplied by Stratagene. The two reactions were combined after heat inactivation of reverse transcriptase, and 2 μl of the cDNA were used for each PCR amplification. PCR primers are described in Table I. cDNA from approximately 200,000 cells was PCR amplified to generate the bands seen in figure 1. Total RNA from freshly isolated NK cells was a kind gift from Dr. Peter Kuppen.

Matrigel invasion assay. The ability of NK cells to traverse basement membrane and degrade ECM components was examined using Matrigel-coated invasion chambers. The assay was performed as previously described (35) with a few modifications. RNK-16 cells grown in culture were harvested and washed three times in RPMI-1640, and were then re-suspended at a concentration of 1.0×10^5 cells/ml in serum free RPMI-1640 media containing 0.1% BSA, 55 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 0.1 mM MEM nonessential amino acids, and 1.0mM sodium pyruvate. Matrigel (11.8 mg/ml Collaborative Biomedical Products, Bedford, MA) was diluted with sterile distilled cold water to 3.0 mg/ml and plasminogen was added to the Matrigel to give a final plasminogen concentration of 500 μg/ml. A 31.18 mm^2, 8 μm pore size transwell filter (Costar, Cambridge, MA) was coated with 10 μl of Matrigel with or without plasminogen, and was allowed to gel and dry at room temperature for 30 minutes. Prior to use, Matrigel covered wells were reconstituted with serum-free RPMI-1640 medium. A total of 50,000 cells in 500 μl were loaded into the top well which contained plasminogen at a final concentration of 500 nM; 750 μl of 24 hour 3T3 conditioned media were added to the lower chamber as a chemoattractant. In one experimental group, aprotinin (Boehringer Mannheim, Indianapolis, IN) was added at a final concentration of 200 μg/ml. Plasminogen-free Matrigel was used to coat the membrane in the control group, and plasminogen was not added to the upper chamber in the control group. The plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. All determinations were performed in triplicate. Photomicrographs were taken at 18 hours using a Spot digital camera (Diagnostic Instruments). To

quantitate the number of cells which had invaded after 48 hours, the non-invading cells in the top well were removed by scrubbing the upper surface of the filter membrane with a cotton swab; invading cells were recovered from the bottom chamber and from the underside of the Matrigel coated membrane by incubating the membrane for 15 minutes in PBS-0.02% EDTA. The recovered cells were counted using a hemocytometer. Invasion index was determined using the following formulas:

$$\% \text{ Invasion} = \frac{\text{Mean number of cells which invaded}}{\text{Mean number of cells which migrated through uncoated transwells}} \times 100$$

$$\text{Invasion Index} = \frac{\% \text{ invasion of experimental cells}}{\% \text{ invasion of control cells}}$$

Fluorescence Microscopy. Cells were harvested and washed three times in PBS. In order to remove uPAR bound uPA, the cells were re-suspended in an acid wash solution for 3 minutes at room temperature as described by Stoppelli et al (36). Cells were washed twice in PBS, spun onto glass slides, and fixed in 10% buffered formalin at 4 °C. After air drying the slides, they were blocked with PBS-0.1% BSA for 2 hours at 4 °C. The slides were then washed twice for 3 minutes in PBS plus 0.05% Tween 80. Staining was performed using 60 nM BODIPY labeled uPA in PBS-0.1% BSA for 2 hours at 4 °C with or without 1.2 μM unlabeled two-chain uPA acting as a competitor to the labeled uPA. The slides were then washed in cold PBS plus 0.05% Tween 80 twice for 3 minutes. After they were air dried, one drop of Vectashield (Burlingame, CA) was placed on the cells, and a cover slip was placed on each slide. The cells were viewed using a FITC filter with a Nikon Microphot FXA fluorescence microscope. Images were

captured using a Roper scientific SenSys cooled slow-scan CCD camera (Phoenix, AZ). Images were enhanced using IPLab software (Scanalytics, Fairfax, VA).

RESULTS

Detection of mRNA for uPA and uPAR. uPA and uPAR mRNA was detected in PCR amplified NK cell cDNA. Figure 1 shows bands indicating the expression of both uPA and uPAR mRNA in YT, RNK-16, and IL-2 stimulated, freshly isolated, human NK cells. The primers used to amplify the cDNA are shown in table I. cDNA from approximately 200,000 cells was used in each PCR reaction.

Detection of uPA associated with cell membranes. Phospholipase C extracts from U937, YT and RNK-16 cells were loaded on 10% SDS PAGE gels. Electrophoresis and immunoblotting were performed as described in Materials and Methods. U937 cells were used as a control for uPA (37,38). Figure 2 indicates the presence of uPA in YT and RNK-16 phospholipase C extracts. Using monoclonal antibody against uPA, we detected a slightly lower M_r for uPA in YT and RNK-16 cells (48-50 kD) than the characteristic M_r for uPA (55 kD) seen in U937 phospholipase C extracts. Furthermore, two bands of an approximate M_r of 83 kD and 26 kD were seen only in YT phospholipase C extracts.

Proteolytic activity of NK cell uPA. To determine the activity of NK cell uPA, zymography using SDS polyacrylamide gels containing casein and plasminogen was performed. Plasminogen-dependent lysis zones were found that coincided with uPA molecular weights, specifically the molecular weights detected in the Western blots described above (figure 3). Plasminogen-free zymography was also performed to

determine whether or not the lysis zones seen were plasminogen dependent (data not shown). Consistent with the Western uPA data for YT and RNK-16 cells, the lysis zones seen for YT and RNK-16 uPA have a slightly lower M_r (48-50 kD) than human recombinant uPA, which displays the characteristic 55kD and 33kD lysis bands. In order to determine whether or not the uPA detected in YT and RNK-16 cell homogenates was uPAR associated, YT and RNK-16 PtdIns-PLC extracts were run along side YT and RNK-16 homogenates on casein-plasminogen zymography (figure 3). The lysis bands seen for YT and RNK-16 PtdIns-PLC extracts had the same M_r (48-50kD) as the lysis zones seen in the cell homogenates.

Detection of uPAR on YT cell membranes. Using fluorescence microscopy with BODIPY labeled uPA as ligand, we were able to detect uPAR on the surface of YT cells (figure 4). U937 cells were used as a control. To determine the specificity of BODIPY-uPA binding to uPAR, unlabeled two-chain uPA was used as a competitive ligand and its use resulted in a dramatic reduction in the fluorescence of control U937 and YT cells (figure 4B).

Role of uPA system in cell invasion. In order to determine whether the uPA system plays a role in NK cell invasion through the extracellular matrix, Matrigel invasion chambers were prepared with or without plasminogen in the matrix. After the addition of plasminogen to the invasion chamber wells, there was a marked increase in RNK-16 cell invasion (figure 5). This invasion was dramatically reduced following the addition of

aprotinin (200 μg/ml), a serine protease inhibitor, emphasizing the importance of the uPA system in the invasion of this cell type. The photomicrographs (figure 5A) were taken 18 hours following the start of the invasion assay. To measure the migratory capacity of the RNK-16 cells, cells were added to uncoated invasion chamber wells and incubated for 2 hours. Approximately 98% of the cells were recovered from the lower chamber indicating the high potential of the RNK-16 cells to migrate in response to 3T3 conditioned media.

DISCUSSION

This report documents for the first time that NK cells utilize both uPA and uPAR for ECM degradation. In addition to identifying both uPA and uPAR in NK cells using enzymatic analysis, protein detection, molecular, and microscopic approaches, we have also investigated the role of the uPA system in NK cell invasion through the ECM. In agreement with our earlier studies with human NK cells (32,39), we detected similar molecular weight species in Western blots for uPA in the RNK-16 and the YT cell lines: 100-105 kD, 79-83kD, 55 kD, 50 kD, and 26 kD.

Our samples were not boiled or reduced prior to loading of the gels in order to retain the structural and functional integrity of uPA which contains two chains held together by a disulfide bond; we therefore believe that the highest uPA band observed on Western blots (100-105 kD) represents either aggregates of various species of uPA or uPA-uPAR aggregates (33). It is also possible that uPA-plasminogen activator inhibitor (PAI) aggregates may also be detected as high molecular weight uPA bands on Western blots. The 79-83 kD band observed in YT cells may be aggregates of various uPA forms, primarily high molecular weight (HMW) uPA which has an M_r of 55 kD, and low molecular weight uPA (LMW) which has a M_r of 33 kD. Lytic bands were not detected on casein/plasminogen zymograms for any uPA species greater than 55 kD which implies that the uPA in these aggregates is inactive. The 55 kD and the 48-50 kD bands were both detected on zymography, with the latter band as the most active form of uPA in YT and RNK-16 cells. As anticipated, we did not find the 26 kD uPA form on zymography

since most commonly the lowest active form of uPA detected is 33 kD. We speculate that this 26 kD form of uPA might be a low yield degradation product of the α–chain of the YT cell-derived uPA, since it also appeared in the phospholipase C extracts.

Two chain active HMW uPA (55 kD) is composed of an α-chain (amino terminal end), and a β-chain (which makes up the majority of the LMW uPA form (33 kD)), held together by a disulfide bond. It has been documented that the amino terminal end of uPA must be present for the enzyme to bind to its phosphatidylinositol anchored receptor uPAR (40,41), which can be cleaved from the cell surface using phospholipase C. Since phospholipase C extracts were used in Western blots for uPA, we presume that all the forms of uPA seen in the Western blots must be bound to the glycolipid anchored receptor uPAR, and therefore must contain the amino terminal end (α-chain) of uPA. In view of the data examining phospholipase C extracts in uPA Western blots and SDS-PAGE zymographies, this is the first documentation of uPA associated with uPAR in NK cells. The RTPCR results clearly show the presence of uPAR and uPA mRNA in freshly isolated NK, YT, and RNK-16 cells. Our results are in contrast to a previous report by Nykjaer et al. (42) which indicated the presence of uPAR, but not uPA in NK cells. Since they failed to detect uPA synthesis in NK cells, they concluded that NK cells relied primarily on uPA synthesized by other cell types.

It has been documented that the uPA system plays an important role in the invasion and migration of tumor cells (27-29,43) and various cells of the immune response including neutrophils (44). In view of our electron microscopy findings showing A-NK cells in contact with tumor cells following basement membrane degradation by the

A-NK cells (15), we hypothesized that A-NK cells may also employ the uPA system, at least in part, for their migration and accumulation into tumor metastases following adoptive immunotherapy. uPA proteolytically converts plasminogen to plasmin, which is then able to degrade ECM components such as laminin and fibronectin. Moreover, uPA-generated plasmin can activate other enzyme systems, such as MMPs (21), for tumor cell invasiveness via degradation of basement membrane type IV collagen. MMPs also appear to contribute to NK cell migration and invasiveness through extracellular matrices (45). Our data documents that NK cell invasion through ECM is augmented by plasminogen (figure 5), and this supports our hypothesis for a role of the uPA system in NK cell invasiveness. Moreover, our findings suggest that the uPA system works in concert with MMPs in ECM degradation (46).

uPAR may be directly involved in the migration of the NK cells as it can localize uPA to the leading edge of the cell, via the so-called invadopodia, as noted in other cell types (47). Components of the uPA system have been detected on the invadopodia of certain cells during invasion and migration (48-50). uPAR may play an important role in increasing directional cellular invasion, allowing NK cells to degrade only ECM components which form a barrier in their path toward target tumor sites.

In regard to cytolysis of tumor cells, increased invasion of A-NK cells into target tissues may be critical for NK cell killing. Previous reports have documented the necessity of cell-cell contact in NK cell-mediated killing (16,17). Indeed NK cells might yield maximal cancer therapy only if they are able to reach and form contacts with their targets (18).

In sum, the urokinase plasminogen activator system appears to have the potential to regulate degradative aspects of NK cell invasion through the ECM and subsequent migration into tumor tissue following NK cell adoptive immunotherapy. This in turn may result in the enhancement of essential NK cell-target cell interactions within tumor metastases. Optimal killing of tumor cells by NK cells within the microenvironment of established metastases may therefore be dependent in part on NK cell uPA-uPAR mediated degradative proteolysis of ECM components.

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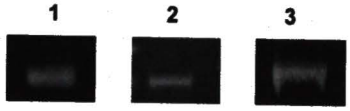
Table I. *Primer sequences used in PCR of NK cell cDNA.*

Primer	**Forward (5' → 3')**	**Reverse (5' → 3')**
Human uPA	AGGAACCCAGACAACCGGAG	GTTATACATCGAGGGCAGGC
Human uPAR	TGTAAGACCAACGGGGATTG	CCTTTGGACGCCCTTCTTCA
Rat uPA	CCATGAGAGTCTGGCTTGCG	CATAGCACCAGGGTCGCCTC
Rat uPAR	ACAGAACGGAGCGTGAAGGA	ATGAGGATAAGATGAGCAGG

Figure 1. uPA and uPAR mRNA detection

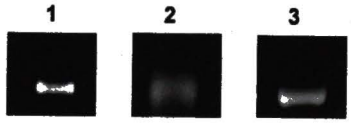
RT-PCR showing uPAR(A) and uPA (B) in freshly isolated (1), YT (2), and RNK-16 (3) cells. mRNA in YT and RNK-16 cells was extracted from approximately 1 X 10^7 cells. Following mRNA extraction, cDNA was generated using reverse transcriptase enzyme, and was then used in PCR. cDNA from 2 x 10^5 cells was used in each PCR reaction.

A



403 BP 403 BP 475 BP

B



549 BP 549 BP 405 BP

Figure 2. Protein detection of uPA in NK cells

Western blot showing uPA in PtdIns-PLC extracts from U937, YT, and RNK-16 cells. Approximately 40 X 10^6 cells (U937,YT, and RNK-16) were incubated in 0.5% BSA-RPMI-1640 plus PtdIns-PLC(1 U/ml) at 37 °C for 45 minutes. The cells were centrifuged and the uPA/uPAR-enriched supernatants were analyzed by Western blot.

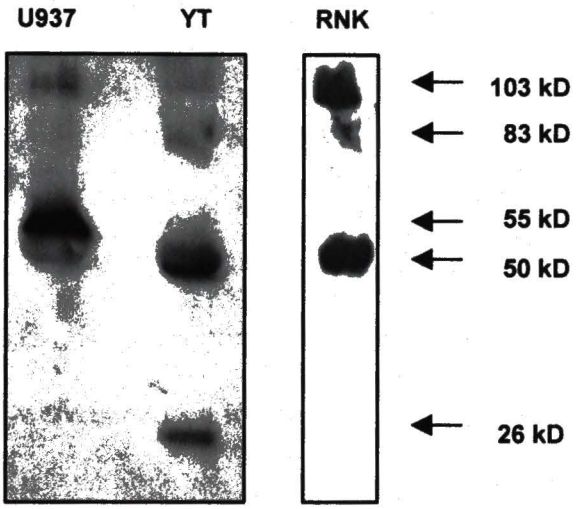

U937
YT
RNK
103 kD
83 kD
55 kD
50 kD
26 kD

Figure 3. Activity of NK cell uPA

Casein + plasminogen zymography showing control uPA (1), YT homogenates (2), YT Phospholipase C extracts (3), RNK-16 homogenates (4), and RNK-16 Phospholipase C extracts (5). The images were enhanced to show contrast between the various lanes.

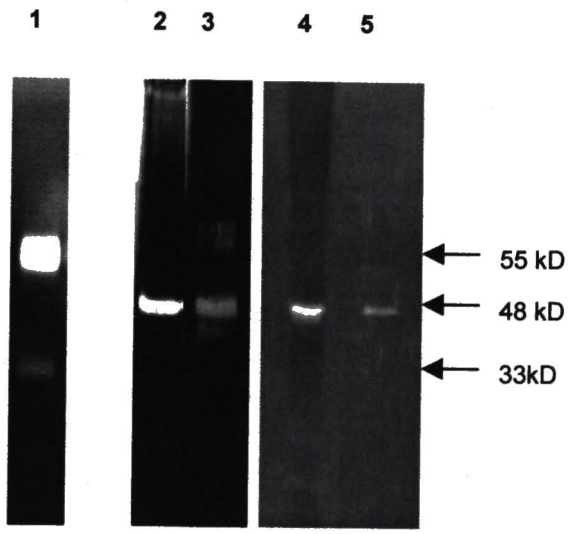

1
2
3
4
5
55 kD
48 kD
33kD

Figure 4. Fluorescence microscopic detection of uPAR on NK cell surface

Photomicrographs showing uPAR on the surface of U937 (control) and YT cells (A). Unlabeled two-chain uPA (1.2 μM) was used as a competitive ligand to the BODIPY-labeled uPA (B). Top figures represent U937 cells. Bottom figures represent YT cells.

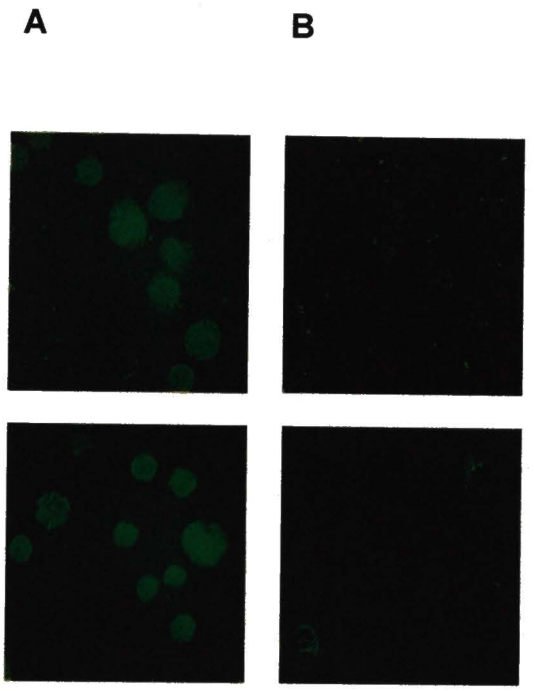

A
B

Figure 5. Invasion of RNK-16 cells under various conditions

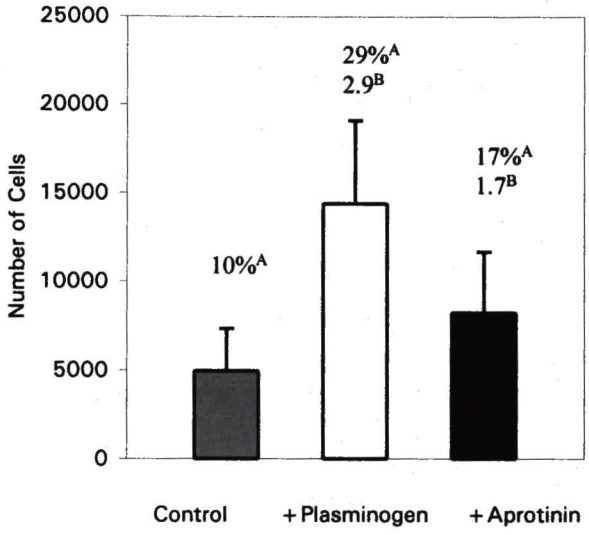
In the control group, cells were examined for their invasive capacity in the absence of plasminogen. In the + plasminogen group, 5μg of plasminogen were added to the Matrigel and 500 nM final plasminogen concentration was added to the upper well. The conditions in the third group are the same as the + plasminogen group with the exception of the added aprotinin (200μg/ml). Photomicrographs (A) showing invading RNK-16 cells on Matrigel covered transmembrane filters were taken 18 hours following the start of the invasion assay. 48 hours following the start of the invasion assay, RNK-16 cells were removed and counted (B). [a]Percent invasion compared to uncoated wells. [b]Invasion index as described in the methods. Statistical significance was obtained between all three groups using Anova and Tukey's tests, $p < 0.05$. Each bar represents the average of triplicate determinations (±SD)

A

Control

+Plasminogen

+ Aprotinin


25000
20000
15000
10000
5000
0
Number of Cells
10%A
29%A
2.9B
17%A
1.7B
Control
+ Plasminogen
+ Aprotinin

CHAPTER III

Cooperation of Urokinase Plasminogen Activator and Matrix Metalloproteinases in NK Cell Invasion

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Key Words: Urokinase Plasminogen Activator (uPA), Matrix Metalloproteinases, Aprotinin, BB-94.

Running title: uPA / Plasmin and MMPs in NK cell invasion

ABSTRACT

We have previously investigated the role of the urokinase plasminogen activator (uPA) system in NK cell invasion. We have also studied NK cell- derived matrix metalloproteinases (MMPs) in extracellular matrix (ECM) degradation. We now report that both enzyme systems cooperate in NK cell invasion. Zymographic analyses detected uPA in RNK-16 cell conditioned media (CM) with the same molecular weights as the uPA we have previously shown to be associated with the rat NK cell urokinase plasminogen activator receptor. The combination of aprotinin, an inhibitor of plasmin, and Batimastat (BB94), an inhibitor of MMPs, in Matrigel invasion assays showed a more potent inhibitory effect on NK cell invasion than either inhibitor alone. Finally, a down regulation of uPA mRNA was noted following RNK-16 stimulation with collagen IV, fibronectin, and laminin.

INTRODUCTION

Immunotherapy using adoptively transferred lymphokine activated killer (LAK) cells has shown promise for the treatment of various forms of cancer (1-8). Activated natural killer (A-NK) cells, a sub-population of LAK cells which become adherent to plastic following stimulation with the lymphokine IL-2, have been shown to be the primary cells responsible for the tumoricidal activity exhibited by LAK cells (9,10). Following their adoptive transfer into tumor bearing animals, A-NK cells have been shown to localize within tumor metastases (11) and establish cell to cell contact with tumor cells (12). Electron microscopy studies have shown that A-NK cells actively penetrate components of the basement membrane/extracellular matrix (ECM) that form barriers in their path toward their target tumor cells. Basement membrane/ECM degradation requires the concerted effort of a variety of proteases that can degrade ECM proteins such as laminin, fibronectin, and collagen types I and IV. Such proteases are used by a variety of cells as they invade and migrate through tissues.

The uPA system and MMPs have been shown to be involved in the invasion of a variety of tumor cell types (13-16). The uPA system is composed of the neutral serine proteases uPA and plasminogen/plasmin, uPAR (uPA receptor), and plasminogen activator inhibitor (PAI). uPA is a highly specific protease that is secreted in a low activity single chain form (scuPA) which has approximately 5% the activity of the two-chain form of the enzyme (tcuPA) (17). The most efficient substrate for uPA is plasminogen (18), which following its conversion to its active form, plasmin, acquires the

enzymatic capacity to cleave a variety of ECM components including fibronectin, laminin, and type IV collagen (19,20). The MMPs constitute a family of proteases that have as substrates a very broad spectrum of ECM components. In addition to the plasmin substrates mentioned above, the MMPs have the ability to degrade a variety of glycoproteins, elastin, proteoglycans, and the majority of collagens (21).

There are a number of ways in which the uPA system and the MMPs interact. During ECM degradation, the cleavage of glycoproteins by plasmin works in concert with the degradation of the collagen components of the ECM by MMPs (22,23). Plasmin appears to have a regulatory role in MMP activation due to its capability to cleave the prodomain of MMP zymogens, specifically proMMP-1, and proMMP-3 (21). Plasmin also has been shown to activate MMP-2 and MMP-9 (24). On the other hand, MMP-3 has been shown to interact with uPA by cleaving the receptor binding domain of uPA (25,26). Moreover, plasmin can regulate MMP activity by its ability to degrade inhibitors of MMPs such as TIMP-2 (21).

In HT1080 fibrosarcoma cells, Ikeda et al (27)showed an augmentation of the anti-invasive effects of a serine protease inhibitor following the addition of an MMP inhibitor. Recently we have reported that NK cells employ uPA and uPAR (Al-Atrash et al., manuscript submitted for publication), and MMPs in their invasion (30,31). In this report, we demonstrate the cooperation of these two enzyme systems in NK cell invasion. We also show the ability of the ECM to regulate the uPA system of NK cells, as has been shown in other cell types (32).

MATERIALS AND METHODS

Cells and Cell culture.

U937 (human monocytic cell line), and RNK-16 (F344 rat leukemic large granular lymphocytic cell line) were cultured in RPMI 1640 with 10% FBS, 55 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 2 mM glutamine, 0.1 mM MEM nonessential amino acids, and 1mM sodium pyruvate. The 3T3 murine fibroblast cell line was grown in DMEM with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 2 mM glutamine. At 80-90% confluency, 3T3 complete media was removed, the cells were washed 3X with RPMI-1640, and were re-incubated in RPMI-1640 with 0.1% BSA, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate for 24 hours. Following the incubation, the media was removed, centrifuged to remove cell debris, and was filtered with a 0.22 μm filter. Stock 3T3 conditioned media (CM) was stored at -80 °C and was used as needed as a chemoattractant in the invasion assays. All cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air at a density of approximately 10^6 cells/ml.

ECM stimulation.

Approximately 10^7 cells were cultured in laminin, fibronectin, and collagen IV coated 25 cm^2 flasks (Becton Dickinson Labware, MA) in OPTI-MEM serum free media (GibcoBRL Life Technologies, MD). After 24 hours of incubation, adherent cells

(approximately 3 x 10^6 cells) were harvested, washed twice with RPMI-1640 media, and then used for RNA extraction as described below.

Preparation of conditioned media (CM).

U937 and RNK-16 cell conditioned media was prepared following 24 hour culturing of approximately 40 x 10^6 cells in OPTI-MEM serum free media (GibcoBRL Life Technologies, MD, USA). The CM was removed from the cell culture flasks and centrifuged at 350 x g to remove cell debris. The pH of the CM was then adjusted to a pH of 3.5 to inactivate protease function. CM was concentrated approximately 30x using Amicon (Beverly, MA, USA) Centriprep-10 concentrators. Aliquots were frozen at –80°C.

Zymographic analyses.

Visualization of protease activity was performed as previously described in Roche et al and Wasserman et al (33,34). Briefly, 0.2% w/v casein Hammarsten was added to 10% SDS-PAGE gels with or without 10 μg/ml lys-plasminogen. Sample proteases in the CM were then loaded onto the gels. To retain enzymatic activity, the samples were not boiled or reduced prior to loading. Following electrophoresis, the gels were incubated in 2% Triton X-100 in H_2O for about 2 hours at room temperature. The gels were then incubated in 0.1M glycine-NaOH buffer pH 8.3 for 4-6 hours at 37 °C and then were stained with 0.025% Coomasie blue G-250, 40% methanol, 7% acetic acid, for 6 hours. The gels were destained for 6 hours in 40% methanol, 7% acetic acid.

Fluorogenic substrate assays

Human plasmin or human uPA (American Diagnostica inc., CT) were preincubated at 37 °C for 30 minutes with or without either 10 μM aprotinin (Boehringer Mannheim, Indianapolis, IN), a plasmin inhibitor, 10 μM 1,5 dansyl-Glu-Gly-Arg-chloromethylketone (CMK) (Calbiochem, La Jolla, CA), a uPA inhibitor, or 10 μM of Batimastat (BB-94) (a generous gift of British Biotech, Oxford, U.K.). The specific plasmin substrate N-Succinyl-Ala-Phe-Lys-7-Amido-4-Mehtylcoumarin (AMC) (Sigma, MO), and the specific uPA substrate Cbz-Gly-Gly-Arg-7-Amido-4-Methylcoumarin (AMC) (Sigma, MO) (35) were used to determine plasmin and uPA activities respectively. 100 μl of enzyme or enzyme-inhibitor mixtures were aliquoted into a 96 well Costar plate (Corning Inc., NY) to give a final enzyme concentration of 37.5 ng/well. This was followed by the addition of 100 μl of 20 μM substrates. Cleavage of plasmin or uPA substrate was detected immediately using a CytoFluor II fluorescent plate reader (PerSeptive Biosystems, Framingham, MA) using an excitation wavelength of 360 nm and an emission wavelengths of 460 nm. Fluorogenic assay reaction buffer (0.1 M Tris-HCl, pH 8.0, 0.1% Triton X-100) was used to dilute all the enzymes and substrates.

Matrigel invasion assay.

The ability of NK cells to traverse basement membrane and degrade ECM components was examined using Matrigel-coated invasion chambers. The assay was performed as previously described (36) with a few modifications. RNK-16 cells grown in culture were harvested and washed three times in RPMI-1640, and were then re-suspended at a

concentration of 1.0 x 10^5 cells/ml in serum free RPMI-1640 media containing 0.1% BSA, 55 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 0.1 mM MEM nonessential amino acids, and 1.0mM sodium pyruvate. Matrigel (11.8 mg/ml Collaborative Biomedical Products, Bedford, MA) was diluted with sterile distilled cold water to 3.0 mg/ml and plasminogen was added to the Matrigel to give a final plasminogen concentration of 500 μg/ml. A 31.17 mm^2, 8 μm pore size transwell filter (Becton Dickinson, MA) was coated with 10 μl of Matrigel with or without plasminogen, and was allowed to gel and dry at room temperature for 20 minutes. Prior to use, Matrigel covered wells were reconstituted with serum-free RPMI-1640 medium. A total of 500 μl (50,000 cells) were loaded into the top well which contained plasminogen at a final concentration of 500 nM, and 750 μl of 24 hour 3T3 conditioned media was added to the lower chamber as the chemoattractant. Aprotinin (75 μg/ml) and/or BB-94 (1.0 μM) were added to the top and lower wells. The plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. To quantitate the number of cells which had invaded after 48 hours, the non-invading cells in the top well were removed by scrubbing the upper surface of the filter membrane with a cotton swab; invading cells were recovered from the bottom chamber and from the underside of the Matrigel coated membrane by incubating the membrane for 15 minutes in PBS-0.02% EDTA. The recovered cells were counted using a hemocytometer. All determinations were performed in triplicate.

Reverse transcriptase (RT)-PCR.

Total RNA was isolated from RNK-16 cells using RNeasy columns (Qiagen, Chatsworth, CA). cDNA synthesis was performed using the RT-PCR kit from Stratagene (La Jolla, CA). For each cDNA synthesis, total RNA from NK cells was reverse transcribed using random hexamer or oligo $(dT)_{16}$ primer in a volume of 50 µl each, according to the protocol supplied by Stratagene. The two reactions were combined after heat inactivation of reverse transcriptase, and 2 µl of the cDNA were used for each PCR amplification. PCR primers are described in Table I. cDNA from approximately 60,000 cells was PCR amplified to generate the bands seen in figure 5.

RESULTS

Proteolytic activity of NK cell uPA.

RNK-16 CM was analyzed for uPA activity using casein zymography. Plasminogen containing casein gels were used, and plasminogen dependent lysis zones were detected in the CM that coincided with uPA molecular weights determined previously for RNK-16 membrane-associated uPA (Al-Atrash et al., manuscript submitted for publication). Plasminogen-free zymography gels were also run to determine whether or not the lysis zones seen were plasminogen dependent (data not shown). Figure 1 shows plasminogen dependent lysis zones in CM of U937 cells, which were used as control cells, at an M_r of 50-52 kD. Another low molecular form of uPA is apparent in U937 CM at an M_r of 43 kD. RNK-16 uPA was detected at its characteristic M_r of 48-50 kD.

BB-94 effects on uPA and plasmin.

To determine whether or not BB-94 had any inhibitory effects on the proteolytic components of the uPA system, specifically the serine proteases uPA and plasmin, fluorogenic substrate assays using the uPA selective substrate Cbz-Gly-Gly-Arg-AMC (35) and the plasmin selective substrate Suc-Ala-Phe-Lys-AMC were performed. As shown in figures 2 and 3, BB-94 appears to have no inhibitory effects on either ability of uPA or plasmin to cleave their specific substrates. Preincubation of uPA with 10 μM 1,5 dansyl-Glu-Gly-Arg-Chloromethylketone (CMK) (Calbiochem, La Jolla, CA), a highly

selective uPA inhibitor (37,38), prior to the addition of the substrate completely ablated the catalytic function of uPA. Similar inhibition was seen following the preincubation of plasmin with 10 μM aprotinin.

Cooperation of the uPA system and the MMPs in NK cell invasion.

Matrigel invasion assays were performed to investigate the roles of the uPA system and MMPs in NK cell invasion. As shown in Figure 4, Following the addition of 1.0 μM BB-94 to the wells, approximately 57% of the cells were able to invade as compared to uninhibited control wells. On the other hand, following the addition of 75 μg/ml of aprotinin, approximately 23% of the cells were able to invade. The combination of both inhibitors almost completely diminished RNK-16 cell invasion, allowing only 6% of the cells to invade.

Regulation of uPA by the ECM.

Following the stimulation of RNK-16 cells with ECM proteins, there is an apparent down-regulation of uPA mRNA by laminin, fibronectin, and collagen IV. Fibronectin appears to down-regulate uPA mRNA to a lesser extent than either laminin or collagen IV (figure 5). All results were compared to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (figure 5).

DISCUSSION

We have previously documented the accumulation of A-NK cells within tumor metastases following their adoptive transfer (11,12). We have also shown that A-NK cells establish cell to cell contacts with target tumor cells prior to lysis (12). These findings suggest that A-NK cells have the ability to penetrate the subendothelial basement membrane, and degrade various ECM components within the tumor interstitium as they approximate their target tumor cells.

The cooperation between the uPA system and MMPs in cellular invasion, specifically in cancer metastasis, has been clearly demonstrated (21,27) In this report, we show additivity of the anti-invasive effects of the serine protease inhibitor aprotinin with the addition of the MMP inhibitor BB-94. This demonstrates for the first time the cooperation of the uPA system and the MMPs in NK cell invasion through the ECM (figure 4). We have previously documented the presence of various MMPs in NK cell conditioned media, and we have also noted the role the MMPs play in NK cell invasion through the ECM (30,31). Herein we document the presence of uPA in NK cell conditioned media at an M_r of 48-50 kD, consistent with the molecular weights that have been previously noted for NK cell uPA (Al-Atrash et al., manuscript submitted for publication, 28, 29). After ruling out any inhibitory effects of BB-94 on uPA and plasmin (figures 2 and 3), we used BB-94 in combination with aprotinin and observed almost a complete ablation of RNK-16 cell invasion (i.e. less than 10% of the invasion compared to control wells) (figure 4), which can be attributed to the independent

inhibition of the plasmin and MMPs by aprotinin and BB-94 respectively. The large inhibitory effects of aprotinin alone on RNK-16 invasion was expected due to the presence of plasminogen in all the invasion wells, thus amplifying the effects of the uPA system. Indeed, we have previously noted a significant increase in RNK-16 invasion following the addition of plasminogen to the invasion wells (Al-Atrash et al., manuscript submitted for publication), presumably due to uPA mediated plasminogen activation.

In addition, we observed a down regulation of uPA mRNA following the stimulation of RNK-16 cells with the ECM proteins laminin, fibronectin, and collagen IV (figure 5). The decrease in uPA mRNA following laminin and fibronectin stimulation is noteworthy in view of the major role the uPA system plays in the degradation of both laminin and fibronectin. However, since MMPs have also been shown to degrade laminin and fibronectin (21), the noted down-regulation of uPA following stimulation with laminin and fibronectin may be a way by which NK cells can maintain fine control over ECM degradation, and thereby prevent the massive degradation of ECM proteins within tumor interstitium which in turn might facilitate tumor metastasis.

In conclusion, we have noted that *in vitro*, NK cells appear to rely on both the uPA system and on MMPs for their degradation of the ECM. We have also observed the regulation of uPA in NK cells by the ECM components laminin, fibronectin, and collagen IV. In sum, our studies suggest that ECM degrading enzymes of both the uPA and MMP systems contribute to the NK cell degradation of the ECM, a process which is most likely a prerequisite for the accumulation of adoptively transferred A-NK cells into established tumor metastases.

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Table I

Primer sequences used in PCR of RNK-16 cell cDNA

Primer	**Forward (5' →3')**	**Reverse (5' →3')**
Rat uPA	CCATGAGAGTCTGGCTTGCG	CATAGCACCAGGGTCGCCTC
Rat uPAR	ACAGAACGGAGCGTGAAGGA	ATGAGGATAAGATGAGCAGG
Rat GAPDH	ACCCCCAATGTATCCGTTGT	TACTCCTTGGAGGCCATGTA

Figure 1. Zymographic analysis of RNK-16 and U937 uPA

Casein-plasminogen zymography revealing uPA lysis zones. Lane 1 shows U937 CM which was used as a control. Lane 2 shows RNK-16 CM. The same samples were analyzed on plasminogen free gels to confirm the plasminogen dependency of the apparent lysis zones.

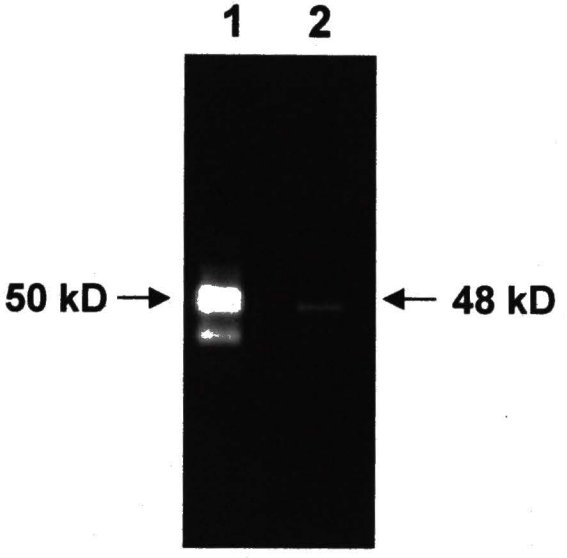

1
2
50 kD
48 kD

Figure 2. Effect of BB94 on fluorogenic substrate cleavage by uPA

Fluorogenic substrate assay showing cleavage of uPA specific substrate Cbz-Gly-Gly-Arg-AMC (10 μM) by uPA (37.5 ng/well). Enzyme samples were preincubated with either BB94 (10μM) or Dansyl-Glu-Gly-Arg-CMK (10 μM) for 30 minutes at 37 °C.

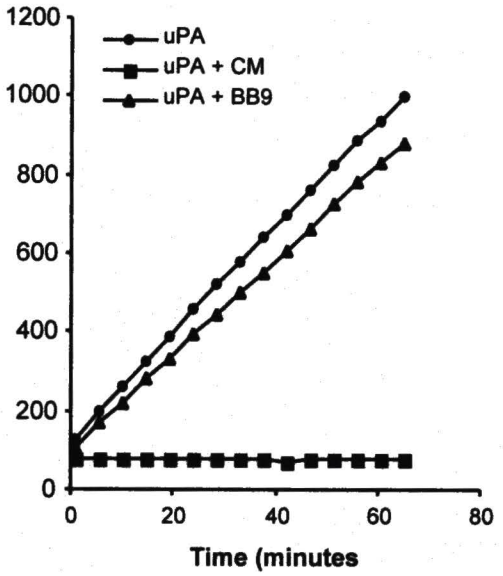

1200
1000
800
600
400
200
0
uPA
uPA + CM
uPA + BB9
0
20
40
60
80
Time (minutes

Figure 3. Effect of BB94 on fluorogenic substrate cleavage by plasmin

Fluorogenic substrate assay showing cleavage of plasmin substrate Suc-Ala-Phe-Lys-AMC (10 μM) by plasmin (37.5 ng/well). Enzyme samples were preincubated with either BB94 (10μM) or aprotinin (10μM) for 30 minutes at 37 °C.

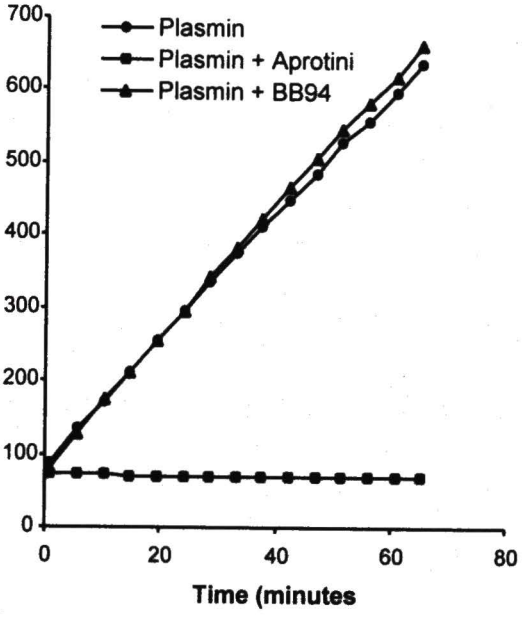

Plasmin
Plasmin + Aprotini
Plasmin + BB94
700
600
500
400
300
200
100
0
0
20
40
60
80
Time (minutes

Figure 4. Effects of aprotinin and BB94 on RNK-16 cell invasion

Invasion assay demonstrating the role of the uPA system and the MMPs in RNK-16 cell invasion. BB-94 (1.0 μM) and aprotinin (75 μg/ml) were added either alone or in combination to the invasion assays. Results are expressed as percentages of untreated control invasion. Experimental significance was determined using ANOVA statistical analysis, $p<0.05$. Tukey's pairwise comparisons confirmed significance between all three groups.

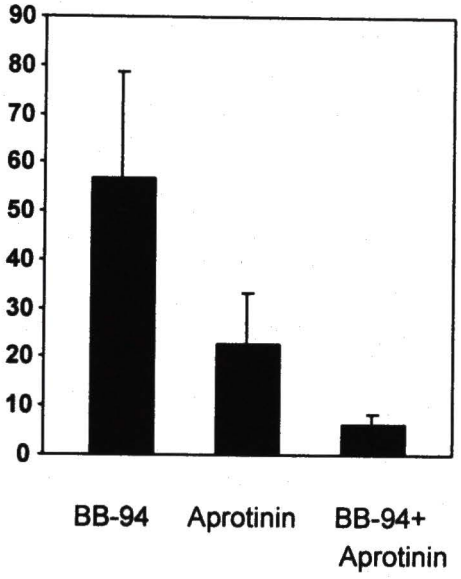
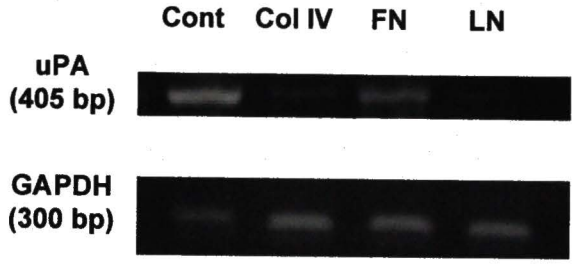

90
80
70
60
50
40
30
20
10
0
BB-94
Aprotinin
BB-94+
Aprotinin

Figure 5. RTPCR analysis of uPA regulation by ECM components

RTPCR showing uPA mRNA expression in un-stimulated control (Cont) RNK-16 cells, and following the stimulation of RNK-16 cells with collagen IV (Col IV), fibronectin (FN), and laminin (LN). GAPDH was used to account for variations in the amount of mRNA extracted.


Cont
Col IV
FN
LN
uPA
(405 bp)
GAPDH
(300 bp)

CHAPTER IV

IL-2 Mediated Upregulation of uPA and uPAR in Natural Killer Cells

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Key Words: NK cells, Urokinase, Interleukin-2, cytokines, gene regulation.

ABSTRACT

Urokinase plasminogen activator (uPA) and its receptor uPAR play a major role in immune cell-mediated, including natural killer (NK) cell-mediated, degradation of extracellular matrices. Herein, we investigate the effects of IL-2 on NK cell uPA and uPAR. RNA and protein analyses showed upregulation of uPA and uPAR following IL-2 stimulation. Gel-shift assays and Western blots detected uPA and uPAR mRNA binding proteins (mRNABPs), previously shown to destabilize uPA and uPAR mRNA. Following IL-2 stimulation, a downregulation of uPAR mRNABP and a reciprocal induction of uPAR mRNA were noted. The increase in uPA following IL-2 stimulation appeared to be more transcriptionally regulated. These data suggest that IL-2 upregulates both uPA and uPAR in NK cells through posttranscriptional as well as transcriptional mechanisms, partially explaining increases in NK cell invasiveness following IL-2 stimulation.

INTRODUCTION

The urokinase plasminogen activator (uPA) system has been shown to play a major role in the extravasation and migration of leukocytes into areas of inflammation (1, 2). In the processes of inflammation, as leukocytes invade into diseased tissues and approach their target cells, various proteases such as uPA, matrix metalloproteinases (MMPs), human leukocyte elastase, and cathepsin G, each displaying a selective specificity for components of the extracellular matrix (ECM), cooperate to degrade ECM proteins that form barriers between the leukocytes and their target cells (3-7). The uPA system is capable of directly degrading glycoprotein components of the ECM such as laminin and fibronectin, as well as interacting with MMPs and their inhibitors in the degradation of various other ECM components (8, 9). One unique feature of the uPA system is the presence of a specific cellular receptor for uPA; uPAR, which is involved in cell migration and invasion independent of its role in the proteolytic pathways (8). uPAR has been shown to be involved in cellular adhesion by its ability to bind to the ECM protein vitronectin (10, 11), and has the capacity to initiate secondary signaling pathways through its interactions with the integrins, thereby promoting cellular movement and migration (12-14). Recently, we have demonstrated the expression of both uPA and uPAR by NK cells, have shown that NK cells employ the uPA system in their *in vitro* invasion through Matrigel (15), and have reported an increase in NK cell invasion through Matrigel following stimulation with IL-2 (16).

NK cells have been shown to exhibit high antitumor properties which has allowed for their successful use in cancer immunotherapy (17-20). Prior to their administration into tumor bearing animals, NK cells are stimulated with IL-2, one of the most potent activators of NK cells, and are termed activated NK (A-NK) cells. NK cells, including the human NK cell line (YT) used in our studies, have been shown to express both the high and moderate affinity IL-2 receptors, and to respond to IL-2 stimulation by increasing their proliferation and cytotoxicity toward other cells (21-23).

Although there have been numerous studies on the MMPs of IL-2 activated NK cells (16, 24, 25), to date there has been very little research on the effects of IL-2 on NK cell uPA and uPAR, and the concomitant effects on NK cell invasiveness. Understanding the effects of IL-2 on NK cell invasiveness is crucial for optimizing NK cell antitumor adoptive immunotherapy. NK cells must be able to traverse the basement membrane (BM)/ECM barriers and establish cell-cell contact with their target cells for effective cytolysis (26). Although the activation of NK cell cytotoxicity by IL-2 has been well established (23, 27-30), the effects of IL-2 on NK cell proteolytic enzymes, and hence their capacity to invade the BM/ECM following IL-2 stimulation, must be addressed to improve the therapeutic efficacy of A-NK cancer immunotherapy.

It has been shown that in lung fibroblasts, mesothelial, epithelial, and carcinoma cells, the regulation of uPA and uPAR expression occurs at a posttranscriptional level through mRNA binding proteins (mRNABPs) that destabilize uPA/uPAR mRNA following mRNA-mRNABP binding. In the case of uPA, a 30 kD protein was detected that binds to 66 nucleotides in the uPA mRNA 3'-untranslated region (UTR) (31).

Likewise, the regulation of uPAR was also shown to be mediated through the interaction of uPAR mRNA with a 50 kD protein that bound to a 51 nucleotide fragment of the uPAR mRNA coding region (32-34).

In this report we demonstrate an increase of both uPA and uPAR following stimulation of NK cells with IL-2. uPA and uPAR mRNABPs were detected in NK cells, and the noted increases in uPA and uPAR following IL-2 stimulation correspond to changes in uPA and uPAR mRNA-mRNABP interactions. Herein we document for the first time the presence of posttranscriptional regulation of both uPA and uPAR in NK cells following IL-2 stimulation.

MATERIALS AND METHODS

Cells and Cell culture

YT cells (human non-ATL leukemic cell line, a kind gift from Dr. Porunelloor Mathew, University of North TX Health Science Center (35)) were cultured in RPMI 1640 with 10% FBS, 55 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 2 mM glutamine, 0.1 mM MEM nonessential amino acids, and 1 mM sodium pyruvate. All cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air at a density of approximately 10^6 cells/ml.

Reverse transcriptase (RT)-PCR

Total RNA was isolated from YT cells using TRI Reagent-chloroform (Molecular Research Center, Inc., Cincinnati, OH). cDNA synthesis was performed using the RT-PCR kit from Stratagene (La Jolla, CA). For each cDNA synthesis, total RNA (5 μg) from NK cells was reverse transcribed using random hexamer or oligo $(dT)_{16}$ primer in a volume of 50 μl each, according to the protocol supplied by Stratagene. The two reactions were combined after heat inactivation of reverse transcriptase, and 2 μl of the cDNA were used for each PCR amplification. uPAR primers (5'-3') forward CCAATGGTTTCCACAACGA and reverse GGTCACACAGCAAGTCTGTA, uPA primers (5'-3') forward GTGGCCAAAAGACTCTGAGG and reverse

ATTTTCAGCTGCTCCGGATA, and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers (5'-3') forward TAGACGGGAAGCTCACTGGC and reverse AGGTCCACCACCCTGTTGCT were added to the PCR reactions at a final concentration of 30 μM. The reactions were carried out using HotStart Taq polymerase (Qiagen, Valencia, CA).

Ammonium sulfate precipitation

Approximately $1x10^8$ cells were serum starved for 12 hours in OPTI-MEM serum free media (Gibco BRL Life Technologies, MD, USA), and stimulated with IL-2 (1000 IU/ml) for 3, 6, and 12 hours. At each time point, the cells were harvested and sonicated in 10 ml of dialysis buffer (2.5mM Tris-HCl pH 7.9, 0.05mM EDTA pH 7.45, 0.1 mM PMSF). Cell lysates were centrifuged at 30,000 xg for 15 minutes at 4 °C. The pellet was discarded and to the supernatant solid $(NH_4)_2SO_4$ crystals (Sigma, St. Louis, MO) were added to yield a final $(NH_4)_2SO_4$ saturation of 40%. The $(NH_4)_2SO_4$ -supernatant mixture was incubated for 30 minutes at 4 °C with continuous stirring. The 40% $(NH_4)_2SO_4$ solution containing precipitated proteins was centrifuged at 30,000 xg for 15 minutes at 4 °C. The pellet containing proteins was discarded, solid $(NH_4)_2SO_4$ crystals were added to the 40% $(NH_4)_2SO_4$ supernatant to yield a final $(NH_4)_2SO_4$ saturation of 60%, and again the 60% $(NH_4)_2SO_4$ supernatant was incubated for 30 minutes at 4 °C with continuous stirring. The 60% $(NH_4)_2SO_4$ solution containing precipitated proteins was centrifuged at 30,000 xg for 15 minutes at 4 °C. The supernatant was discarded and

the 40-60% $(NH_4)_2SO_4$ precipitated proteins were collected, redissolved, and exhaustively dialyzed against extraction buffer containing 10% glycerol.

Cytosolic Protein Extraction

Approximately 5 x 10^7 cells were harvested, washed 3X with PBS, resuspended in 5 ml of hypotonic buffer (10mM Tris-HCl pH 7.5, 1.5 mM MgCl2, 10 mM KCl, 1mM PMSF), and kept on ice for 15 minutes. The cells were centrifuged at 500 xg for 8 minutes, and resuspended in 200-400 μL of hypotonic solution. The cells were lysed using a motorized homogenizer, and were centrifuged at 6000 xg for 8 minutes. The post-nuclear supernatants were collected and used as the cytosolic extracts following protein estimation.

Cell Homogenization

Approximately 5 x 10^7 cells were harvested, washed 3X with PBS, and resuspended in 300 μL of lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl pH 7.4, 15% glycerol, 1 mM Na_3VO_4, 1 mM EDTA, and 1 mM PMSF. Cells underwent 5 freeze/thaw cycles using dry ice and 37 °C with continuous vortexing. Cell lysates were then centrifuged at 1000 xg for 8 minutes. The pellet was discarded and the supernatants were used in western blot analyses.

Western Blotting

Cell homogenates and 40-60% $(NH_4)_2SO_4$ precipitated protein extracts were electrophoresed on 10% SDS-polyacrylamide gels under non-reducing conditions. Gels were then electroblotted onto nitrocellulose membranes, which were then blocked for 1 hour with 1% BSA in wash buffer containing 0.6% w/v NaCl, 10 mM Tris-HCL pH 7.4, 0.025% Tween-20. For uPA detection, mouse anti-human uPA monoclonal antibody (American Diagnostica, Greenwich, CT) was used at a final concentration of 66 pg/ml. For uPAR mRNABP detection, guinea pig anti-rabbit uPAR mRNABP polyclonal antibody was used at a final concentration of 100 ng/ml. Goat anti-mouse peroxidase conjugate (Pierce Chemical, Rockford, IL) and goat anti-guinea pig peroxidase conjugate (Rockland, Gilgertsville, PA) were used as secondary antibodies. The resulting chemiluminescence was recorded on Fuji film (Medical Systems U.S.A. Inc, Stamford, CT).

Gel mobility shift assays

RNA-protein binding assays were performed using ^{32}P uniformly-labeled transcripts corresponding to the uPA mRNA 3'-untranslated region (UTR) (31-33). ^{32}P-labeled uPA transcripts (30,000 cpm) were incubated with the cytosolic extracts (50 μg) in 15 mM KCl, 5 mM $MgCl_2$, 0.25 mM EDTA, 0.25 mM dithiothreitol (DTT), 12 mM HEPES, pH 7.9, 10% glycerol, and *E. coli* tRNA (200 ng/μL) in a total volume of 60 μL at 30 °C for 30 minutes. To degrade free ^{32}P-labeled mRNA, reaction mixtures were then treated with 50 units of RNase T_1 (Life Technologies Inc., Grand Island, NY) for 30 minutes at 37 °C.

Heparin (final concentration 5 mg/ml) (Sigma, St. Louis, MO) was added to the reaction mixtures and incubated at room temperature for 10 minutes to eliminate nonspecific protein binding. Samples were loaded on to 5% native polyacrylamide gels with 0.25 X TBE running buffer and separated by electrophoresis. The gels were then dried and autoradiographed at -70 °C using Kodak X-AR film (Eastman Kodak, Rochester, NY).

Steady-state mRNA assessment by transcription chase

Following 12 hours of serum starvation in OPTI-MEM serum free media, the cells in the experimental group were stimulated with IL-2 (1000 U/ml) for 12 hours in OPTI-MEM, while those in the control group were incubated in OPTI-MEM for 12 hours. Actinomycin-D (Sigma, St. Louis, MO) was then added to the cell cultures at a final concentration of 10 μg/ml, to inhibit ongoing transcription. RNA was extracted as described above immediately following the addition of actinomycin-D and at times 3, 6, 12, and 24 hours following the addition of actinomycin-D. The RNA was then reverse transcribed and PCR amplified using the uPA primers shown above.

RESULTS

Effects of IL-2 on uPA expression.

The effects of IL-2 on uPA mRNA expression was studied using RT-PCR and Western blotting. Stimulation of YT cells with IL-2 for 3, 6, 12, and 24 hours resulted in a time-dependent increase in uPA mRNA as detected by RT-PCR (Figure 1A). The maximal uPA induction occurs at 12 hours following IL-2 stimulation, and very little upregulation is seen between 12 and 24 hours. Western blot analysis using anti-uPA monoclonal antibody was used to show the effects of IL-2 on uPA protein level (Figure 1B). Consistent with the RT-PCR data, there also appears to be a time dependent upregulation of uPA protein levels with a peak induction 12 hours following IL-2 stimulation.

Effects of IL-2 on uPA mRNABP.

We investigated the presence of uPA mRNABP in YT cells to determine whether or not the uPA mRNA-mRNABP interactions caused the noted increase in uPA expression following IL-2 stimulation. YT cytosolic extracts were analyzed using gel shift assays to determine the effects of IL-2 on uPA mRNA-mRNABP interactions. Following the stimulation of YT cells with IL-2 (1000 U/ml), the capacity of the uPA mRNABP to bind to uPA mRNA decreases (Figure 2). This decrease appears to be time

dependent with a maximum decrease occurring 24 hours following IL-2 stimulation. Cold competition assays using 200-fold molar excess unlabeled probe confirmed the specificity of the uPA mRNA-mRNABP interactions (data not shown).

Decay of uPA mRNA.

Transcription chase experiments were conducted to determine the effects of transcriptional inhibition on uPA mRNA stability. Figure 3 compares the degradation of uPA mRNA in the IL-2 stimulated cells with the uPA mRNA degradation in unstimulated cells. The half-life of uPA mRNA following IL-2 stimulation (≈7 hrs) appears to be slightly longer than the half-life in the unstimulated group (≈5 hrs).

Effects of IL-2 on uPAR mRNA.

Figure 4 demonstrates the effects of IL-2 on uPAR mRNA of YT cells. Cells were serum starved in OPTI-MEM serum free media for 12 hours. Following IL-2 (1000 U/ml) stimulation for 3, 6, 12, and 24 hours, RNA was extracted and RT-PCR was conducted as described above. The results (Figure 4) show a time-dependent increase in the uPAR mRNA compared with unstimulated cells (time 0). GAPDH levels were examined to control for variations in the RNA used in the RT-PCR.

Effects of IL-2 on uPAR mRNABP.

Western blot analysis was used to determine whether or not YT cells expressed uPAR mRNABP, and to investigate the effects of IL-2 on uPAR mRNABP. Following the stimulation of YT cells with IL-2 (1000 U/ml), Western blot analysis of protein samples precipitated from the 40%-60% $(NH_4)_2SO_4$ fraction, which has been shown to contain the uPAR mRNABP in human pleural mesothelial cells (33), was utilized to determine if the noted increases in the uPAR mRNA following IL-2 stimulation of YT cells coincided with changes in the expression of uPAR mRNABP. Consistent with the uPAR mRNA increases noted via RT-PCR (Figure 4), Western blot analysis demonstrate a time dependent decrease in the level of expression of uPAR mRNABP following IL-2 stimulation. We also found similar results with gel mobility shift assays.

DISCUSSION

The uPA system appears to play a major role in the accumulation of immune cells into inflamed pathologic tissues (1, 2). IL-2 activated NK cells have shown potential for use in antitumor therapy (17-19). Although IL-2 is primarily known for its ability to activate NK cell-mediated cytotoxicity (23, 27-30, 36), in the present study we report an increase in both uPA and uPAR mRNA and protein following stimulation of NK cells with IL-2, possibly contributing to the previously observed increases in NK cell invasion through Matrigel following IL-2 stimulation (16).

Previous reports have shown that uPA and uPAR are regulated posttranscriptionally through interaction of uPA and uPAR mRNA with their respective destabilizing mRNABPs (31-34). In this report, we document the presence of both uPA and uPAR mRNABPs in NK cells. The uPA mRNABP was detected in cytosolic extracts of YT cells. The interaction of the uPA mRNABP decreased with time following YT cell stimulation with IL-2 (Figure 2). In spite of the changes detected in uPA mRNABP levels, transcription chase experiments showed that the upregulation of uPA by IL-2 is primarily controlled transcriptionally, although a slight posttranscriptional component is also noted. This dual regulation of components of the uPA system is not unique to NK cells. Maity et al. have also detected both transcriptional and posttranscriptional regulation of uPAR mRNA following hypoxia in human MCF7 breast carcinoma cells (37). As demonstrated by Western blot analysis (Figure 5), there appears

to be a downregulation of uPAR mRNABP expression following IL-2 stimulation. This downregulation of uPAR mRNABP may be a major contributor to the noted increase in uPAR mRNA following IL-2 stimulation of YT cells (Figure 4). Even though we infer that IL-2 downregulates the production of uPAR mRNABP by YT cells, we can not exclude other mechanisms such as structural modifications to the uPAR mRNABP that could also contribute to the noted increase in uPAR mRNA following IL-2 stimulation.

Our data indicate that the posttranscriptional regulation of uPAR in YT cells following IL-2 stimulation appears to be controlled at least in part through destabilizing mRNABP interactions. Furthermore, in contrast to T-lymphocyte posttranscriptional regulation of uPAR mRNA, the destabilizing protein detected in YT cells has been shown to interact with nucleotides within the uPAR mRNA coding region (195-246), rather than the AU rich motif of the uPAR mRNA 3'UTR (32, 34). An understanding of these events may allow for the design of better approaches for cell-mediated immunotherapy, e.g. for the development novel approaches that will allow the generation of potent NK cells without the use of toxic immunostimulants and cytokines (e.g. Il-2) for their activation.

In view of reports showing the capacity of ECM proteins and protease inhibitors within the tumor microenvironment to form barriers preventing NK cell entry into tumors (38, 39), we speculate that under such conditions, modifying uPA and/or uPAR mRNABP levels in NK cells prior to their administration, can potentially lead to enhanced tumor infiltration by NK cells, and consequently more effective tumor destruction. Since the infiltration of NK cells into tumor tissues may be required for

effective antitumor therapy, and since NK cell-target cell contact may be essential for NK cell killing (26, 40-42), increasing NK cell invasiveness prior to NK cell administration for cancer immunotherapy, by upregulating uPA and/or uPAR, may lead to increased tumor infiltration and eradication by NK cells.

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Figure 1. Effects of IL-2 on uPA expression.

A, RT-PCR showing an increase in uPA mRNA following 3, 6, 12, and 24 hours of stimulation with IL-2 (1000 U/ml). GAPDH was used to account for the variations in the mRNA extracted. *B*, Western blot analysis demonstrating the effects of IL-2 stimulation on the uPA protein levels in YT cells at 3, 6, 12, and 24 hours. β-actin protein levels are used as loading controls. Time 0 refers to unstimulated control cells. Experiments were performed in duplicate. Con = 2.0 μg uPA positive control (American Diagnostica, Greenwhich, CT).

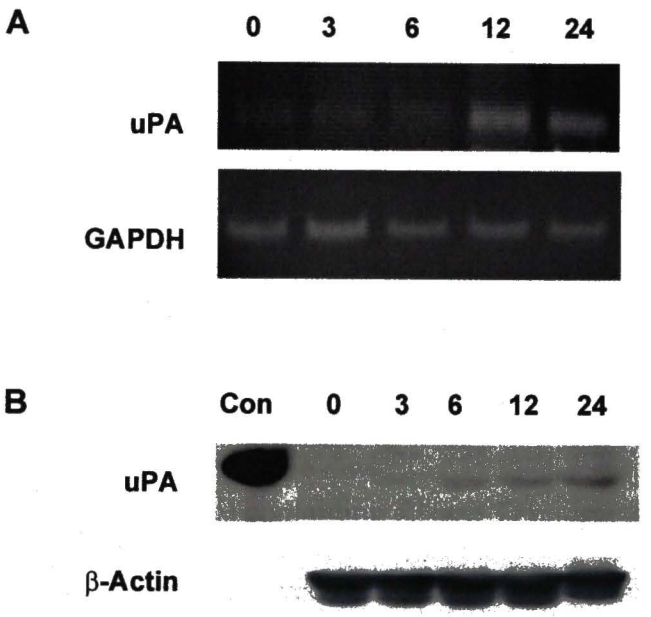

A
0
3
6
12
24
uPA
GAPDH
B
Con
0
3
6
12
24
uPA
β-Actin

Figure 2. uPA mRNA-mRNABP interactions.

YT cytosolic extracts (50 μg) containing RNase T_1 resistant ^{32}P-labeled uPA mRNA-mRNABP complexes were resolved on a 5% polyacrylamide gels, dried, and autoradiographed. The bands reflect the degree of uPA mRNA-mRNABP interactions following 3, 12, and 24 hour IL-2 (1000 U/ml) stimulation compared with control unstimulated cells (time 0). FP refers to free probe without any sample. Experiments were performed in duplicate.

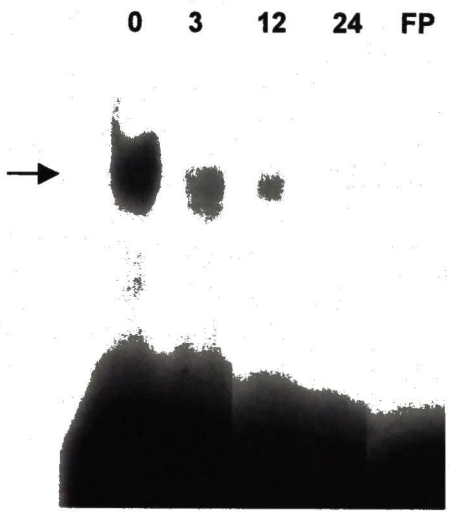
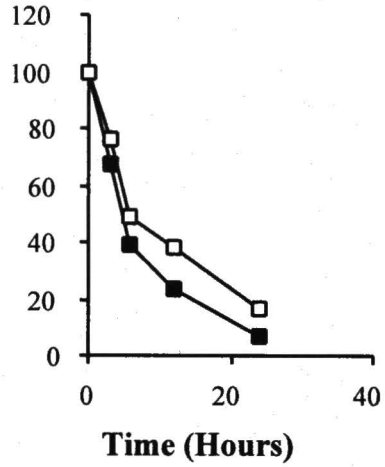

0
3
12
24
FP

Figure 3. Stability of uPA mRNA following IL-2 stimulation.

Following serum starvation, IL-2 (1000 U/ml) stimulation, and actinomycin-D treatment, RNA was extracted and RT-PCR was conducted. *A,* Data was transformed and graphed over 24 hours to compare the rate of uPA mRNA decay following IL-2 stimulation (□) to control unstimulated cells (■). *B,* RT-PCR showing the decay of the uPA mRNA over time 3, 6, 12, and 24 hours following actinomycin-D treatment. GAPDH shows equal amounts of RNA used in the RT-PCR. Experiments were performed in triplicate.

A


120
100
80
60
40
20
0
0
20
40
Time (Hours)

B

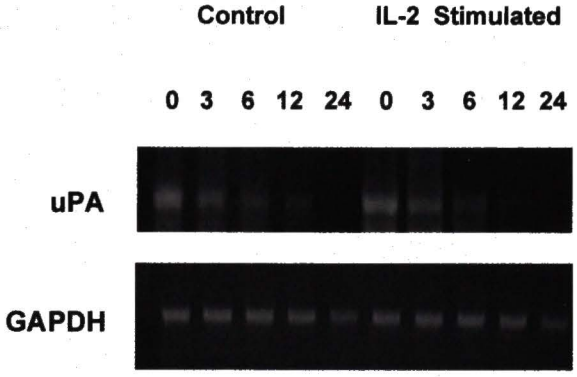

Control
IL-2 Stimulated
0 3 6 12 24 0 3 6 12 24
uPA
GAPDH

Figure 4. Effects of IL-2 on uPAR expression.

Cells were serum starved for 12 hours, and were then stimulated with IL-2 (1000 U/ml) for 3, 6, 12, and 24 hours. RNA was extracted and RT-PCR was used to analyze the uPAR mRNA levels following IL-2 stimulation. Time 0 refers to unstimulated control cells. GAPDH was used to account for the variations in the mRNA extracted. Experiments were performed in duplicate.

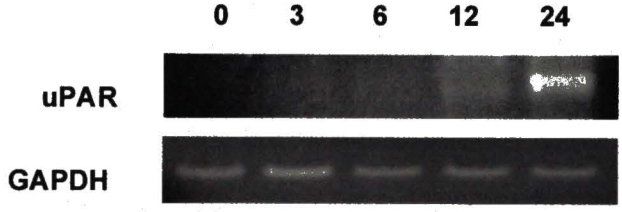
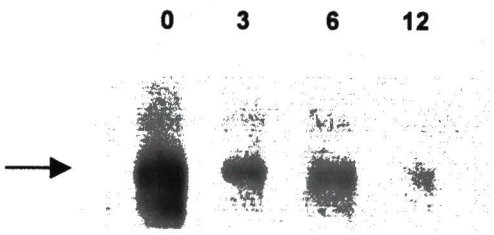

0
3
6
12
24
uPAR
GAPDH

Figure 5. Effects of IL-2 on uPAR mRNABP.

Western blot analysis showing the effects of IL-2 on uPAR mRNABP expression. Protein samples (20 μg) were precipitated from YT cell 40%-60% $(NH_4)_2SO_4$ fractions 3, 6, and 12 hours following IL-2 stimulation. Time 0 refers to control unstimulated cells. Experiments were performed in duplicate.


0
3
6
12

CHAPTER V

A NOVEL DRUG DELIVERY SYSTEM USING IL-2 ACTIVATED NK CELLS AND ZYN-LINKED DOXORUBICIN

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Running Title: A-NK cell drug delivery

Type of Study: Experimental

Keywords: NK cells, drug delivery, doxorubicin

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ABSTRACT

Adoptively transferred IL-2 activated NK (A-NK) cells selectively accumulate within tumor metastases which recommends them as vehicles for locoregional drug delivery. Zyn-Linkers® are membrane-binding lipophilic dyes which can be coupled by a variety of conjugation chemistries to therapeutic agents. We have previously demonstrated that A-NK cells labeled with PKH26 are able to accumulate within established B16 melanoma pulmonary metastases by 16 h at a concentration of over 600 cells/mm^2 of tumor tissue (Basse et al. *J. Exp. Med.* 174:479 1991). Zyn-205 is the same molecule conjugated to doxorubicin with an acid-sensitive bond. We have optimized the *ex vivo* labeling conditions and found that a 10 min incubation with 25 μM Zyn-205 results in the uptake of over 10^8 drug molecules per cell with no effect on either cell viability or cytolytic activity up to 24 h after labeling. Given these parameters, the amount of drug which may be carried to and concentrated in metastatic lesions represents a local concentration of ~15 μM. In addition, A-NK cells carrying doxorubicin at an equivalent dose of 25 μg/kg was therapeutically comparable to a systemic dose of 8 mg/kg in the 3LL model of experimental metastasis . These data indicate that A-NK cells bearing Zyn-linked chemotherapeutic agents represent a unique and feasible method to target chemotherapeutic agents to cancer metastases.

INTRODUCTION

Adoptively transferred IL-2 activated natural killer (A-NK) cells have the capacity to infiltrate tumor metastases (1,2). Therefore, A-NK cells in addition to their direct or indirect cytolytic effector mechanisms might also constitute a novel delivery vehicle for the locoregional delivery of anti-tumor drugs. This might be accomplished by either attaching such drugs to the surface of A-NK cells, or loading them within A-NK cells, to allow for selective delivery to established micrometastatic lesions (1,2). Indeed, standard LAK cells have already been employed to deliver ricin or 4'-deoxy-4'-iododoxorubicin (IDX) to animals bearing metastases (3,4). IDX-loaded LAK cells used for delivery to tumor-bearing mice led to a significant reduction in the number of lung metastases in contrast to control mice given injections of higher doses of free drug (4). Another recent study has also attempted to simply load A-NK cells with methoxymorpholinyl doxorubicin for delivery to hepatic metastases (5). These results indicate that A-NK cells are suitable for use as drug delivery vehicles.

Zyn-Linkers® are membrane-binding lipophilic dyes linked to a fluorescent head-group which can be coupled by a variety of conjugation chemistries to therapeutic agents. We have previously used the fluorescent Zyn-linker PKH26 to localize A-NK cells within B16 melanoma pulmonary metastases in mice (1). These dyes are readily observable in metastases 24 hours after adoptive transfer of labeled A-NK cells. Using these dyes coupled to therapeutic agents would enable direct delivery of these agents into tumor metastases at concentrations which could exceed that obtainable by systemic

means. This might obviate the need for systemic delivery of toxic doses of chemotherapeutic anticancer drugs and allow for efficient non-toxic locoregional drug delivery of anticancer agents to the site of advanced cancer yielding enhanced nontoxic therapy of established cancer metastases.

MATERIALS AND METHODS

Animals: Specific pathogen-free C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME) and housed in a specific pathogen free animal facility.

Reagents and Chemicals: Tissue culture medium and fetal bovine serum were obtained from Gibco (Grand Island, NY). Recombinant interleukin-2 and polyethylene glycol IL-2 were generous gifts of the Chiron Corp. (Emeryville, CA). All other reagents were of the highest available commercial purity.

Preparation and Labeling of Mouse A-NK Cells: Mouse A-NK cells were prepared from the spleens of C57BL/6 mice as previously described (6). After 6 days of culture the cells were removed from the tissue culture flasks by the addition of 0.02% EDTA, washed and resuspended in 0.25 M sucrose + 5 mM HEPES (pH 7.3) at a concentration of 10^7 cells/ml. For labeling purposes these cells were mixed with an equal volume of Zyn-205 in the same buffer. After incubation for 10 min the reaction is quenched by the addition of a 10 fold excess of media containing 10% serum. The cells were then centrifuged and washed 3x with media.

Tumor Cell and A-NK Cell Injection: B16 melanoma or Lewis lung carcinoma (3LL) tumor cells were injected into the tail veins of C57BL/6 at a concentration of 1-2 x 10^5 cells/mouse on day 0. On day 4 or day 8 25-35 x 10^6 A-NK cells labeled with or without Zyn-205 were injected into the tail vein of tumor bearing mice in a final volume of 0.2 ml of RPMI containing 120,000 IU of rh-IL-2. Subsequently 120,000 IU of PEG IL-2 was administered i.p. directly following the adoptive transfer of A-NK cells. Mice

with day 8 metastases were sacrificed 16 h later, their lungs removed and snap frozen in hexane. Sections were cut and digitized images of tumors were obtained. Mice with day 4 tumors were followed for an additional 45 days at which time all remaining mice were sacrificed. Animals which showed signs of distress during the experiment were sacrificed and their death was recorded as occurring one day later.

RESULTS

Effect of Zyn-205 on A-NK cell function

Zyn-205 (see structure in Figure 1) is a Zyn-linker coupled to doxorubicin through an acid sensitive bond. We have used this prototype to determine that the binding of this molecule to the A-NK cell plasma membrane does not effect either the functionality or viability of A-NK cells. In order to examine the effect of the binding of Zyn-205 on A-NK cell functionality, changes in viability, proliferative capacity and cytolytic activity were examined. There was no change in *in vitro* viability over 48 h after labeling of A-NK cells with Zyn-205 (controls and cells labeled with 10 - 50 μM Zyn-205 were ~85% viable at 48 h). Cytolytic activity was measured 24 h after labeling with 25 μM Zyn-205. Control A-NK cells exhibited 74% lysis against Yac-1 targets at an effector: target ratio of 25:1, while A-NK cells labeled with Zyn-205 gave 88% lysis under the same conditions. In addition there was no change in proliferative capacity of A-NK cells over a 48 h time period after labeling with Zyn-205 (Figure 2).

To determine the optimal concentration of Zyn-205 for labeling A-NK cells, cells were labeled using 10, 25, 50 and 75 μM Zyn-205. Some of the cells were analyzed immediately; however, the remainder were returned to culture and analyzed at 3, 6, 24 and 48 h for the retention of Zyn-205 (Figure 3). Concentrations higher than 25 μM Zyn-205 showed a rather steep decline in bound Zyn-205 at 3 h. At 24 h, there was little difference among the 3 higher concentrations (25, 50 and 75 μM). Therefore 25 μM was chosen as the working concentration for future studies.

Localization of Zyn-205 labeled A-NK cells in tumor metastases:

To determine whether A-NK cells labeled with Zyn-205 could still accumulate within tumor metastases, labeled cells were adoptively transferred into mice bearing 8 day B16 melanoma pulmonary metastases. After 16 h the animals were sacrificed and the lungs removed, sectioned and examined by fluorescent microscopy. As shown in Figure 4 A-NK cells labeled with Zyn-205 have the capacity to accumulate within pulmonary B16 melanoma metastases. Examination of a number of sections yielded an average number of A-NK cells per tumor area of 51. If the average volume of the tumor area in each section is 55×10^{-5} µl and the average number of molecules of Zyn-205 per cell is 10^8, then the concentration of doxorubicin delivered to the tumor would be ~15 µM. This compares favorably with a tumor concentration of 0.5-5 µM when doxorubicin is delivered systemically.

Therapeutic efficacy of Zyn-205 labeled A-NK cells:

In order to determine whether Zyn-205 labeled A-NK cells would be therapeutic against pulmonary metastases, tumor-bearing mice were treated with a therapeutic dose of doxorubicin (8 mg/kg), A-NK cells alone and A-NK cells bearing Zyn-205. After treatment, animals were followed for 45 days. At the end of that time the remaining animals were sacrificed. As shown in Figure 5, animals treated with A-NK cells bearing Zyn-205 compared favorably with doxorubicin administered at 8 mg/kg and was more effective than A-NK cells alone.

DISCUSSION

These studies have demonstrated the capacity of A-NK cells to deliver doxorubicin to tumor cells. Moreover, our preliminary evidence indicates that this treatment modality has some therapeutic efficacy in the 3LL model of experimental metastasis. Previous studies have indicated that LAK cells and A-NK cells can deliver doxorubicin and its derivatives therapeutically into tumors; however these studies passively loaded the chemotherapeutic agents into cells (4,5). The present technique has advantages over the passive loading method in a number of areas. First, the amount of loaded drug may be more accurately controlled and also the amount of drug bound to cells may be accurately quantitated particularly for those agents which are neither fluorescent nor have significant absorbance outside of the UV. Second, the use of Zyn-linker to bind the chemotherapeutic agent to the cells will enable the use of agents which are toxic to the A-NK cells themselves. Finally, the Zyn-linker coupling chemistry allows for the release of the drug under specific conditions, e.g. in the presence of a specific enzyme. These factors make the use of Zyn-linkers a superior method for loading A-NK cells with chemotherapeutic agents. This current study has proved the feasibility of this technique by demonstrating (a) that A-NK cells retain their viability and cytolytic activity when bound to Zyn-205; (b) that A-NK cells carrying Zyn-205 can still localize in tumor metastases and; (c) that A-NK cells bearing Zyn-205 are therapeutic for 3LL tumor metastases.

Doxorubicin, although a very effective anticancer agent, has to date been limited

in its usefulness by severe cardiotoxicity at higher concentrations (7). It is therefore difficult to use this particular agent in an aggressive manner without careful monitoring of both total dose and cardiac function. The dosage of doxorubicin coupled to the Zyn-linker and injected with the A-NK cells amounted to approximately 25 μg of doxorubicin/kg of mouse body weight. This is far less than the dose of 8 mg/kg which was administered to the doxorubicin alone treatment group; however it was equal in therapeutic efficacy. Our calculations indicated that the concentration of doxorubicin delivered to a tumor by A-NK cells would be in the order of 15 μM which assumes that all of the doxorubicin is released at once. Although this in probably not the case, this treatment was equivalent to a systemic dose of 8 mg/kg. Since the total amount of doxorubicin administered is significantly less when delivered by cells, it should be possible to administer multiple rounds of doxorubicin via this route and thereby avoid the dose limiting cardiotoxicity of doxorubicin. Finally modulation of the linkage connecting doxorubicin to the Zyn-linker may enable speedier release of doxorubicin once the A-NK cells reach the tumor. These possibilities as well as a direct comparison of A-NK cells ligated to doxorubicin with liposome encapsulated doxorubicin are currently under investigation in our laboratory.

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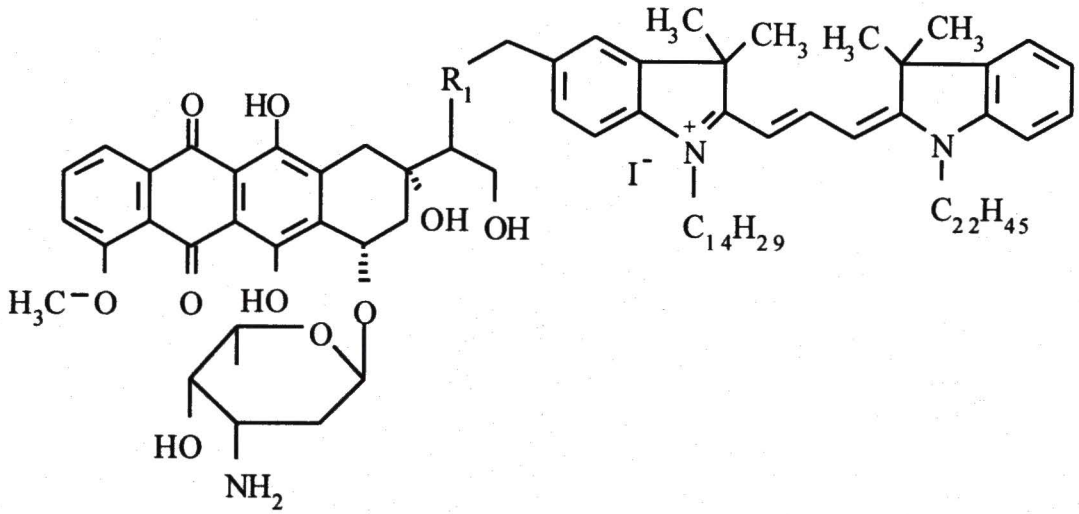
Schwartz P, Gottschalk A, Zaret BL: Serial assessment of doxorubicin cardiotoxicity with quantitative radionuclide angiocardiography. N Engl J Med 300:278-83, 1979.

Figure 1. Structure of Zyn-205. R_1 represents an acid sensitive linkage.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CH3
H3C
CH3
R1
O
HO
OH
OH
I-
N+
N
C14H29
C22H45
H3C−O
O
HO
O
O
HO
NH2

Figure 2. Effect of Zyn-205 on the proliferative capacity of A-NK cells. Approximately 10^7 cells were incubated with the indicated concentrations of Zyn-205, washed and placed back into culture. At 3, 6, 24 and 48 h, a small aliquot of cells was removed and counted.

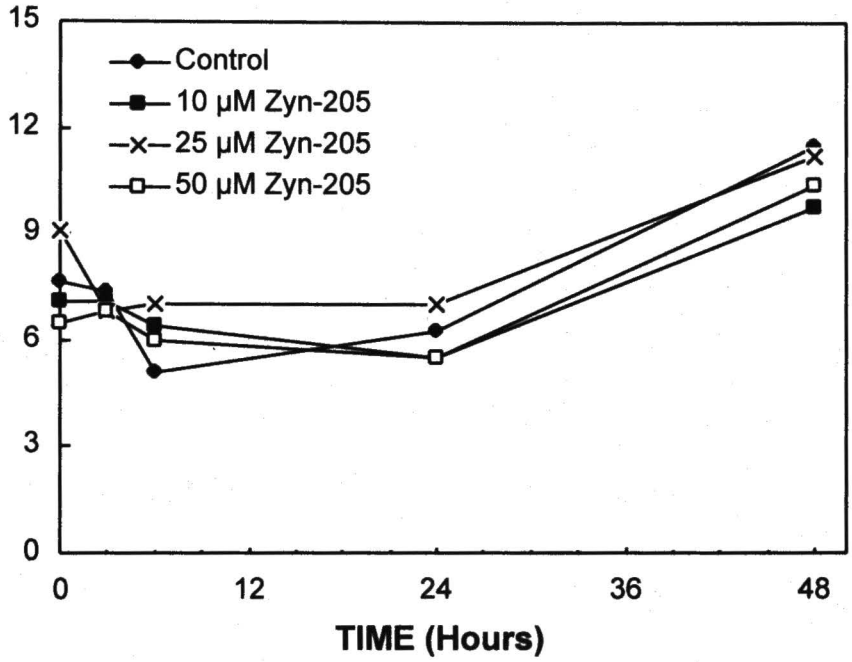

15
12
9
6
3
0
Control
10 µM Zyn-205
25 µM Zyn-205
50 µM Zyn-205
0
12
24
36
48
TIME (Hours)

Figure 3. Retention of Zyn-205 by A-NK cells over time. A-NK cells were incubated with the indicated concentrations of Zyn-205, washed and returned to culture. At 0, 3, 6, 24 and 48 h, an aliquot of the cells was removed and the amount of Zyn-205 bound to the cells was determined.

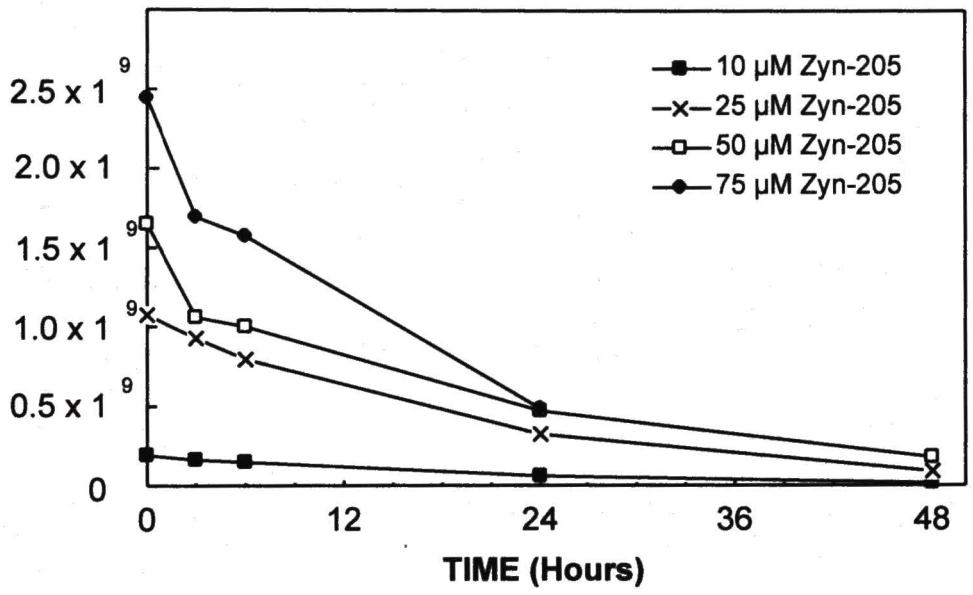

10 µM Zyn-205
25 µM Zyn-205
50 µM Zyn-205
75 µM Zyn-205
2.5 x 1 9
2.0 x 1
1.5 x 1 9
1.0 x 1 9
0.5 x 1 9
0
0
12
24
36
48
TIME (Hours)

Figure 4. Accumulation of A-NK cells labeled with Zyn-205 into mouse B16 melanoma metastases. A-NK cells labeled with Zyn-205 were injected into mice bearing 8 day B16 melanoma metastases. After 16 h, the mice were sacrificed and the lungs removed, snap frozen and sectioned. **A.** Differential interference contrast image. **B.** Fluorescence image. **C.** Overlay

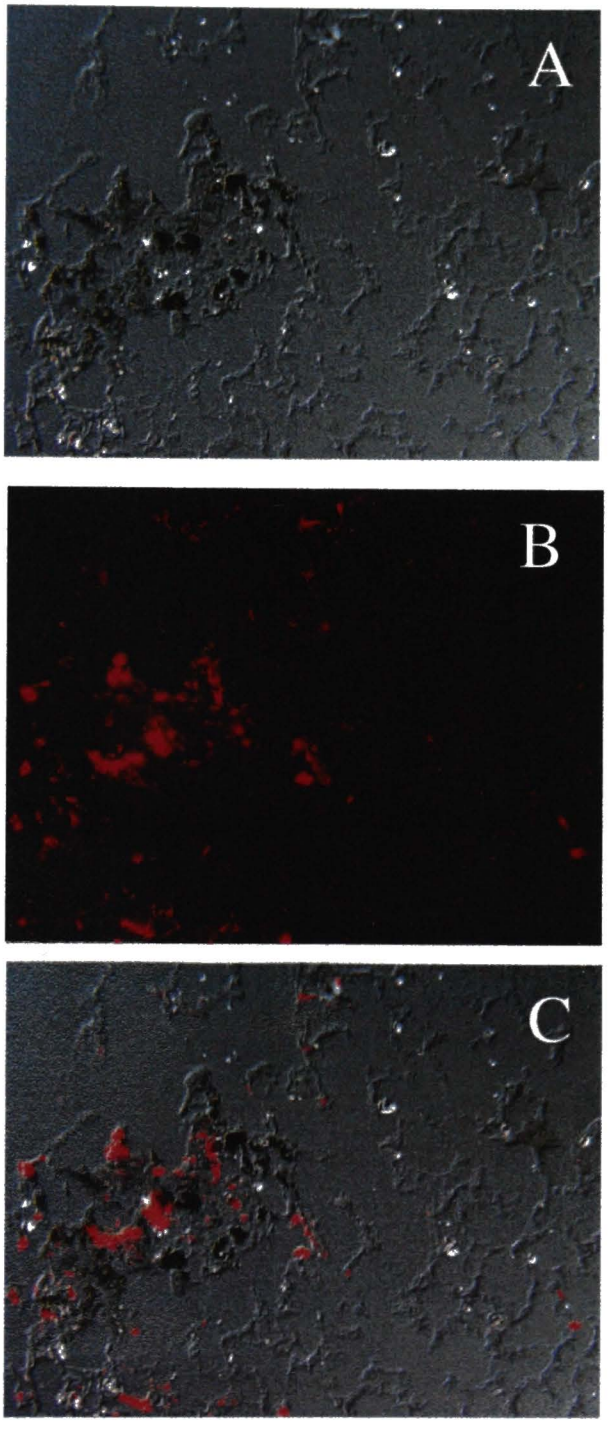
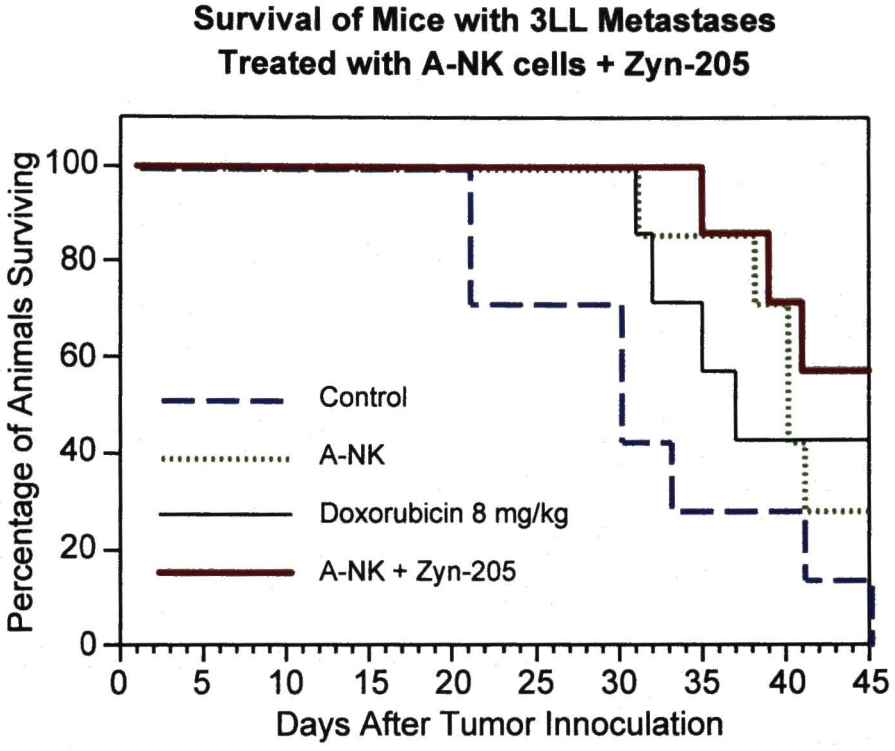

A
B
C

Figure 5. Therapy of 3LL lung metastases using adoptively transferred A-NK cells bearing Zyn-205. Animals were injected with 3LL tumor cells (2 x 10^5 cells per mouse) on day 0. Therapy was started on day 4 . Groups of 7 animals were dosed with either vehicle alone, doxorubicin at 8 mg/kg, A-NK cells or A-NK cells bearing Zyn-205.


Survival of Mice with 3LL Metastases
Treated with A-NK cells + Zyn-205
Percentage of Animals Surviving
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
Days After Tumor Innoculation
Control
A-NK
Doxorubicin 8 mg/kg
A-NK + Zyn-205

CHAPTER VI

CONCLUSION

Standard cancer therapy using conventional protocols consisting of chemotherapy, surgery, radiotherapy, and combinations of the three, has improved the survival of many patients afflicted with cancer, and in a many cases has even led to cures (1,2). The limitations encountered by these forms of cancer therapy--such as host toxicity and tumor resistance to treatment, as is the case for chemotherapy and radiotherapy, and anatomical barriers rendering tumors inaccessible to surgery--has emphasized the urgency for the development of other forms of therapy for the treatment of cancer. Although tremendous progress has been encountered in the improvement of the prognosis and the survival of cancer patients following treatment, there has been much less success achieved in curing and completely eradicating this disease. In the year 2000, following many decades of intensive and diligent research in the cancer field, it is predicted that approximately 552,200 Americans will die of cancer, more than 1,500 people a day. This haunting statistic highlights the need for further cancer research to address novel forms of cancer therapy offering different approaches to handling and attacking this disease.

Immunotherapy using exogenously activated immune cells has shown promise in cancer therapy. Although clinical trials conducted throughout the last two decades

showed immunotherapy to offer little or no great advantages over standard methods of cancer therapy (3), research in the field of cancer immunotherapy is still in its infancy and far from reaching its peak impact. Given our only fragmentary and incomplete knowledge to date, the efficacy achieved using anti-tumor adoptive immunotherapy has been quite remarkable. The numerous types of immune cells that can be used in adoptive cellular immunotherapy such as monocytes (4,5), LAK and/or A-NK cells (1,2,6-8), T-cells (9,10) and dendritic cells (11,12), and the unique antitumor behavior these cells display, make the field of adoptive cellular immunotherapy for the treatment of cancer more promising than ever before.

The ability of A-NK cells to kill target cells without prior sensitization makes A-NK cells highly attractive for use against malignantly transformed tumors. In A-NK cell immunotherapy, cell activation using IL-2 is the technically rate limiting step prior to A-NK cell administration into tumor bearing hosts, whereas in other cell types such as T-lymphocytes, the complexity of immune cell-target cell interactions necessitates the sensitization of T-cells to tumor cells in addition to their activation. The sensitization step, which is essential in T-cell preparation prior to immunotherapy, limits the utility of immunogenicity of T-cells only to those tumors which express antigens that can be recognized by T-cells, and must be sufficiently immunogenic to create a T-cell response(13,14). Furthermore, T-cells must receive secondary signals from other cell types such as B-cells and helper T-cells for their effective activation (14), a process which is not necessary for the NK cell-mediated immune response.

In addition to our previous reports showing the role of the MMPs in NK cell invasion (15-17), the evidence presented in this dissertation documenting for the first time the presence of both uPA and uPAR in NK cells **(Chapter II)**, the employment of the uPA system in NK cell invasion **(Chapter II)**, and the cooperation of the uPA system with MMPs in NK cell invasion **(Chapter III)**, creates a clearer picture of the mechanisms used by NK cells in the processes of tumor invasion and infiltration. Understanding these processes is essential for enhancing NK cell cancer immunotherapy, including the improved use of NK cells for locoregional transport of anti-cancer drugs into cancer metastases **(Chapter V)**.

In view of the need for A-NK cells to be in close proximity and possibly in contact with tumors for target cell killing (18, 34), the lack of optimal tumor infiltration by A-NK cells obviously hinders efficient A-NK cell functions. One factor that may possibly influence tumor infiltration by A-NK cells is the tumor production of uPA inhibitors/serine protease inhibitors. The presence of such inhibitors in tumor ECMs containing large amounts of laminin and fibronectin, two ECM proteins susceptible to degradation by the uPA system, may impede or even completely block A-NK cell movement within the milieu of the tumor. This may in turn render A-NK cell immunotherapy useless under such conditions without appropriately addressing and managing the presence of such inhibitors. On the other hand, in tumors that produce high amounts of uPA (19-22), A-NK cell immunotherapy may yield better therapeutic results by possibly enabling A-NK cells to employ tumor produced uPA to degrade the ECM, thereby leading to increased tumor infiltration by A-NK cells.

The presence of uPAR in NK cells as documented in **chapter II** is also extremely valuable in evaluating the efficacy of A-NK cell adoptive immunotherapy. uPAR's ability to function in cell signaling during cellular migration (21,23), in addition to its functions in proteolysis, amplifies its role in cell migration and invasion. Immunogenic tumor tissues display a high degree of inflammation caused by the entry of various immune cells into the site of the tumor. Interactions of NK cells with other immune cells, and the exposure of NK cells to various cytokines and other stimuli within the tumor environment may modulate the level of expression or function of uPA and uPAR, therefore affecting the infiltration and the movement of NK cells within the tumor tissues. For example, Tumor necrosis factor-α (TNF-α), a cytokine produced by both helper and cytotoxic T-cells, has been shown to downregulate the expression and function of uPAR in dendritic cells, resulting in a decrease in dendritic cell motility (24). On the other hand, we have documented an increase in uPAR following stimulation of NK cells with IL-2 **(Chapter IV)**, a cytokine commonly produced by immune cells such as helper T-cells, which resulted in increased NK cell invasion (15). Furthermore, uPAR's existence as a purely extracellular receptor attached to the cell surface by a glycosyl phosphatidylinositol anchor renders it very susceptible to cleavage by proteases such as uPA and plasmin (25), and by other enzymes such as Phospholipase C (**Chapter II**) (26,27), thereby modulating its effects on cellular migration. Evaluating the presence of these uPAR modulating factors within the environment of the tumor prior to the administration of A-NK cells can be a crucial step in enhancing the efficacy of A-NK cell adoptive immunotherapy.

The protein composition of the tumor ECM may also play a major role in the success of A-NK cell immunotherapy by either facilitating or inhibiting tumor infiltration by the effector cells (28-30, 53). Tumor ECMs rich in laminin (LN), fibronectin (FN), and vitronectin (VN) may facilitate A-NK cell tumor infiltration due to the susceptibility of such proteins to degradation by the uPA system (i.e. LN and FN), or due to the invasion promoting interactions of the ECM proteins with uPAR (i.e. VN). Conversely, A-NK cell infiltration into tumors embedded in ECM proteins not susceptible to the uPA system may be significantly hindered. In view of the numerous reports showing the ability of uPA and plasmin to activate MMPs, degrade MMP inhibitors, and cooperate with MMPs in invasion through ECM **(Chapter III)** (31-34), and considering our previous reports demonstrating the role of MMPs in the degradation of the collagenous components of the ECM by NK cells (15-17), it is reasonable to speculate that even in cases where the tumor ECM can not be directly degraded by either uPA or plasmin, the capacity of NK cells to use MMPs and other proteases in their invasion through ECM enables NK cells to adapt to a variety of tumor microenvironments. The ability of NK cells to adapt to various ECMs is also highlighted by the noted regulation of uPA following NK cell exposure to various ECM proteins **(Chapter III)**. ECM regulation of NK cell uPA may be a means by which NK cells maintain fine control over ECM degradation, a process which can facilitate tumor metastasis if not closely regulated.

Since the *in vitro* enhancement of NK cell cytotoxicity (e.g. via stimulation with IL-2) prior to their administration into tumor bearing hosts is a common practice, engineering NK cells with high invasive capacities is not a farfetched task. Although

during the past years the focus has consistently been on manipulating A-NK cells to posses exceptional cytotoxic abilities (35-37), it is equally important, if not more important, to generate A-NK cells with high invasive capacities that can efficiently enter tumors and establish cellular contacts with their target cells. The results presented in **chapter IV** demonstrating the presence of posttranscriptional components in the regulation of uPA and uPAR following IL-2 stimulation of NK cells lay the foundation for the development of novel approaches to upregulate NK cell uPA and uPAR without the use of IL-2, which has been shown to be very toxic to patients, and is a major limiting factor in NK cell cancer immunotherapy (3). One possibility for enhancing the invasiveness of NK cells would be to alter the posttranscriptional regulation of uPA/uPAR by modulating mRNA binding protein (mRNABP)-uPA/uPAR mRNA interactions. Since both uPA and uPAR mRNABPs were detected in NK cells **(Chapter IV)**, and because these mRNABPs have been shown to cause a destabilization of the uPA/uPAR mRNA (38-41), introducing mRNA sequences that can compete with uPA/uPAR mRNA-mRNABP interactions may lead to a larger number of free uPA/uPAR mRNA transcripts, and therefore a higher expression of either uPA or uPAR by the NK cells, ultimately leading to more tumor invasion.

In addition to potential increases in NK cell invasion that can occur following the upregulation of the uPA system, there can also be detrimental effects of increased invasion and ECM degradation of tumor tissues. The uPA system has been clearly shown to play a role in the invasion and metastases of tumors (42-45). In fact, numerous studies have demonstrated a decrease in tumor invasion and metastases following the

inhibition of tumor cell uPA and/or uPAR (46-52). In order to achieve optimal therapeutic efficacy using NK cell adoptive immunotherapy, the susceptibility of tumors to NK cells, the protein and protease inhibitor composition of tumor ECMs, and the use of the uPA system and other ECM degrading proteases by tumor cells must be taken into account prior to A-NK administration.

In the work presented in this dissertation we have shown that: 1) NK cells produce both uPA and uPAR **(Chapter II)**, 2) NK cells employ the uPA system in their invasion through the ECM **(chapter II)**, 3) NK cell uPA and MMPs cooperate in ECM degradation **(Chapter III)**, 4) ECM proteins are capable of regulating uPA in NK cells **(Chapter III)**, 5) IL-2 upregulates both uPA and uPAR in NK cells via transcriptional and post-transcriptional mechanisms **(Chapter IV)**, and 6) NK cells can potentially be used as drug delivery vehicles in cancer immunotherapy **(Chapter V)**. Regardless of the ultimate goal of A-NK adoptive immunotherapy, whether the NK cells are used to directly attack and destroy tumor cells or whether they are used as drug delivery vehicles based on their ability to selectively accumulate into tumor metastases, the ability of A-NK cells to infiltrate tumors should not be underestimated. Instead, the capacity of A-NK cells to infiltrate tumors, and the potential to upregulate A-NK cell tumor infiltration by upregulating protease expression should be further explored to help achieve peak therapeutic efficacy using A-NK cells optimally upregulated for ECM invasiveness for adoptive immunotherapy in the treatment of cancer.

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