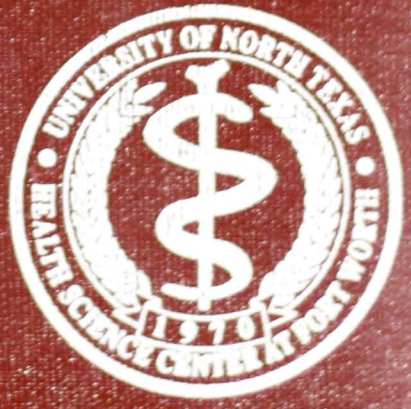
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The urokinase-type plasminogen activator receptor (uPAR) is a GPI-anchored receptor, devoid of an intracellular domain, but nevertheless initiates signaling, possibly through lateral interactions with integrins. Since adoptively transferred interleukin-2 (IL-2) activated natural killer (A-NK) cells can accumulate within established cancer metastases, these A-NK cells may integrate components of adhesion and proteolysis to facilitate their infiltration into tumors. The work in this dissertation investigates the hypothesis that uPAR directly interacts with and regulates the expression of integrins on the surface of NK cells in the potential modulation of NK cell migration and invasion. Crosslinking studies have revealed a relationship between the integrins and uPAR on the surface of the human NK cell line, YT. Crosslinking uPAR, which mimics uPAR clustering at focal adhesion sites, caused an increase in the expression of the α_M, α_V and β_2 integrins. Although uPAR is GPI-linked to the plasma membrane and has no direct means of initiating intracellular signaling, crosslinking uPAR activated the MEK/ERK signaling cascade, as phosphorylation of both MEK 1/2 and ERK 1/2 occurred following receptor clustering. The MEK-specific inhibitors PD98059 and U0126 blocked MAP kinase phosphorylation, and PD98059 inhibited the increase in integrin expression induced by uPAR crosslinking. Furthermore, the binding of urokinase plasminogen

activator (uPA) to uPAR also activated the MEK/ERK signaling pathway. Fluorescence microscopy revealed the cocapping of uPAR with the α_V integrin, a process inhibited *N*-acetyl-D-glucosamine, which abrogates the lectin-like interactions that have been suggested to exist between uPAR and integrins. The work presented herein indicates that signaling initiated either by uPAR crosslinking, leading to increased integrin surface expression, or by uPAR occupancy with uPA may depend on the physical association of uPAR with integrins. These studies will enhance our understanding of the mechanisms utilized by NK cells for their adhesion to tumor vasculature and accumulation within established cancer metastases, thereby potentially identifying targets for enhancing their effectiveness during adoptive immunotherapy.

UPAR INTERACTION AND REGULATION OF NATURAL KILLER CELL INTEGRINS: IMPLICATIONS FOR THE MODULATION OF NK CELL MIGRATION AND INVASION

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UPAR INTERACTION AND REGULATION OF NATURAL KILLER CELL INTEGRINS: IMPLICATIONS FOR THE MODULATION OF NK CELL MIGRATION AND INVASION

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COMMONLY USED ABBREVIATIONS

A-NK:	Interleukin-2 activated NK cells
ECM:	Extracellular matrix
ERK:	Extracellular signal-related kinase
FITC:	Fluorescien isothiocyanate
GPI:	Glycosylphosphatidylinositol
IL-2:	Interleukin-2
LAK:	Lymphokine-activated killer
MAPK:	Mitogen-activated protein kinase
MEK:	Mitogen-activated protein kinase/extracelluar signal-regulated kinase kinase
MMP:	Matrix metalloproteinase
NK Cells:	Natural killer cells
TRITC:	Tetramethyl-rhodamine isothiocyanate
uPA:	Urokinase-type plasminogen activator
uPAR:	Urokinase-type plasminogen activator receptor

CHAPTER I

INTRODUCTION TO THE STUDY

Natural Killer Cells and Adoptive Immunotherapy

Natural killer (NK) cells, derived from bone marrow precursors, comprise a small population of peripheral blood lymphocytes that play a major role in killing virally infected cells and tumor cells without prior sensitization and also regulate other immune effector cells via the secretion of cytokines (1). NK cells are large granule lymphocytes (LGLs), as their morphology shows distinct large cytoplasmic granules. These cells kill by granule exocytosis of perforins, serine esterases and other enzymes, which lead to DNA fragmentation of the target cell (1). In addition, NK cells are capable of antibody-dependent cellular cytotoxicity (ADCC), in which the CD16/FcγRIII receptor on NK cells binds to the Fc portion of antibody-coated target cells causing target cell lysis (1). Several cytokines have been shown to effect NK cell proliferation and cytotoxicity against target cells. For example, both IL-2 and IFN-γ enhance NK cell cytolytic activity, and IL-2 also augments natural killer cell proliferation (2). Since NK cells often are the first line of defense against tumor cells, they have been used, in combination with IL-2, for treatment of various cancers, a process called adoptive immunotherapy (3-7). During adoptive immunotherapy, peripheral blood leukocytes (PBLs) are isolated from cancer

patients, stimulated with IL-2 *in vitro* and re-administered back into the patient. Herberman *et al.* first indicated that most lymphokine-activated killer (LAK) activity mediated by PBLs was attributable to NK cells (8). LAK cell therapy has been shown to be useful in controlling metastatic tumors in both animal models and human cancer patients (3-7, 9, 10). Moreover, LAK cells can become adherent to plastic following IL-2 treatment and expand to generate high levels of broad anti-tumor activity and thus are termed adherent LAK (A-LAK) cells (11, 12). A-LAK cells, in combination with IL-2, have been shown to be effective in reducing established lung and liver metastases in rat models (9). In mice, adoptive immunotherapy involving LAK cells and recombinant IL-2 resulted in reducing the number and size of established pulmonary sarcoma metastases (10) and the number of liver metastases (13). Furthermore, adoptive transfer of $NK1.1^+$ $CD3^-$ NK cells into NK cell-deficient mice led to tumor regression and suppression of tumor metastases and outgrowth (14). In humans, LAK therapy in combination with IL-2 has demonstrated regression of solid tumors in some patients with certain advanced cancers, particularly melanoma and renal cell cancer (3-7).

NK Cell Accumulation Within Tumors

In hopes of facilitating the use of adoptive immunotherapy of NK cells and improving their anti-tumor effectiveness for cancer treatment, investigation of the mechanism of NK cell infiltration into established tumor metastases is under current examination. It has been well documented that adoptively transferred IL-2 activated NK

cells (A-NK cells) bind to tumor vasculature and accumulate within established tumor metastases (15, 16). Furthermore, IL-2 activated NK cells have been reported to infiltrate into the lung metastases of colon carcinoma in rats (17). One study showed that A-NK cells arrest for an extended period of time in the tumor microvasculature and become deformed as they infiltrate into tumors (18). *In vitro*, A-NK cells not only bind to microvascular endothelial cells (19) but also invade B16 melanoma tumor cell aggregates (20). Recent studies have involved the investigation of possible resistance of NK cell infiltration by tumor metastases and the role that tumor structure may play during adoptive immunotherapy. For example, patterns of NK cell accumulation within tumors have been documented, as A-NK cells demonstrated increased accumulation in loose metastatic lesions as compared to dense metastases (21). Moreover, the extracellular matrix (ECM) has been reported to be a barrier against immune cells (22), thus possibly altering the effectiveness of NK cell accumulation within advanced cancers. Overcoming these barriers warrants the need for examination of molecular mechanisms, such as the use of proteases and adhesive molecules, employed by NK cells as they extravasate from the vasculature and accumulate within cancer metastases. Recent studies have shown that NK cells secrete the proteolytic enzymes urokinase-type plasminogen activator (uPA) and matrix metalloproteinases (MMPs), which contribute to their migration and invasion through the ECM (23-27); therefore, these enzymes may be an essential requirement for NK cell infiltration into tumors.

uPA system - uPA

Generally, the first step of leukocyte migration during processes of immunity and inflammation is adhesion to the vasculature, which is largely mediated by integrins. Following firm adhesion, leukocytes can secrete various proteases, such as uPA and MMPs, to degrade different components of the ECM, i.e. laminin, fibronectin and collagen. uPA is part of the uPA system, which is comprised of the serine protease uPA, the uPA receptor (uPAR), and plasminogen activator inhibitors (PAIs). Both uPA and uPAR are expressed by many cell types and are implicated in ovulation, wound healing, angiogenesis, and tumor cell invasion and metastasis (28-32). uPA is a highly specific 54 kD protease that converts the zymogen plasminogen to plasmin through cleavage of the Arg561-Val562 peptide bond. Plasmin is able to degrade components of the ECM, including laminin and fibronectin (33, 34). In addition, plasmin can activate MMPs, which can lead to the degradation of collagen components of the ECM. Like plasminogen, uPA is secreted in zymogen form and is proteolytically converted from single chain uPA (scuPA) to active two-chain uPA (tcuPA), a product of two chains held together by a disulfide bond. Activation of uPA is achieved by plasmin, which cleaves scuPA at the Lys158-Ile159 bond, thus resulting in a positive-feedback mechanism and cascade amplification. The general structure of uPA consists of the C-terminal catalytic domain and the N-terminal region, which contains a growth factor domain that mediates the binding of uPA to uPAR and a triple disulfide kringle domain. Furthermore, uPA can be cleaved between the Glu143-Leu144 bond by MMPs (35) or the Lys135-Lys136 bond

by uPA itself or plasmin (36), resulting in a low molecular weight uPA (LMW-uPA), which retains the serine protease domain, and releasing the amino-terminal uPA (ATF-uPA), which is catalytically inactive but retains the uPAR-binding domain (growth factor domain).

The uPA System – uPAR

The high affinity cell surface receptor for uPA is a 55-60 kD, heavily glycosylated protein, uPAR, which is attached to the outer leaflet of the cell membrane by a glycosylphosphatidylinositol (GPI) anchor. Therefore, a major function of uPAR is to bind uPA thereby localizing proteolysis to the pericellular surface, thus promoting cellular migration and invasion. The general structure of uPAR is divided into three internally repeating domains joined by two linker regions. Domain 1 (D1) mediates the binding of uPA (37), and domains 2 and 3 (D2 and D3) are important for uPA binding affinity and also recognize and bind vitronectin (38-42). Therefore, uPAR is multifunctional in that it binds uPA, consequently concentrating proteolysis at the cell surface, and binds vitronectin, thus playing a role in cell adhesion. To enhance these functions, uPAR has been shown to promote cellular adhesion and migration by aggregating at cell-substratum interfaces, focal adhesion sites, and leading edges of migrating cells, including monocytes, endothelial cells and vascular smooth muscle cells (43-48). Furthermore, uPAR has the ability to be recycled. Once uPA is bound to uPAR and activated, PAI binds to the uPA/uPAR complex and inhibits uPA. The

uPAR/uPA/PAI complex is capable of internalization; uPA and PAI are subjected to lysosomal degradation, and uPAR is then recycled back to the cell surface (49). Since uPAR is GPI-linked to the plasma membrane, its internalization is aided by other receptors, such as the α-2 macroglobulin receptor/LDL-receptor related protein (50).

High levels of uPA and uPAR expression in certain cancers have been correlated with poor patient prognosis (51). The contribution of uPA to increased cancer invasion is not surprising, as its proteolytic activity leads to matrix degradation and enhances the migratory potential of many cell types. uPA activity is elevated in many types of cancers, including colorectal, breast, lung, and gastric carcinoma cancers, (52-56). Following the comparison of four different bladder cancer cell lines, it was found that the cells with the highest levels of uPA demonstrated the greatest propensity for invasion (57). In addition to uPA, uPAR is also increased in other various cancers, such as ovarian, breast, lung and hepatocellular carcinoma cancers (58-61).

Similarly, there is a strong correlation between uPA/uPAR expression and leukocyte migration during processes of immunity and inflammation (62-65). For instance, uPAR-deficient mice have decreased recruitment of leukocytes to inflamed peritoneum (62). Mice lacking uPA show inadequate immune response to bacterial infection and are subsequently predisposed to infection (63, 65). Moreover, in NK cells, uPA and uPAR have currently been shown to contribute to cellular invasion through the extracellular matrix, either alone or in combination with MMPs (26, 27). Therefore, the expression of both uPA and uPAR appear to play a major role in the invasive potential of leukocytes during the immune response.

uPAR-mediated signaling

Despite the lack of an intracellular domain, uPAR is nevertheless able to initiate signal transduction leading to changes in adhesion and migration of certain cell types. In addition to the appearance of a vitronectin-binding epitope following a conformational change induced by the binding of uPA to uPAR (66), uPAR ligation with uPA also induces a conformational change that reveals a chemotactic epitope (67). Similarly, chymotryptic cleavage in a region between D1 and D2 on the soluble form of uPAR (suPAR), an area shown to be sensitive to enzymatic cleavage (68), also exposes a chemotactic epitope (67). Furthermore, the chemotactic properties of uPAR occupancy with uPA and chymotrypsin-cleaved uPAR lead to activation of tyrosine kinases, such as p56/p59^{hck} (67, 69). The chemotactic effect appears to be mapped to a small linker region $S_{88}R_{89}S_{90}R_{91}Y_{92}$, which connects D1 and D2 and is exposed by either a conformational change induced by uPA binding or by chymotrypsin cleavage at Tyr87 (69). In addition, it has been well documented that the binding of uPA to uPAR on various tumor cells, vascular smooth muscle cells, and endothelial cells leads to activation of numerous signaling pathways, including the Ras/ERK, JAK/STAT and calcium signaling pathways (70-78). Many studies report that uPAR immunoprecipitates from monocytes and epithelial cells have associated signaling components, such as non-receptor tyrosine kinases of the Src family and tyrosine-phosphorylated proteins (76, 79, 80). Moreover, signal transduction initiated by the binding of uPA to uPAR often favors adhesion, cell migration and appears to be chemotactic (48, 71, 75-78, 81-85). In tumor

cells, Nguyen *et al.* demonstrated that uPA stimulation promoted ERK1 phosphorylation and MCF-7 cell migration via a FAK-dependent mechanism (81). Furthermore, disruption of the binding of uPA to uPAR blocked cellular migration on vitronectin in human pancreatic carcinoma cells (86).

In certain leukocytes, similar findings have been reported. For example, in monocytes, uPA treatment resulted in calcium signaling, increased cellular adhesion via activation of cAMP (75, 87) and stimulated a chemotactic response (67). uPA treatment has been associated with enhanced superoxide production in neutrophils (88) and has stimulated neutrophil chemotaxis *in vitro* (89). Nonetheless, the most extensive studies investigating the promotion of migration following uPA treatment have been done in tumor cells.

In addition to uPAR occupancy with uPA, other mechanisms leading to activation of intracellular signaling by uPAR have also been reported, including increased signaling following uPAR clustering, binding to vitronectin or uPAR overexpression. Crosslinking uPAR, which mimics uPAR clustering at focal adhesion sites, results in calcium signaling in human monocytes and neutrophils (90, 91) and JAK/STAT signal transduction in a human kidney epithelial tumor cell line (92). Furthermore, the interaction of uPAR with vitronectin has also been reported to initiate p130Cas and Rac-dependent signaling leading to actin reorganization and increased cell motility (93). Another study showed that high levels of uPAR expression promoted its interaction with integrins and generated activation of ERK signaling (94). Although uPAR is GPI-anchored to the plasma membrane, uPAR-mediated signaling has been well documented. Therefore, these

findings suggest that uPAR must form cis interactions with transmembrane adaptors, such as integrins, on the cell surface to facilitate intracellular signaling.

Integrins

Integrins encompass a family of extracellular matrix (ECM) receptors that mediate cell-cell and cell-matrix interactions, contributing to physiological processes, such as cellular migration, proliferation, survival, differentiation, angiogenesis and tumor cell metastasis. They are heterodimers of α and β subunits, which noncovalently associate to form an extracellular ligand-binding region for matrix proteins, such as laminin, fibronectin, and vitronectin. The integrins were originally grouped into three major families: 1) the VLA (very late antigen) integrins, which are the β1 integrins that were found to appear on the cell surface very late after activation, 2) the LEU-CAMs (leukocyte cell adhesion molecules) that are β2 integrins found primarily on leukocytes, and lastly, 3) the β3 integrins that were originally associated with platelets. However, to date, there are currently about 19 different α subunits and 8 β subunits resulting in approximately 25 various integrins (95). Almost all of the α subunits associate with only one particular β subunit; however, α_V appears to be an exception, showing more promiscuity by associating with various β subunits. Different associations of the α and β subunits determine the numerous ligand specificities for integrins, many of which show redundancy by having the ability to bind to the same ligands. Integrins have a large NH_2-terminal extracellular domain, a single membrane-spanning domain and a short C-

terminal cytoplasmic domain, which associates with the cytoskeleton and various signaling molecules resulting in activation of numerous intracellular signaling pathways.

Integrins on NK Cells

Integrins are expressed by an extensive variety of cell types, commonly showing constitutive expression of certain integrins, and, likewise, a diverse repertoire of integrins are typically expressed by a single cell. On NK cells, many different integrins are expressed, including members of the β_1, β_2, and β_3 families (96). During processes of immunity, integrins play a large role in the arrest of leukocytes, including NK cells, to the vascular endothelium, followed by migration of immune cells to locations of tissue injury, inflammation or tumor sites. Moreover, integrin expression and ligation with their physiological ligands has been correlated with increased adhesion, migration and cytotoxicity of NK cells. For example, NK cell infiltration into lung, liver, and subcutaneous B16 melanoma in mice is mediated by the $\alpha_4\beta_1$ integrin (97). Furthermore, the α_4 and α_5 integrins are constitutively expressed on freshly isolated human NK cells, thus mediating NK cell adhesion to fibronectin (98), and their ligation with immobilized fibronectin augments cytotoxicity of NK cells (99, 100). Blocking antibodies directed against the α_L and β_2 integrin subunits abrogated the cytotoxicity of A-NK cells against tumor targets (101). Furthermore, pretreatment of A-NK cells with β_2 or α_4 blocking antibodies significantly reduced the number of NK cells binding to endothelial cells during physiological flow conditions (102). Similarly, blocking the $\alpha_5\beta_1$ integrin

completely abrogated LAK activity against target cells (103). Engagement of $\alpha_V\beta_3$ with vitronectin co-stimulated both cytokine production and proliferation of NK cells (104).

IL-2 treatment, which enhances NK cell cytotoxicity and migration, causes an increase in the expression of various integrins on the surface of human NK cells, including $\alpha_L\beta_2$ (105, 106), the α_1, α_2, and α_3 integrins (106), and $\alpha_4\beta_7$, which possibly increases human NK cell adhesion to the ECM and endothelial cells (107). In many instances, increased integrin expression upon treatment with IL-2 augmented NK cell adhesion to target cells or endothelial cells, transendothelial migration, and/or enhanced killing of target cells (105, 108-110). Further evidence supports this phenomenon by showing that antibodies that block integrin function also inhibit NK cell migration across endothelial cell monolayers and inhibit NK cell cytotoxicity against target cells, such as fresh uncultured endometrial carcinoma (110-113).

Integrin Structure

The structures of the α and β subunits differ from each other and play an essential role in controlling the ligand-binding properties of integrins. While the β subunits of integrins are remarkably similar, showing 40-48% amino acid sequence homology, the α subunits share weak homology to one another (114). The α subunits generally consist of seven repeats that fold into a β-propeller configuration (115). Within the third β-propeller repeat exists a metal ion-dependent adhesion site (MIDAS) that contains the cation-binding DXSXS motif, which contributes to the ligand-binding properties of

integrins by coordinating bound cations (116). In certain α subunits, an I-domain (inserted domain) is present and has been proposed to be the major ligand-binding site in these I-domain-containing integrins (116). The structure of the β subunits reveals 56 conserved cysteine residues, most of which are found in four cysteine-rich repeats in the extracellular domain close to the C-terminal. At the N-terminus, there are 7 cysteine residues, one of which forms an intrachain disulfide bond with a cysteine residue within the cysteine-rich repeat at the C-terminal extracellular segment of the β subunit (117). The β subunits have an I-like domain, which also contains the DXSXS motif and appears to regulate the ligand binding properties of integrins (95). Therefore, there are three major regions of the integrin subunits that appear to control ligand binding with integrins: the β-propeller regions on the α subunit, the I-domain of certain α subunits, and the DXSXS I-like domain of the β subunit. Conformational changes in these areas appear to regulate the affinity of integrins for their ligands.

To enhance affinity for ligand binding, integrins can initiate inside-out signaling; integrins exist in a non-active state on the cell surface, and local stimuli, such as cytokines, activate the integrins from the inside to change conformation and bind to matrix proteins. The open active conformation of integrins results in high affinity binding to ligands, while the closed conformation either does not bind to ligands or binds with low affinity (95). Inside-out signaling occurs very quickly, as the ability of integrins to bind to ligands can be activated by signals within the cell on a timescale of <1 second (95). This expeditious feature of inside-out signaling enables leukocytes to quickly bind to the vasculature and subsequently migrate across endothelium to participate in immune

and inflammatory processes (95). Furthermore, integrins have been reported to be found in subcellular storage vesicles in certain leukocytes, and cellular activation results in their rapid translocation to the plasma membrane (118-121). In monocytes, $\alpha_X\beta_2$ was shown to be stored in intracellular vesicles and peroxidase negative granules, which were quickly mobilized to the cell surface following inflammatory stimuli (120). Likewise in neutrophils, the $\alpha_M\beta_2$ integrin surface expression was increased following chemotactic stimulation as a result of exocytosis (121). Therefore, inside-out signaling can result in integrin conformational changes and also fusion of subcellular vesicles containing integrins with the plasma membrane, thereby allowing cells to quickly respond to their surroundings and promptly control their adhesive capabilities.

Integrin Signaling

Upon ligand binding, integrins are capable of initiating many complex intracellular signaling cascades. Many of these pathways lead to assembly and reorganization of the cytoskeleton resulting in integrin clustering at the site of cell contact, thus enhancing binding strength. Integrins commonly co-localize with actin-binding proteins, such as α-actinin, talin, vinculin and tensin, and assembly of these structural proteins appears to regulate cellular adhesion, morphology and mobility (122). Formation of focal adhesions generally leads to activation of many tyrosine kinases, including Src kinases, focal adhesion kinase (FAK), and protein kinase C (PKC) (123). Other signaling pathways initiated by integrins following their activation by ECM

proteins include the Rho family of GTPases, PI3K, and MAPK signaling (124-127). Activation of the MAPK signaling cascade that occurs following integrin interaction with the ECM appears to be regulated by FAK, which activates Ras, thus leading to the MAPK signaling cascade (122, 127). In the MAPK pathway, Ras is directly upstream of Raf-1, which phosphorylates MEK 1/2, on Ser217 and Ser221 (128). Studies have shown that dual specificity MEK 1/2 is highly specific for the downstream MAPK, ERK 1/2, and activation of ERK 1/2 occurs following the phosphorylation of threonine and tyrosine residues in a -TXY- motif (128). While MAPK/ERK 1/2 has the ability to translocate to the nucleus and phosphorylate transcription factors, cytoplasmic substrates have also been reported to be phosphorylated by MAPK, including the EGF receptor, cytoplasmic phospholipase A_2, and microtubule-associated proteins (129). While integrin-induced signaling often leads to changes in migration, differentiation, growth and survival, recent studies have indicated a novel role for integrins, which involves their physical association with and modulation of certain proteases, namely MMPs and uPA/uPAR (130-135).

Physical Association of uPAR with Integrins

Recent studies show that uPAR co-immunoprecipitates and co-localizes with integrins on the surface of various tumor cells to enhance cellular migration and invasion (73, 77, 82, 86, 94, 136-139). The binding of uPA to uPAR has been shown to induce physical association of uPAR with β_1 integrins and enhance tumor cell migration via FAK/p130Cas signaling (77). Furthermore, uPAR has been reported to interact with the

$\alpha_V\beta_5$ integrin, promoting migration of the MCF-7 human breast carcinoma cell line (82). Fluorescence microscopy and resonance energy transfer have also been utilized to demonstrate the close proximity of uPAR and integrins on the surface of human fibrosarcoma cells (136). Recently, Simon *et al.* have identified a binding sequence, M25, for uPAR on the α subunit of $\alpha_M\beta_2$ and showed the site to be critical for human vascular smooth muscle cell migration (140). Additionally, trans-interactions between the integrins and uPAR have been identified, as both β_1 and β_3 integrin-expressing cells were adherent to uPAR-coated surfaces and could not bind in the presence of integrin blocking antibodies (141).

The interaction between uPAR and integrins is not limited to tumor cells, as studies in human neutrophils and monocytes have also revealed the physical association of these receptors (44, 79, 142). On human neutrophil membranes, fluorescence microscopy showed the cocapping of uPAR with the $\alpha_M\beta_2$ integrin (142). In monocytes, the coupling of $\alpha_M\beta_2$ and uPAR not only occurred on the cell surface but also facilitated monocyte adhesion to fibrinogen (44). Another group used immunoprecipitation to show that uPAR exists in a single receptor complex with β_2 integrins and Src-kinases, such as $p60^{fyn}$, $p53/56^{lyn}$, $p58/64^{hck}$ and $p59^{fgr}$, on the surface of human monocytes (79). Since uPAR has been shown to induce cell signaling, discovery of the close lateral associations between uPAR and integrins and the complexes of uPAR, integrins and protein kinases, suggest that integrins may be the likely candidates linking the GPI-anchored uPAR with intracellular signaling molecules. Nevertheless, the functional importance of the uPAR/integrin association is currently under intense investigation, as macromolecular

complexes of multiple proteins in close and physical proximity at the cell surface apparently regulate certain aspects of cellular adhesion, migration and proteolysis.

Additionally, integrins play a role in modulating members of the uPA/uPAR system. Stimulating the integrins either by crosslinking or by ligand binding also leads to changes in uPA and uPAR expression. Increases in uPAR expression following co-clustering of both β_1 and CD3 on T cells have been documented (143). In oral keratinocytes, clustering of the α_3 and β_1 integrin subunits enhanced uPA secretion and resulted in the redistribution of uPAR to sites of clustered integrins, thereby promoting their physical association as shown by co-immunoprecipitation (144). Likewise, stimulating the integrins with ECM components or their peptide fragment RGD also results in an increase in uPA expression in tumor cells (130, 131). Moreover, changes in integrin expression appear to have an effect on the expression of uPA/uPAR (145, 146). The use of the α_V-specific antisense oligonucleotide inhibited uPA expression in invasive breast cancer cells (146). In metastatic melanoma cells, elevated levels of $\alpha_V\beta_3$ correlated with high levels of uPAR mRNA and increased amounts of surface-bound uPA (145). Therefore, there appears to be a highly coordinated interplay in various cell types between cellular adhesion via the integrins and pericellular proteolytic activity mediated by the uPA system.

Concluding Remarks

While there has been extensive work done on uPAR signaling and its physical association with integrins in tumor cells, neutrophils and monocytes, no studies have been employed on establishing uPAR as a signaling receptor in NK cells. In fact, expression of uPAR has only recently been shown in NK cells (27). Since adoptively transferred A-NK cells can bind to tumor vasculature and accumulate within established cancer metastases following their adoptive transfer (15, 16), intense investigation of NK cell proteolytic and adhesive machinery may lead to the enhancement of the infiltration of these effector cells during adoptive immunotherapy. uPAR-mediated signaling and the coupling of uPAR with integrins on the surface of tumor cells appears to increase their migratory potential (77, 82); therefore, this may be a key strategic mechanism utilized by NK cells as they traverse the ECM and subsequently accumulate within tumor metastases. **Therefore, the work presented herein investigates the hypothesis that uPAR directly interacts with and regulates expression of integrins on the surface of NK cells in potential modulation of NK cell migration and invasion.** There are three major aims of this study. **Specific Aim 1** is to evaluate the relationship between the integrins and uPAR. For example, does uPAR have the potential to modulate integrin surface expression although it is a GPI-anchored receptor? In **Specific Aim 2,** signaling events activated by uPAR crosslinking, which mimics uPAR clustering at focal adhesion sites between the cell and the matrix, are investigated. In particular, the signaling pathways that lead to the increase in integrin surface expression shown in Specific Aim 1

are identified. Furthermore, in addition to uPAR clustering, does the binding of its natural ligand, uPA, activate signal transduction? Lastly, in **Specific Aim 3** the physical association between uPAR and integrins on the cell surface is investigated, as the integrins may serve as the transmembrane adapters for uPAR-induced signaling following uPAR clustering or occupancy with uPA. Therefore, identifying uPAR as a signaling receptor, which communicates to the cell to increase the adhesive capacity and possibly increase the migratory and invasive potential of NK cells, leads to a better understanding of the molecular mechanisms utilized by NK cells that may serve as a requirement for NK cell accumulation within tumors. Results from this study will contribute to the long-term goal of identifying targets for devising novel strategies to increase the therapeutic utility of NK cells during their adoptive transfer for the treatment of cancer metastases.

Chapter II

Urokinase-type Plasminogen Activator Receptor Crosslinking in an NK Cell Line Increases Integrin Surface Expression by the MAP Kinase/ERK 1/2 Signaling Pathway

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ABSTRACT

Urokinase-type plasminogen activator receptor (uPAR) is attached to cell membranes by a glycosylphosphatidylinositol (GPI) anchor, and as such is devoid of an intracellular domain, but is nevertheless able to initiate signal transduction. Herein, we report a relationship between integrins and uPAR on the surface of the human NK cell line, YT. Our data reveals that crosslinking uPAR, which mimics uPAR clustering at focal adhesion sites, causes increases in expression of the α_M, α_V and β_2 integrins on the surface of YT cells. Activation of the MEK/ERK signaling cascade occurs following uPAR crosslinking, as phosphorylation of both MEK 1/2 and ERK 1/2 results from receptor clustering. The MEK-specific inhibitors PD98059 and U0126 blocked MAP kinase phosphorylation; furthermore, PD98059 inhibited the increase in integrin expression induced by uPAR clustering. This study suggests that uPAR is a signaling receptor and regulator of integrins in NK cells and may impact on NK cell function, including the potential for their accumulation within tumor metastases following adoptive transfer.

INTRODUCTION

Natural killer (NK) cells comprise a small population of peripheral blood lymphocytes and play a major role in killing virally infected cells and tumor cells without prior sensitization. A number of studies have shown the potential utility of the adoptive transfer of lymphokine-activated killer (LAK) cells, including IL-2 activated NK cells (A-NK cells) (8), for treatment of both animals bearing cancer metastases as well as in cancer patients (3-6, 15). We have previously reported that adoptively transferred A-NK cells can accumulate within established cancer metastases in a time and IL-2-dependent manner (15). Moreover, A-NK cells bind to tumor vasculature and accumulate within established tumor metastases (16). Our laboratory has reported that NK cells produce and regulate urokinase-type plasminogen activator (uPA) and urokinase-type plasminogen activator receptor (uPAR), both of which appear to contribute to the ability of NK cells to accumulate within cancer metastases (27).

uPAR is a glycosylphosphatidylinositol (GPI)-anchored receptor that binds uPA. uPA is a serine protease that cleaves plasminogen to plasmin, contributing to extracellular matrix proteolysis, thus promoting cellular migration and invasion (e.g. (27)). Furthermore, uPAR has been shown to promote adhesion and localize matrix degradation by aggregating at the site of cell-matrix interactions, focal adhesion sites, and leading edges of migrating monocytes and endothelial cells (43-47).

Although uPAR lacks an intracellular domain, the binding of uPA to uPAR has been shown to activate numerous intracellular signaling cascades, including Ras/ERK,

JAK/STAT, and calcium signaling pathways (70, 73, 74). Moreover, signaling induced by uPAR ligation with uPA leads to enhanced adhesion and migration of various tumor cells, human vascular smooth muscle cells and cultured endothelial cells (48, 71, 72, 75-77, 81, 84, 85, 147). In addition to uPAR occupancy by uPA, crosslinking of uPAR, which mimics uPAR clustering at focal adhesion sites, also results in calcium signaling in neutrophils and monocytes (90, 91) and JAK/STAT signal transduction in a human kidney epithelial tumor cell line (92). Although uPAR is GPI-anchored to the plasma membrane, both uPAR occupancy by uPA and uPAR clustering results in initiation of cell signaling, most likely via a transmembrane adaptor, such as the integrins (77, 90, 92).

Integrins are a family of cell adhesion receptors that bind to various components of the extracellular matrix. Integrins play a role in cell adhesion, stimulating the clustering of various extracellular matrix (ECM) receptors, and signaling, which can lead to changes in cell migration, proliferation and survival. Recent studies suggest a novel role for the integrins, which involves physically associating with and modulating members of the uPA/uPAR system (142). Integrins have been shown to co-immunoprecipitate and co-localize with uPAR on the surface of neutrophils, monocytes and certain tumor cells to enhance cellular adhesion, migration and uPAR polarization (44, 73, 77, 79, 82, 136, 139, 142, 148). Moreover, Bohuslav *et al.* have reported the interaction of uPAR, β_2 integrins and protein tyrosine kinases within a single receptor complex on the surface of human monocytes (79). Thus, it appears that macromolecular complexes of multiple proteins in close and physical proximity at the cell surface regulate certain aspects of cellular migratory and proteolytic function. Identification of an

interaction site between uPAR and integrins has recently been reported, and blockage of this site leads to impaired migration of human vascular smooth muscle cells (140). Furthermore, complexes of integrins also modulate members of the uPA/uPAR system. Bianchi *et al.* reported the induction of uPAR expression following coclustering of CD3 and β_2 integrins on the surface of T cells (143).

We have previously reported that uPA is expressed by freshly isolated and IL-2 cultured NK cells (27, 149). Moreover, we have recently shown that uPA and uPAR are employed by NK cells for their invasion and migration through the extracellular matrix (27), either alone or in combination with matrix metalloproteinases (MMPs) (26). This proteolytic activity leading to extracellular matrix degradation is an important function of NK cells, as the ECM has been shown to be a barrier for therapeutic approaches using immunotherapy in certain cancers (22). Herein, we provide evidence that uPAR clustering leads to intracellular signal transduction and changes in integrin expression, suggesting that it may be part of a large complex on the surface of NK cells that regulates aspects of migration and accumulation within tumor metastases following their adoptive transfer. Therefore, understanding the coordinated and multifaceted complex of adhesive and proteolytic molecules utilized by NK cells as they migrate into tumors may identify potential targets for improving the effectiveness of their adoptive immunotherapy. Our studies strongly suggest that the interactions between uPAR and integrins are particularly worthwhile for further investigation for devising novel strategies to enhance the accumulation into and cytolytic properties of A-NK cells within established cancer metastases.

MATERIALS AND METHODS

Materials and antibodies - FITC-labeled α_M, FITC-labeled β_2, unlabeled β_2 and α_M integrin antibodies were all purchased from Chemicon International (Temecula, California). The α_V-FITC integrin antibody was obtained from Immunotech (Fullerton, California). Murine isotype IgG_1 controls were purchased from Caltag Laboratories (Burlingame, California). Phospho-MAPK monoclonal antibody, phospho-MEK and anti-MEK polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, Massachusetts). Anti-MAPK polyclonal antibodies were obtained from Upstate Biotechnology (Lake Placid, New York). Monoclonal mouse anti-uPAR antibodies were purchased from American Diagnostica (Greenwich, Connecticut). Secondary goat anti-mouse-HRP antibodies were purchased from Pierce Chemical (Rockford, Illinois), and secondary goat anti-rabbit-HRP antibodies were obtained from Sigma (St. Louis, Missouri).

Cell Culture - The human NK cell line, YT, a kind gift from Dr. Porunellor Mathew, was cultured in RPMI-1640 supplemented with 10% FBS, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate.

Cell Homogenization - Cells were incubated in lysis buffer (10 mM Tris pH 7.4, 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 20 mM NaF, 1 mM EGTA, 1 mM EDTA, 0.2

mM PMSF, 1 U/ml aprotinin, and 1 mM $NaVO_4$) for 15 minutes on ice with continuous vortexing. Lysates were then centrifuged at 1000 x g for 8 minutes. The supernatant was saved and stored at –20 °C.

uPAR Crosslinking - Crosslinking was performed as described in similar studies by Sitrin *et al.* (91). To crosslink uPAR, cells were washed three times in RPMI and incubated with 150 μg/ml murine IgG Fc fragment (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) for 15 minutes at 4 °C to block binding of primary antibodies to Fc receptors. Following two washes in 0.1% PBS-BSA, cells were incubated with monoclonal mouse anti-uPAR antibodies or control mouse IgG antibodies for 1 hour at 4 °C. Cells were washed twice, then warmed to 37 °C for 5 minutes, followed by addition of 100 μg/ml of $F(ab')_2$ fragment of goat anti-mouse IgG antibodies (Jackson ImmunoResearch) to initiate crosslinking for 15 and 30 minutes and analyzed as described below by flow cytometry. For western blotting, the crosslinking was performed as above for 0, 1, 5, 15, and 30 minutes at 37 °C. Cells were immediately centrifuged at 5000 rpm for 30 seconds to stop the reaction and washed twice in cold 1 mM sodium orthovanadate-PBS prior to cell lysis as described above. The MEK kinase inhibitors PD98059 (Calbiochem, LaJolla, California) and U0126 (Cell Signaling Technology) were incubated with the cells at the concentration of 50 μM for 30 minutes prior to addition of the crosslinking antibodies. Cell viability following incubation with the MEK inhibitors PD98059 and U0126 was assessed using Trypan Blue dye exclusion.

Flow Cytometry - Cells were washed three times in RPMI, and resuspended at 5 x 10^5 cells/tube in 0.1% PBS-BSA. The cells were incubated with the indicated monoclonal antibodies for one hour at 4 °C, followed by two washes in cold 0.1% PBS-BSA. Cells were incubated with 10 µl of goat anti-mouse FITC-conjugated secondary antibody (Molecular Probes, Eugene, Oregon) for one hour at 4 °C. Cells were washed twice in cold 0.1% PBS-BSA and fixed in 0.5 ml of 10% formalin. For uPAR aggregation, crosslinking was performed as described above. Following two washes in cold 0.1% PBS-BSA, FITC-integrin antibodies were incubated for 1 hour at 4 °C. After final washing, cells were fixed in 0.5 ml of 10% formalin. Purified mouse IgG_1 isotype antibodies were used as controls. Data was analyzed using a Beckman Coulter EPICS XL flow cytometer.

Western blotting - Samples were resolved by 10% or 4-12% SDS-polyacrylamide gel electrophoresis (precast gels from Bio-Whittaker Molecular Applications, Rockland, Maine) at a concentration of 10 µg of protein per lane and transferred to PVDF membranes. The membranes were blocked in 5% nonfat dry milk in TBS + 0.05% Tween 20 for one hour. The membranes were incubated in the indicated primary antibodies at 1:1000 dilutions in blocking buffer overnight at 4 °C with gentle agitation. Membranes were washed and incubated with 1:25,000 dilution of secondary antibody for 45 minutes, followed by another wash. Bands were detected using SuperSignal CL-HRP substrate System (Pierce Chemical), and chemiluminescence was recorded on Hyperfilm ECL (Amersham Pharmacia Biotech, Piscataway, New Jersey). In some cases,

membranes were stripped for 30 minutes in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol) at 55 °C and reprobed as above with indicated antibody.

RESULTS

uPAR crosslinking increases integrin expression

uPAR has been shown to aggregate at the site of cell-matrix interactions and polarize to the leading edge of migrating monocytes (44, 45). Moreover, uPAR co-localizes with integrins at cell-substratum sites as shown by immunofluoresence and confocal microscopy (44, 136, 139). To mimic uPAR clustering *in vitro*, we used antibody-mediated crosslinking of uPAR, and evaluated changes in integrin expression to determine the relationship between uPAR and integrins. The α_M, α_V and β_2 integrins have been shown to be expressed by NK cells and have been reported to be in physical association with uPAR on the surface of certain cell types other than NK cells (79, 82, 106) therefore, we chose to evaluate changes in surface expression of these particular integrins following uPAR crosslinking on the surface of YT cells. Cells were first incubated with uPAR monoclonal antibodies, followed by addition of crosslinking antibodies, which was carried out at 37 °C for 15 and 30 minutes. We evaluated changes in integrin surface expression by flow cytometry using FITC-conjugated integrin monoclonal antibodies. IgG_1 isotype antibodies were used as controls. Upon uPAR clustering, an increase in α_M, α_V and β_2 integrin surface expression occurred as indicated by a shift to the right (Fig. 1B, C and D). Data was quantitated by calculating the percent change in the mean fluorescence intensities between integrin expression before and after uPAR crosslinking. The mean fluorescence intensity was 2.6 fold higher at 15 minutes and 3 fold higher at 30 minutes of uPAR crosslinking for α_M (Fig. 2A). For α_V, the mean

fluorescence intensity was 4.7 fold higher at both 15 minutes and 30 minutes of uPAR crosslinking (Fig. 2B). The mean fluorescence intensity for β_2 was 1.5 fold and 1.7 fold higher at 15 and 30 minutes of uPAR crosslinking, respectively (Fig. 2C). The increases in α_M, α_V and β_2 integrin expression following uPAR clustering on the surface of YT cells were shown to be significantly greater using a paired t-test, $p < 0.05$, n = 3.

Activation of MAPK/ERK1/2 by uPAR clustering

We next investigated whether uPAR aggregation was capable of initiating signal transduction. Crosslinking was performed as described above. Cells were incubated with the crosslinking antibodies for the indicated time periods in minutes and lysed. Samples were resolved on 10% SDS-PAGE gels and western blotting was performed using a phospho-specific MAPK/ERK1/2 antibody to evaluate changes in phosphorylation of MAPK/ERK1/2. An increase in phosphorylation of MAPK was seen at 15 and 30 minutes of uPAR clustering as shown by bands at 42 kDa, and a weaker signal at 44 kDa, indicating phospho-ERK 2 and phospho-ERK 1, respectively (Fig. 3A). Membranes were stripped and reprobed for total MAPK protein, confirming phosphorylation of MAPK was not due to an alteration in MAPK protein (Fig. 3B). As controls, isotype antibodies were crosslinked, and as expected, there was no phosphorylation of ERK1/2, thus the phosphorylation of MAPK is specific for uPAR crosslinking (Fig. 3C and 3D).

MAPK/ERK1/2 phosphorylation is completely inhibited by PD98059 and U0126

Prior to crosslinking, YT cells were incubated with 50 μM of the MEK-specific inhibitor PD98059 or U0126 for 30 minutes. Crosslinking of uPAR was carried out for the indicated times. For samples with the MEK inhibitors added, uPAR was crosslinked for 30 minutes. Samples were resolved on 4-12% gradient gels. Western blotting was performed using anti-phospho-MAPK/ERK1/2 antibodies. The phosphorylation of MAPK/ERK1/2 was completely inhibited by the MEK-specific inhibitors PD98059 or U0126 (Fig. 4A) while no change in total MAPK protein was observed (Fig. 4B).

uPAR clustering leads to activation of MEK

MEK1/2 lies upstream of MAPK in the signaling cascade and directly phosphorylates MAPK/ERK1/2; therefore, we sought to determine whether MEK1/2 is phosphorylated upon uPAR crosslinking. Following uPAR aggregation, MEK1/2 was phosphorylated at 15 and 30 minutes as indicated by a 45 kDa band (Fig. 5A). Control extracts from serum-treated NIH-3T3 cells (Cell Signaling Technology) were run as positive controls. We performed western blotting using antibodies against total MEK1/2 protein to confirm equal amounts of MEK in each lane (Fig. 5B).

PD98059 inhibits the increase in integrin expression induced by uPAR clustering

Since PD98059 inhibited the activation of the MAP kinase signaling pathway induced by uPAR clustering, we sought to determine whether it would also prevent the increase in integrin surface expression that occurs as a result of uPAR crosslinking. Prior to addition

of the crosslinking antibody, cells were incubated for 30 minutes with 50 μM PD98059. Following addition of the crosslinking antibodies, integrin expression was evaluated on cells that were both untreated and treated with the MEK-specific inhibitor PD98059. Flow cytometry data shows that PD98059 inhibits the increase in integrin expression that is seen upon uPAR clustering. Upon uPAR crosslinking, a shift to the right, indicating increasing fluorescence, occurred for the α_M, α_V and β_2 integrins; however, following incubation for 30 minutes with PD98059 prior to addition of the crosslinking antibody, the increase in integrin expression on the cell surface was inhibited (Fig. 6). The mean fluorescence intensity of α_M was 2-fold less in samples that were incubated in the presence of PD98059 during uPAR crosslinking (Fig. 7A). For α_V and β_2 integrins, the mean fluorescence intensities were 3.5 fold and 1.5 fold less, respectively, following uPAR crosslinking in the presence of PD98059 (Fig. 7B and 7C). Experimental significance was determined using a paired t-test, $p < 0.05$. Experiments were done at least three times ($n = 3$ for α_M and β_2, $n = 4$ for α_V). Therefore, this data demonstrates a novel role for uPAR on NK cells, involving activation of the MAP kinase signaling cascade, which results in increased integrin expression, and may therefore impact on NK cell function.

DISCUSSION

In addition to its role in invasion and extracellular matrix degradation, the uPA receptor has been implicated in cell signaling leading to changes in adhesion and migration of certain cells types by both ligation with uPA (48, 71, 72, 75-77, 81, 84, 85, 147) and receptor clustering (90-92). We have previously documented that IL-2 activated NK cells can accumulate within tumor metastases following their adoptive transfer (15, 16). Moreover, we have recently identified a role for uPA and uPAR in NK cell invasion through basement membrane-like extracellular matrices both alone and in cooperation with MMPs (26, 27). It was of interest to determine whether uPAR-mediated signal transduction is utilized in NK cell functions, including enhancement of their adhesion and migration into established tumor metastases. Herein, we show for the first time that uPAR is capable of carrying out intracellular signaling in NK cells and displays a novel function as a regulator of integrin expression on the surface of the NK cell line, YT.

Our studies show that uPAR clustering leads to an increase in the expression of the α_M, α_V and β_2 integrins on the surface of YT cells. It has been well documented that integrins on tumor cells cluster at cell-matrix interaction sites to enhance adhesion (150). Similarly, others have demonstrated uPAR polarization to the leading edge of migrating monocytes and endothelial cells (43-47). Furthermore, it has been reported that uPAR and integrins are in close proximity on the surface of certain cell types, such as neutrophils, monocytes and various tumor cells; this physical relationship between the

two receptors leads the cellular changes, including enhanced adhesion, migration and uPAR polarization (44, 79, 82, 86, 136, 139, 142, 148). Therefore, it is not surprising that clustering of uPAR promotes an increase in integrins on the surface of NK cells, as it appears that these two receptors act in coordination to enhance cell adhesion and movement of other migratory cell types. Nevertheless, cooperation between these two receptors may be a key mechanism to facilitate NK cell accumulation in and within microvessels of cancer metastases following adoptive therapy, a distinguishing feature for these effector cells in the immune response (15, 16).

We have further shown that uPAR crosslinking activates members of the MAPK/ERK1/2 pathway. This signaling cascade has been implicated in uPAR signal transduction in other cell types leading to a number of cellular changes, such as increases in adhesion, migration and differentiation (151). Treatment of YT cells with PD98059 not only prevents phosphorylation of MAPK/ERK1/2 but also results in inhibition of the increase in integrin expression induced by uPAR clustering. The ability of uPAR to stimulate cell signaling relies on adapter proteins, since the receptor is GPI-linked to the plasma membrane. The requirement of a transmembrane adaptor has been indicated in studies done by Resnati *et al.* in which a soluble form of uPAR induced chemotaxis in cells lacking endogenous uPAR (67). The evidence that uPAR laterally associates with integrins on the cell surface suggests that an integrin may be a likely candidate for uPAR signaling of NK cells.

MAP kinase signaling initiated by the binding of uPA to uPAR on various tumor cells has been well documented (73, 74, 81, 85). While in this study we report that

crosslinking of uPAR activates MAP kinase signal transduction, we have preliminary data showing that the binding of uPA to uPAR also results in phosphorylation of MAP kinase in YT cells (152). In tumor cells, the binding of uPA to uPAR has been correlated with increased cell adhesion and migration (73, 81, 85); therefore, we suspect similar changes may occur in NK cells, however, this remains to be determined.

In this study, we have shown that uPAR-induced signal transduction leads to increased integrin expression on the surface of the human NK cell line, YT. These results suggest that uPAR physically associates with a membrane component, such as an integrin, enabling uPAR to initiate cell signaling upon receptor clustering, a process that may be a prerequisite for NK cell migration and a key regulatory check point for their accumulation into cancer metastases. Therefore, uPAR and integrins may act in coordination to enhance NK cell migration and accumulation within established cancer metastases. Recent studies have shown that the extracellular matrix surrounding certain tumors provides a barrier for the tumor cells against immune cells, including NK cells, during immunotherapy (22). Our studies suggest that NK cells integrate aspects of adhesion and extracellular matrix degradation in order to overcome this impediment during cancer immunotherapy. Our data strongly suggests that these mechanisms may be employed by adoptively transferred NK cells as they traverse extracellular matrices and subsequently accumulate within cancer metastases following their adoptive transfer. Therefore, understanding the molecular mechanisms, including the mechanism by which uPAR and integrins regulate each other, utilized by NK cells to accumulate within

advanced tumors may represent a novel approach in hopes of increasing their therapeutic utility for treatment of established cancer metastases.

Figure 1 of Chapter II. uPAR crosslinking increases integrin expression.

Control peaks represent cells alone without antibody treatment. **A:** YT cells (5 x 10^5 cells/tube) were incubated with uPAR antibody to show uPAR expression. **B-D** YT cells were incubated with uPAR antibody for 1 hour, followed by addition of crosslinking antibody for 30 minutes at 37 °C. Changes in integrin surface expression with and without uPAR crosslinking were evaluated by flow cytometry using β_2-FITC (**B**), α_M-FITC (**C**), or α_V-FITC (**D**) monoclonal antibodies.

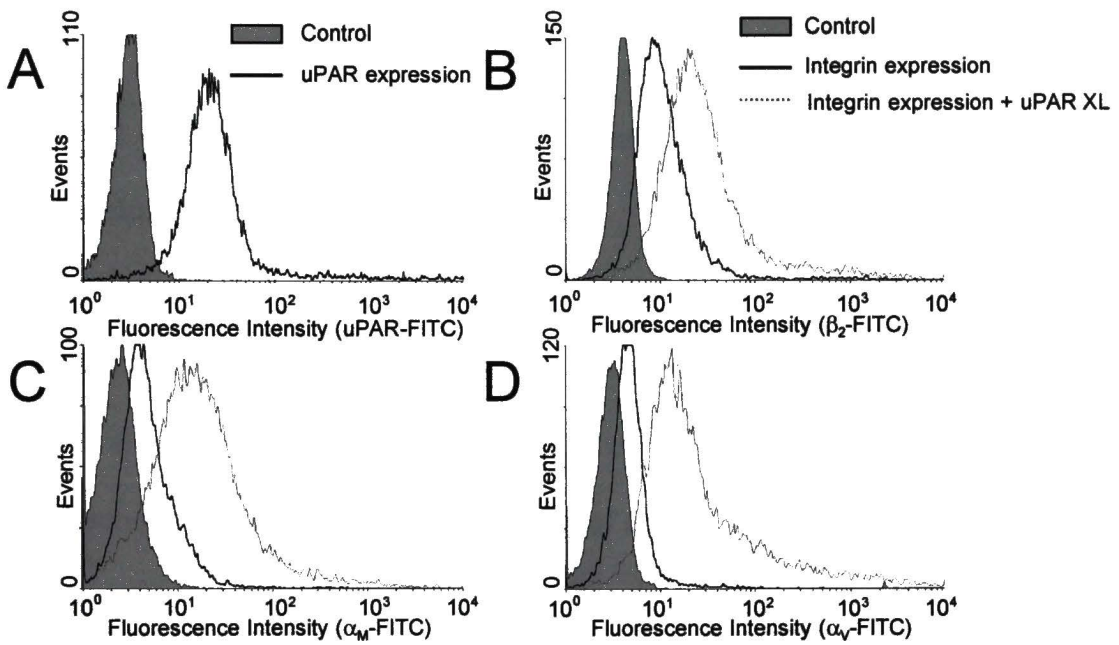
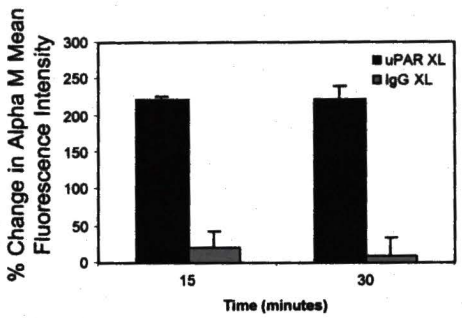

A
Control
uPAR expression
Events
110
0
10^0 10^1 10^2 10^3 10^4
Fluorescence Intensity (uPAR-FITC)
B
Control
Integrin expression
Integrin expression + uPAR XL
Events
150
0
10^0 10^1 10^2 10^3 10^4
Fluorescence Intensity (β_2-FITC)
C
Events
100
0
10^0 10^1 10^2 10^3 10^4
Fluorescence Intensity (α_M-FITC)
D
Events
120
0
10^0 10^1 10^2 10^3 10^4
Fluorescence Intensity (α_V-FITC)

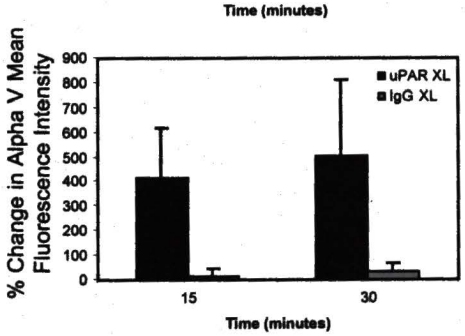
Figure 2 of Chapter II. Percent change in integrin mean fluorescence intensity upon uPAR clustering.

YT cells were incubated with uPAR antibody for 1 hour, followed by addition of crosslinking antibody for 15 and 30 minutes at 37 °C. Changes in integrin expression were evaluated using flow cytometry. uPAR crosslinking resulted in a 2.6 fold and 3 fold increase in the mean fluorescence intensity of α_M at 15 and 30 minutes, respectively (**A**). The percent change in mean fluorescence intensity of α_V was 4.7 fold higher at both 15 and 30 minutes of uPAR crosslinking (**B**) and 1.5 and 1.7 fold higher for β_2 at 15 and 30 minutes of uPAR crosslinking, respectively (**C**). All experiments were done at least three times. Experimental significance was determined using a paired t-test, $p < 0.05$.

A


% Change in Alpha M Mean Fluorescence Intensity
300
250
200
150
100
50
0
uPAR XL
IgG XL
15
30
Time (minutes)

B


% Change in Alpha V Mean Fluorescence Intensity
900
800
700
600
500
400
300
200
100
0
uPAR XL
IgG XL
15
30
Time (minutes)

C

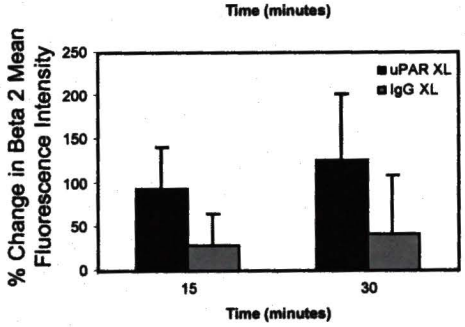

% Change in Beta 2 Mean Fluorescence Intensity
250
200
150
100
50
0
uPAR XL
IgG XL
15
30
Time (minutes)

Figure 3 of Chapter II. Activation of MAPK/ERK1/2 by uPAR clustering.

A: Cells were incubated with uPAR antibody for 1 hour, followed by incubation with crosslinking antibody for the indicated times in minutes. Cell lysates were loaded on 10% SDS-PAGE gels and immunoblotted with anti-phospho-p44/42 MAPK antibody. **B:** The stripped membrane was reprobed with anti-MAPK/ERK1/2 antibody to confirm equal amounts of MAPK protein. **C:** Isotype-matched antibody was crosslinked as a control for the indicated time periods in minutes, and cell lysates were immunoblotted with anti-phospho-p44/42 MAPK antibody. **D:** The stripped membrane was reprobed with anti-MAPK/ERK1/2 antibody to confirm equal amounts of MAPK protein.

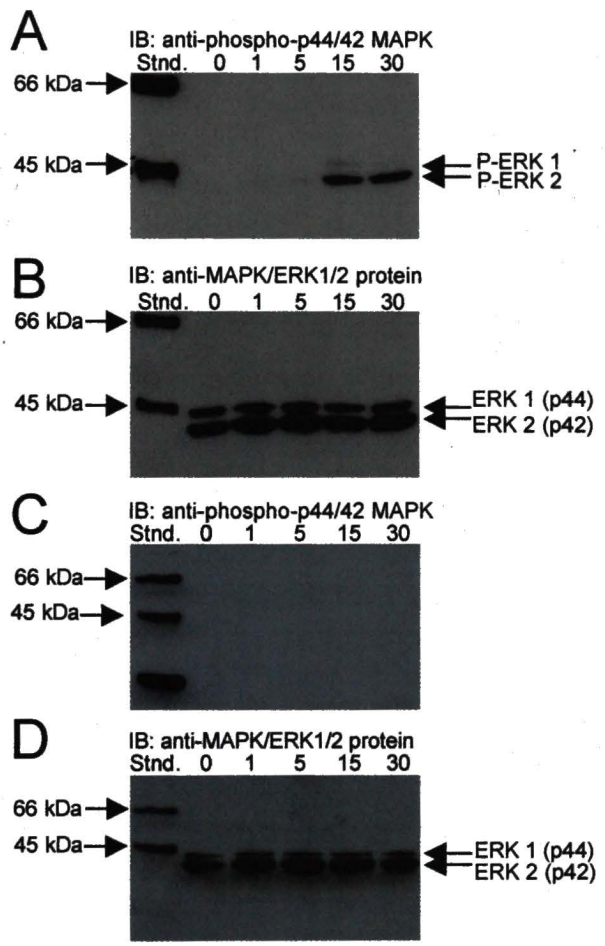

A
IB: anti-phospho-p44/42 MAPK
Stnd. 0 1 5 15 30
66 kDa
45 kDa
P-ERK 1
P-ERK 2
B
IB: anti-MAPK/ERK1/2 protein
Stnd. 0 1 5 15 30
66 kDa
45 kDa
ERK 1 (p44)
ERK 2 (p42)
C
IB: anti-phospho-p44/42 MAPK
Stnd. 0 1 5 15 30
66 kDa
45 kDa
D
IB: anti-MAPK/ERK1/2 protein
Stnd. 0 1 5 15 30
66 kDa
45 kDa
ERK 1 (p44)
ERK 2 (p42)

Figure 4 of Chapter II. PD98059 and U0126 inhibit the phosphorylation of MAPK induced by uPAR clustering.

A: YT cells were incubated with uPAR antibody for 1 hour, followed by incubation with crosslinking antibody for the indicated times in minutes (lanes 1-5). In lanes 6-7, YT cells were incubated with uPAR antibody for 1 hour and then pretreated with 50 μM of either PD98059 (lane 6) or U0126 (lane 7) MEK inhibitor for 30 minutes prior to crosslinking for 30 minutes. Cell lysates were prepared and separated on 4-12% gradient gels. Immunoblotting using an anti-phospho-p44/42 MAPK antibody was performed. **B:** The membrane was stripped and reprobed using anti-MAPK/ERK1/2 antibody to confirm equal amounts of MAPK protein in each lane.

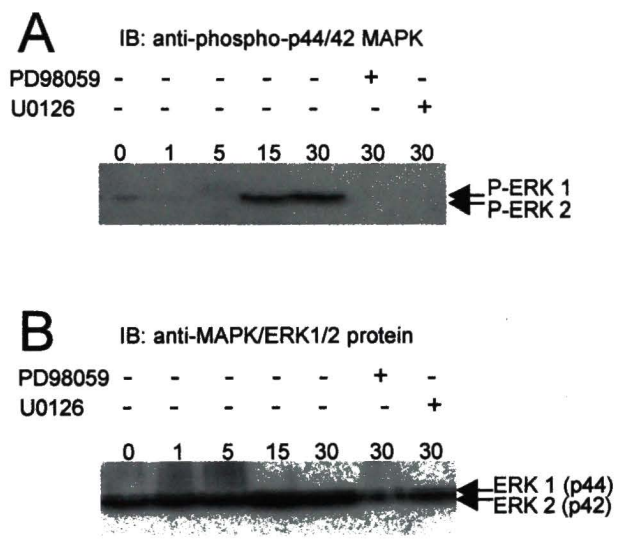

A
IB: anti-phospho-p44/42 MAPK
PD98059 - - - - - + -
U0126 - - - - - - +
0 1 5 15 30 30 30
P-ERK 1
P-ERK 2
B
IB: anti-MAPK/ERK1/2 protein
PD98059 - - - - - + -
U0126 - - - - - - +
0 1 5 15 30 30 30
ERK 1 (p44)
ERK 2 (p42)

Figure 5 of Chapter II. Activation of MEK by uPAR clustering.

A: YT cells were incubated with uPAR antibody for 1 hour, followed by incubation with crosslinking antibody for the indicated time periods in minutes. Cell lysates were immunoblotted with anti-phospho-MEK1/2 (Ser217/221) antibody. NIH-3T3 total cell extracts prepared with serum treatment were used as positive controls (lane 7). **B:** The membrane was stripped and reprobed with anti-MEK1/2 antibody to confirm equal amount of MEK protein.

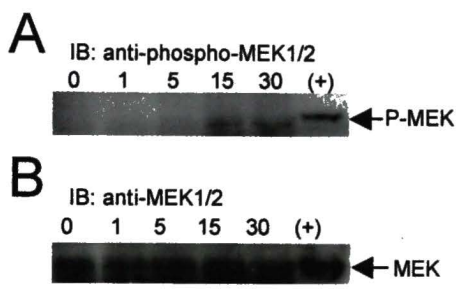

A
IB: anti-phospho-MEK1/2
0 1 5 15 30 (+)
P-MEK
B
IB: anti-MEK1/2
0 1 5 15 30 (+)
MEK

Figure 6 of Chapter II. PD98059 inhibits the increased integrin expression induced by uPAR crosslinking.

A-C: YT cells were incubated at a concentration of 5 x 10^5 cells per tube with uPAR antibody for 1 hour and pretreated with 50 μM of the MEK-specific inhibitor PD98059 at 37 °C for 30 minutes prior to crosslinking uPAR for 30 minutes. Following uPAR crosslinking, changes in integrin expression with and without PD98059 treatment were evaluated by flow cytometry using FITC-labeled integrin antibodies. The increases in the α_M (**A**), α_V (**B**) and β_2 (**C**) integrins following uPAR clustering were inhibited by PD98059.

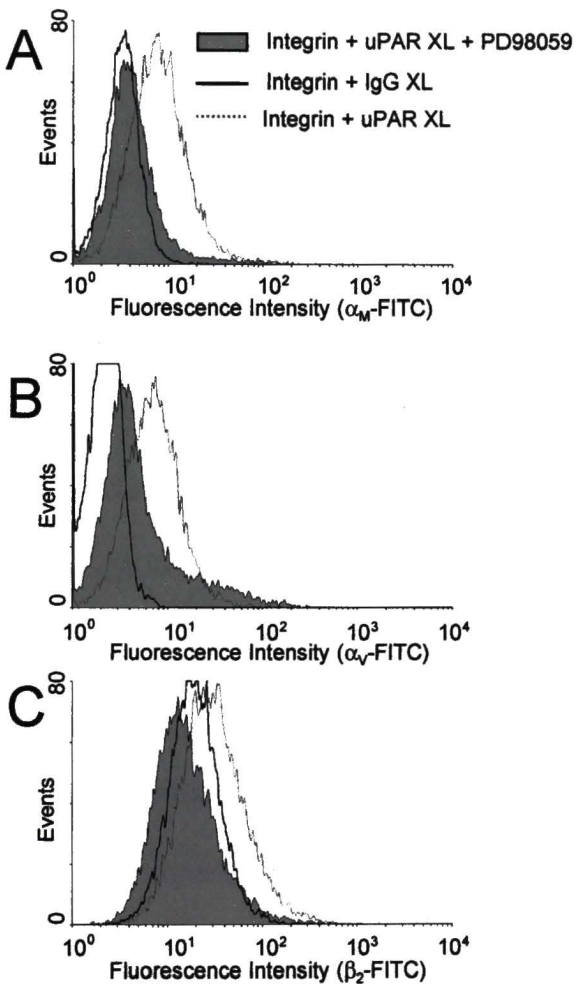
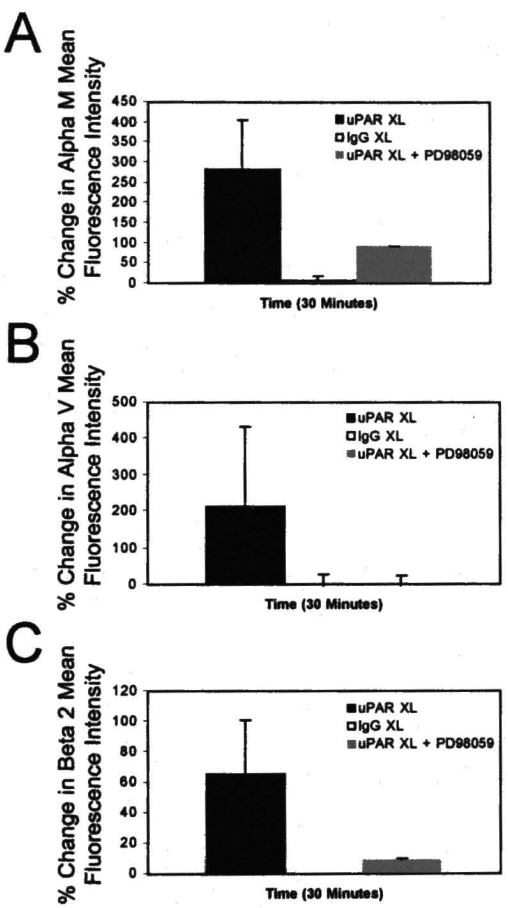

A
Integrin + uPAR XL + PD98059
Integrin + IgG XL
Integrin + uPAR XL
Events
80
0
10^0 10^1 10^2 10^3 10^4
Fluorescence Intensity (α_M-FITC)
B
Events
80
0
10^0 10^1 10^2 10^3 10^4
Fluorescence Intensity (α_V-FITC)
C
Events
80
0
10^0 10^1 10^2 10^3 10^4
Fluorescence Intensity (β_2-FITC)

Figure 7 of Chapter II. PD98059 inhibits the increased integrin mean fluorescence intensity following uPAR crosslinking.

YT cells were incubated with uPAR antibody for 1 hour and pretreated with 50 μM of PD98059 at 37 °C for 30 minutes prior to crosslinking uPAR for 30 minutes. Changes in integrin expression were evaluated using flow cytometry. Data was quantitated by calculating the percent change in mean fluorescence intensities between samples with and without PD98059. The mean fluorescence intensities for α_M (**A**), α_V (**B**) and β_2 (**C**) integrins were 2 fold, 3.5 fold, and 1.5 fold less, respectively, in samples that were incubated in the presence of PD98059 during uPAR crosslinking. All experiments were done at least three times. Experimental significance was determined using a paired t-test, $p < 0.05$.


A
% Change in Alpha M Mean Fluorescence Intensity
450
400
350
300
250
200
150
100
50
0
uPAR XL
IgG XL
uPAR XL + PD98059
Time (30 Minutes)
B
% Change in Alpha V Mean Fluorescence Intensity
500
400
300
200
100
0
uPAR XL
IgG XL
uPAR XL + PD98059
Time (30 Minutes)
C
% Change in Beta 2 Mean Fluorescence Intensity
120
100
80
60
40
20
0
uPAR XL
IgG XL
uPAR XL + PD98059
Time (30 Minutes)

Chapter III

Physical Association of uPAR with the α_V Integrin and Induction of the MAP Kinase Signaling Pathway by the Binding of uPA to uPAR on the Surface of Human NK Cells

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ABSTRACT

The urokinase-type plasminogen activator receptor (uPAR) serves as a receptor for the enzyme urokinase plasminogen activator (uPA) and plays a role in the invasiveness and migration of certain immune cells, including NK cells. Although uPAR is anchored to the plasma membrane via a glycosylphosphatidylinositol lipid moiety, and is devoid of an intracellular domain, we have previously shown that uPAR crosslinking results in MAP kinase signaling, leading to an increase in integrin expression on the surface of the human NK cell line, YT. In addition to receptor clustering, we report, herein, that the binding of uPA to uPAR also activates the MAP kinase signaling cascade. Phosphorylation of both MEK kinase and MAP kinase occurs following uPA stimulation. Furthermore, we show the physical association between uPAR and integrins on the surface of YT cells using fluorescence microscopy to show the cocapping of BODIPY-labeled uPA, which labels uPAR, with the FITC-labeled α_V integrin. N-acetyl-D-glucosamine blocks such association, as it has been suggested that uPAR and integrins associate via lectin-like interactions. These results suggest that signaling initiated by either uPAR binding to uPA or by uPAR clustering may depend on the physical association of uPAR with integrins, a process that may be a prerequisite for NK cell accumulation within established tumor metastases during adoptive therapy.

INTRODUCTION

Urokinase plasminogen activator receptor (uPAR) serves as a receptor for the enzyme urokinase plasminogen activator (uPA), which converts the zymogen plasminogen into the active enzyme plasmin leading to cleavage of certain components of the extracellular matrix (ECM). Therefore, one of the major functions of uPAR is to bind uPA and localize ECM and basement membrane degradation to the pericellular surface, thus promoting cellular migration and invasion. uPAR binds both inactive single-chain uPA (scuPA) and active two-chain uPA (tcu-uPA), which results from the cleavage of scuPA by plasmin at the lysine 158-isoleucine 159 peptide bond (153). Furthermore, uPA can be cleaved between amino acids 135 and 136 resulting in a low molecular weight form of uPA (LMW-uPA), which retains catalytic activity but is devoid of the uPAR-binding domain, and amino-terminal uPA (ATF-uPA), which is catalytically inactive but contains the uPAR-binding domain. uPAR is a GPI-anchored protein and lacks an intracellular domain. Nevertheless, recent studies in tumor cells have shown that uPAR is capable of initiating various intracellular signaling cascades following uPA stimulation resulting in changes in cellular migration (77, 81, 83-85, 147). Disruption of the binding of uPA to uPAR in human pancreatic carcinoma cells blocked cellular migration on vitronectin (86). Similarly in certain leukocytes, uPA has been reported to activate cell signaling leading to both a chemotactic response and increased adhesion of monocytes (67, 75). In human neutrophils, uPA treatment has been associated with enhanced superoxide production (88) and has stimulated neutrophil chemotaxis *in vitro*

(89). In addition to uPA binding, antibody-mediated crosslinking of uPAR, which mimics uPAR aggregation at the site of cell-matrix interactions, also resulted in cell signaling in certain leukocytes, such as neutrophils and monocytes (90, 91). We have recently reported the initiation of MAP kinase signaling, which leads to increased integrin expression following uPAR crosslinking in NK cells (154). Since uPAR cannot initiate signal transduction alone, signaling must rely on the physical association of uPAR with other transmembrane proteins, such as integrins.

Previous studies have shown that uPAR co-precipitates with members of the integrin family in monocytes (79) and various tumor cells (73, 77, 82, 137-139). The integrins are heterodimeric receptors for extracellular matrix proteins and therefore play a role in adhesion of migratory cells, such as leukocytes and tumor cells. Integrins are capable of initiating various signaling cascades, which lead to changes in cellular migration, proliferation and survival. These receptors also contribute to the firm adhesion of leukocytes to the vasculature during migration throughout processes of immunity. In addition to co-immunoprecipitation, studies using fluorescence microscopy have also revealed the close physical association of uPAR with integrins on the surface of monocytes, neutrophils and certain tumor cells (44, 79, 136, 142). Recently, an interaction site between uPAR and integrins was identified and shown to be critical for human vascular smooth muscle cell migration (140). Moreover, trans interactions between integrins and uPAR have been reported, as both β1 and β3 integrin-expressing cells were adherent to uPAR-coated surfaces, a process blocked in the presence of integrin-blocking antibodies (141). The connection between integrins and uPAR is not

unanticipated since cellular migration and invasion depends on the close association of certain aspects of adhesion and extracellular proteolysis; therefore, complexes of uPAR and integrins may be a critical molecular feature used by cells during movement.

We have previously shown that uPA and uPAR are produced by NK cells and contribute to NK cell invasion through extracellular matrix, either alone, or in combination with matrix metalloproteinases (MMPs) (26, 27). Production of uPA and MMPs equip NK cells with proteolytic machinery that may be a key feature used by these cells during adoptive immunotherapy for cancer treatment. Immunotherapy using lymphokine-activated killer (LAK) cells, which are primarily comprised of NK cells (8), have been used for treating various cancer metastases (5, 6). The use of NK cells for treatment of tumor metastases has great potential, as marked tumor regression of certain cancers has occurred in patients receiving LAK therapy (4). We have previously reported that adoptively transferred IL-2 activated NK (A-NK) cells can bind to tumor vasculature and accumulate within established cancer metastases (15, 16). Therefore, our current studies involve investigating aspects of NK cell adhesion and proteolysis, in particular uPAR and integrins, which may contribute to the migratory and ECM-degrading properties of these cells during immunotherapy. Herein, we show that the binding of uPA to uPAR activates MAP kinase signaling, a process that may depend on the physical association of uPAR with integrins. Using fluorescence microscopy and cocapping, we show the physical association of uPAR with the α_V integrin. This data reveals that there are complexes of adhesive and proteolytic molecules, i.e. integrins and uPAR, functioning on the surface of NK cells, thus possibly serving as a key molecular feature

of these cells during NK cell accumulation within tumor metastases. A better understanding of the migratory processes of NK cells following their adoptive transfer may lead to improved approaches for the use of immune effector cells, including NK cells, in immunotherapy for the treatment of advanced cancer.

MATERIALS AND METHODS

Materials and antibodies - The α_V integrin monoclonal antibody was purchased from Chemicon International (Temecula, California). ChromoPure mouse IgG Fc fragments and AffiniPure F(ab')$_2$ goat anti-mouse IgG fragments were both purchased from Jackson ImmunoResearch (West Grove, Pennsylvania). Murine isotype IgG_1 controls were purchased from Caltag Laboratories (Burlingame, California). High molecular weight uPA (HMW-uPA), ATF-uPA and LMW-uPA were all purchased from American Diagnostica (Greenwich, Connecticut). BODIPY-uPA and diisopropyl fluorophosphate-inactivated uPA (DFP-uPA) were kind gifts from Dr. Andrew Mazar. Phospho-MAPK monoclonal antibodies and phosphoPlus MEK 1/2 antibody kit (containing phospho-MEK and anti-MEK polyclonal antibodies, goat anti-rabbit-HRP antibodies, anti-biotin antibodies, and Phototope-HRP Detection System) were purchased from Cell Signaling Technology (Beverly, Massachusetts). Anti-MAPK polyclonal antibodies were obtained from Upstate Biotechnology (Lake Placid, New York). Secondary goat anti-mouse-HRP antibodies were purchased from Pierce Chemical (Rockford, Illinois), and secondary goat anti-rabbit-HRP antibodies were obtained from Sigma (St. Louis, Missouri). CD56-FITC was purchased from Southern Biotechnology Associates, Inc. (Birmingham, Alabama), and CD71-FITC was obtained from Cymbus Biotechnology (Chandlers Ford, UK).

Cell Culture - YT cells (human NK cell line, a kind gift from Dr. Porunellor Mathew) were cultured in RPMI-1640 supplemented with 10% FBS, 0.1 mM MEM nonessential

amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate.

uPA stimulation - Cells were washed three times and resuspended in RPMI-0.1% BSA at a concentration of 10 x 10^6 cells per 200 μl. Cells were stimulated with 50 nM HMW-uPA, ATF-uPA, DFP-uPA, or LMW-uPA for 0, 1, 5, 15, and 30 minutes at 37 °C. To inhibit phosphorylation, 50 μM of the MEK kinase inhibitor U0126 (Cell Signaling Technology, Beverly, Massachusetts) or 1:200 (v/v) DMSO was incubated with the cells for 30 minutes at 37 °C prior to addition of uPA. To stop the reaction, cells were immediately centrifuged at 5000 rpm for 30 seconds, washed twice in cold 1 mM sodium orthovanadate-PBS and lysed as described below.

Cell Homogenization - Cells were incubated in lysis buffer (10 mM Tris pH 7.4, 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 20 mM NaF, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 1 U/ml aprotinin, and 1 mM $NaVO_4$) for 15 minutes on ice with continuous vortexing. Lysates were then centrifuged at 1000 x g for 8 minutes. The supernatant was saved and stored at –20 °C.

Western blotting - Samples were resolved on 10% precast gels (Bio-Whittaker Molecular Applications, Rockland, Maine) at a concentration of 20 μg of protein per lane and transferred to PVDF membranes. The membranes were blocked in 5% nonfat dry milk in Tris-buffered saline (TBS) + 0.1% Tween 20 for one hour. The membranes were

incubated with the indicated primary antibodies at 1:2000 dilutions in blocking buffer overnight at 4 °C with gentle agitation. Membranes were washed and incubated with 1:25,000 dilution of secondary antibody for 45 minutes, followed by another wash. Bands were detected using SuperSignal CL-HRP substrate System (Pierce Chemical, Rockford, Illinois), and chemiluminescence was recorded on Hyperfilm ECL (Amersham Pharmacia Biotech, Piscataway, New Jersey). In some cases, membranes were stripped for 30 minutes in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol) at 55 °C and reprobed as above with the indicated antibody. For phospho-MEK and MEK, western blotting was performed according to manufacturer's instructions. Briefly, membranes were blocked as above, incubated with primary antibodies at 1:2000 dilutions in 5% BSA + 0.1% TBS-T overnight, washed and incubated with 1:2000 goat anti-rabbit and anti-biotin antibodies in 5% nonfat dry milk + 0.1% TBS-T. Bands were detected using Phototope-HRP detection system.

Cocapping Assays - To remove uPAR-bound uPA, cells were first subjected to acid treatment as described by Stoppelli *et al.* (155). Following two washes in PBS-0.1% BSA, cells were allowed to attach to poly-L-lysine-coated slides (10 μg/ml) for 1 hour at 4 °C. After brief washing, cells were incubated with 150 μg/ml murine IgG Fc fragment for 30 minutes at 4 °C to block binding of primary antibodies to Fc receptors. Cells were again washed and incubated with a 1:50 dilution of primary antibodies in PBS-0.1% BSA for 1 hour at 4 °C. After washing, cells were incubated with 1:50 $F(ab')_2$ fragment of goat anti-mouse IgG-TRITC antibodies (Jackson ImmunoResearch) in PBS-0.1% BSA

+/- 0.15 M N-acetyl-D-glucosamine (NADG) or sucrose for 15 minutes at 4 °C, then shifted to 37 °C for 45 minutes to induce capping. Slides were washed in ice cold PBS with 0.1% BSA and 0.1% sodium azide. Cells were next incubated with 60 nM BODIPY-uPA in PBS-0.1% BSA for 2 hours at 4 °C with or without 1.2 μM unlabeled HMW-uPA acting as a competitor to the BODIPY-uPA. To investigate cocapping of α_V with control surface proteins, cells were incubated with CD71-FITC or CD56-FITC monoclonal mouse antibodies both in the presence of and following one-hour blocking with mouse IgG antibodies to block any remaining secondary antibody goat anti-mouse $F(ab')_2$ fragments . Following washing, cells were fixed in 10% formalin for 15 minutes and one drop of Vectashield (Vector Laboratories, Inc., Burlingame, California) was placed on the cells and covered with a coverslip. Cells were analyzed using fluorescence microscopy.

Fluorescence Microscopy and Data Quantification - Cells were examined using an Olympus Provis AX microscope (Olympus America Inc., Melville, New York) and images were collected using an Olympus D811 camera. Random coordinates of cells were selected, and all of the cells viewed in that area were counted. The percentage of capping was obtained by dividing the number of cells exhibiting capping by the total number of cells counted. The percentage of cocapping was determined by dividing the number of cells displaying overlapping of the double labels by the number of capped cells.

RESULTS

uPAR occupancy by uPA results in phosphorylation of MAPK

We have previously reported that antibody-mediated uPAR clustering results in phosphorylation of MAP kinase (154); therefore, we investigated whether the binding of the physiological ligand, uPA, to uPAR also results in activation of the MAP kinase signaling pathway. Cells were stimulated for the indicated time in minutes with 50 nM of the active full-length two-chain HMW-uPA, the catalytically inactive ATF-uPA, DFP-inactivated uPA, or LMW-uPA, which retains activity but lacks the uPAR-binding domain. Following HMW-uPA stimulation, an increase in phosphorylation of MAP kinase, preferentially ERK 2, was seen (Fig. 1A, top panel). Similarly, ATF-uPA and DFP-uPA, both of which bind uPAR but are proteolytically inactive, resulted in the phosphorylation of MAP kinase (Fig. 1B and C, top panels). Incubating cells with the MEK-specific inhibitor U0126 for 30 minutes at 37 °C prior to each 30-minute uPA stimulation inhibited the phosphorylation (last lane). In contrast, LMW-uPA, which lacks the uPAR-binding domain, was unable to initiate MAP kinase phosphorylation (Fig. 1D, top panel); therefore, it appears that the cell signaling response mediated by uPA stimulation results from uPAR occupancy, rather than uPA enzymatic activity. All membranes were stripped and reprobed for total MAP kinase protein to ensure equal amounts of protein were loaded (Fig. 1A-D, bottom panels). In control samples, cells were incubated with 1:200 (v/v) DMSO, which was used to dissolve U0126, and did not alter the increased phosphorylation of MAP kinase in response to uPA (data not shown).

Activation of MEK following uPA stimulation

To determine whether MEK is activated following uPA stimulation, cells were stimulated as indicated above. Cells were incubated with ATF-uPA, DFP-uPA or LMW-uPA for 30 minutes prior to cell lysis. In lanes 1-5, an increase in MEK phosphorylation occurred at 15 and 30 minutes. For ATF-uPA (lane 6) and DFP-uPA (lane 7), similar phosphorylation was seen at 30 minutes of incubation; however, LMW-uPA failed to stimulated MEK phosphorylation (lane 8). As a positive control, serum-treated NIH-3T3 cell extracts were used and showed notable phosphorylation of MEK (lane 9).

uPAR cocaps with the α_V integrin

Since uPAR is GPI-anchored to the cell membrane, signaling initiated by the binding of uPA to uPAR must rely on a transmembrane adapter. Integrins are likely candidates for transmitting uPAR-related signals, as many co-precipitation and co-localization studies have shown the close proximity and physical association of the two receptors in tumor cells and certain leukocytes (44, 73, 77, 79, 82, 136-139, 142). Therefore, we wished to determine whether uPAR was in close proximity with integrins on the surface of NK cells. In particular, we chose to investigate the cocapping of uPAR with the α_V integrin, which we have previously demonstrated to be increased by uPAR crosslinking (154) and has been shown to co-immunoprecipitate with uPAR in human monocytes (79). Cells were first incubated with α_V integrin monoclonal antibodies, followed by incubation with TRITC-conjugated $F(ab')_2$ secondary antibodies either at 4 °C or 37 °C, which induces

capping. Cells were then labeled with BODIPY-uPA to detect uPAR. Under resting conditions (4 °C), a uniform distribution of α_V and uPAR was observed (Fig. 3A and B). However, on cells that were incubated at 37 °C to promote capping of α_V, a clear codistribution of uPAR with the α_V integrins occurred (Fig. 3C and D), thus indicating that the two receptors are physically linked together on the surface of YT cells. As a control, no cocapping occurred between the α_V integrin and CD71, the transferrin receptor, (Fig. 3E-H) or CD56, an NK cell marker, (Fig. 3I-L). Data was quantitated in Table I. From the total number of cells counted, α_V was capped in 93% of the cells, and uPAR was found to cocap in 86% of the total cells, whereas, the control surface proteins CD71 and CD56 failed to show considerable cocapping with the α_V integrin. The fact that CD56 showed slightly higher cocapping than CD71 may be due to the possible binding of the mouse anti-CD56 antibodies to the remaining goat anti-mouse $F(ab')_2$ secondary antibodies as a result of improper blocking by mouse IgG antibodies. In addition, as a control, fluorescence did not occurred when cells were incubated with isotype-matched control antibodies rather than the α_V monoclonal antibody or when incubated with the secondary TRITC-labeled goat anti-mouse $F(ab')_2$ fragments alone (data not shown). Fluorescence of BODIPY-uPA was diminished in cells that were not acid-treated or in cells that were co-incubated with BODIPY-uPA and unlabeled HMW-uPA acting as a competitor (data not shown).

Cocapping of uPAR with α_V integrins is inhibited by NADG

Previous studies have indicated that lectin-like interactions may exist between uPAR and integrins, as the cocapping of β1 or β3 integrins and uPAR on fibrosarcoma cells and β2 integrins with uPAR on the surface of neutrophils has been shown to be inhibited by certain saccarides, such as NADG, but not by sucrose or fructose (136, 142). To examine whether uPAR's interaction with the α_V integrins could be inhibited on the surface of YT cells, we performed the capping portion of the cocapping experiment described above in the presence of 0.15 M NADG or 0.15 M sucrose, similar to studies done by Xue *et al.* (142). The NADG saccharide inhibited the cocapping of α_V and uPAR (Fig. 4A and B), while sucrose failed to inhibit such association (Fig. 4C and D). Quantitative data shows that only 17% of the capped cells counted exhibited cocapping of uPAR with α_V when treated with 0.15 M NADG, while sucrose appears to have no effect, as 85% of cells show cocapping of uPAR and α_V (Table II.).

DISCUSSION

Herein, we show that the binding of uPA to uPAR is capable of activating MAP kinase signaling, regardless of the enzymatic activity of uPA and despite the structure of the GPI-anchored uPAR, which lacks an intracellular domain. We have previously shown that uPA and uPAR are important in the invasiveness of NK cells through ECM (27). Moreover, we have shown that the proteolytic activity of uPA in combination with MMPs leads to increased ECM degradation by NK cells, a process that may be a prerequisite for NK cell migration and invasion during adoptive immunotherapy for cancer treatment (26). There is a strong correlation between members of the uPA system and leukocyte migration during immunity and inflammation (62-65). For example, uPAR-deficient mice have decreased recruitment of leukocytes to inflamed peritoneum (62). Studies have shown that uPA-deficient mice are more predisposed to bacterial infections, thus supporting the important role of uPA proteolysis involved in migration and invasion of immune cells during infection and disease (63, 65). Conversely, other reports indicate that uPA and uPAR strongly correlate with the invasive potential of various tumor cells and malignant cells both *in vitro* and during cancer metastasis (52-55, 57, 156). Nevertheless, a novel functional role of uPA has emerged, independent of its proteolytic activity, involving the binding of uPA to uPAR leading to changes in adhesion and migration of various tumor cells (77, 81, 83-85, 147). Herein, we show that not only does full-form active uPA initiate MAP kinase signaling, but the catalytically inactive uPA derivative, ATF-uPA, and the DFP-inactivated uPA also effectively cause

phosphorylation of MEK and MAP kinase. This data suggests that uPAR signaling in NK cells is stimulated by receptor occupancy alone, independent of uPA enzymatic activity, and may be a crucial process in NK cell migratory potential and invasiveness into tumors during adoptive immunotherapy. We have previously shown that adoptively transferred A-NK cells can bind to tumor vasculature and accumulate within established cancer metastases (15, 16) and have more recently shown that uPA and uPAR are employed by NK cells to invade through extracellular matrix (27). In addition, we have reported that uPAR crosslinking activates intracellular signaling leading to increased integrin surface expression on NK cells (154); therefore, the investigation of uPAR signaling in NK cells is of particular interest in order to further elucidate the mechanism by which NK cells move from the vasculature and into tumor metastases.

Additionally, we have shown that uPAR cocaps with the α_V integrin on the surface of YT cells, a process that is abrogated by NADG but not by sucrose. Our data demonstrating the cocapping of uPAR with integrins leads to speculation that the signaling induced by uPA stimulation in NK cells may occur through lateral associations between uPAR and integrins. In human monocytes, uPAR has been shown to be part of a large receptor complex with β_2 integrins and Src-kinases, thus linking uPAR with signaling components (79). As mentioned, a number of studies have shown the functional consequence of uPAR signaling to be instrumental in controlling the migratory processes of tumor cells (77, 81, 83-85, 147). Moreover, uPA-dependent cell migration has been largely correlated with the direct interaction of uPAR and integrins in various cancer cell lines (77, 82, 86). These findings imply that molecular complexes of

components of adhesion and proteolysis, which we have shown to exist on NK cells, may also be a key mechanism used by NK cells as they bind to tumor vasculature (19) and accumulate within cancer metastases (15). Therefore, the functional relevance of the physical interaction between uPAR and integrins on the surface of NK cells may be especially important during NK cell migration and accumulation within established metastases during immunotherapy.

Our previous studies have demonstrated that crosslinking uPAR activates MAP kinase signaling, and subsequently leads to increased α_M, α_V and β_2 integrin expression on the surface of NK cells (154). Therefore, together with results presented here, signaling induced by the GPI-anchored uPAR on NK cells is activated by both receptor clustering and occupancy with its physiological ligand, uPA. We have found that uPA stimulation did not result in increased α_M, α_V and β_2 integrin surface expression (data not shown), as we have previously shown to occurr with uPAR crosslinking. Hence, we are still elucidating the functional outcome following uPA stimulation in NK cells and suspect that such stimulation may nonetheless lead to changes that favor NK cell migration. The finding that uPAR cocaps with integrins on the surface of NK cells enhances the possibility that uPAR-initiated signaling may rely on its physical interaction with integrins. With regards to similar studies in tumor cells, these findings support the hypothesis that uPA stimulation leads to intracellular signaling possibly via the direct interaction of uPAR with integrins and thereby conceivably increasing NK cell migration, a process critical for NK cell accumulation within tumor metastases during cancer therapy. Investigation of these processes and discovery of the molecular mechanisms

underlying the regulation of NK cell migration and accumulation into advanced tumors are essential in hopes of developing novel targets for improving the effectiveness of NK cells during adoptive immunotherapy.

Figure 1 of Chapter III. uPA stimulation leads to MAPK phosphorylation.

Cells were incubated with HWW-uPA (**A**), ATF-uPA (**B**), DFP-uPA (**C**) or LMW-uPA (**D**) for the indicated times in minutes. In the last lane, cells were preincubated with U0126 and then stimulated with the indicated forms of uPA in the presence of the inhibitor for 30 minutes. Lysates were subjected to Western blotting using an anti-phospho-p44/p42 MAPK antibody (**A-D, top panels**). **A-D, bottom panels:** Stripped membranes were reprobed with anti-MAPK/ERK1/2 antibody to confirm that equal amounts of MAPK protein were loaded.

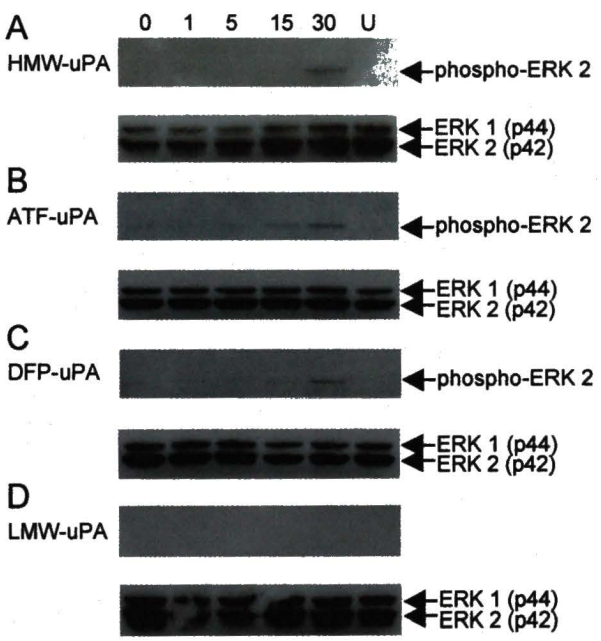

A
0 1 5 15 30 U
HMW-uPA
phospho-ERK 2
ERK 1 (p44)
ERK 2 (p42)
B
ATF-uPA
phospho-ERK 2
ERK 1 (p44)
ERK 2 (p42)
C
DFP-uPA
phospho-ERK 2
ERK 1 (p44)
ERK 2 (p42)
D
LMW-uPA
ERK 1 (p44)
ERK 2 (p42)

Figure 2 of Chapter III. Activation of MEK following uPA stimulation.

Cells were stimulated with HMW-uPA for the indicated times in minutes (lanes 1-5) or with ATF-uPA (lane 6), DFP-uPA (lane 7), or LWW-uPA (lane 8) for 30 minutes. Serum-treated NIH-3T3 total cell extracts were run as a positive control (last lane). Lysates were immunoblotted with anti-phospho-MEK1/2 (Ser217/221) antibody (top panel) or with anti-MEK1/2 antibody to ensure equal protein loading in each lane (bottom panel).

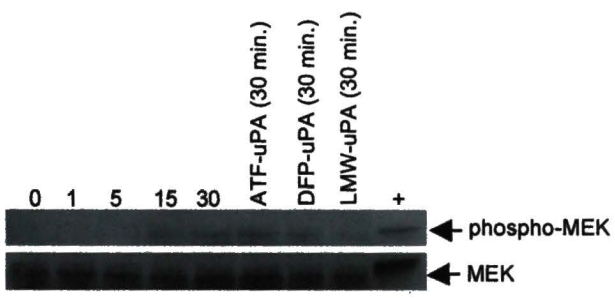

0
1
5
15
30
ATF-uPA (30 min.)
DFP-uPA (30 min.)
LMW-uPA (30 min.)
+
phospho-MEK
MEK

Figure 3 of Chapter III. Cocapping of α_V with uPAR.

A-D: Cells were labeled with α_V antibody followed by TRITC-conjugated F(ab')$_2$ secondary antibodies in absence (**A**) and presence (**C**) of capping. The same cells were also labeled with BODIPY-uPA to detect uPAR (**B** and **D**). **E-L:** Cells were labeled with _$_V$ and TRITC-labeled secondary antibodies in absence (**E** and **I**) and presence (**G** and **K**) of capping. Prior to fixation, cells were also labeled using either CD71-FITC (**F** and **H**) or CD56-FITC (**J** and **L**).

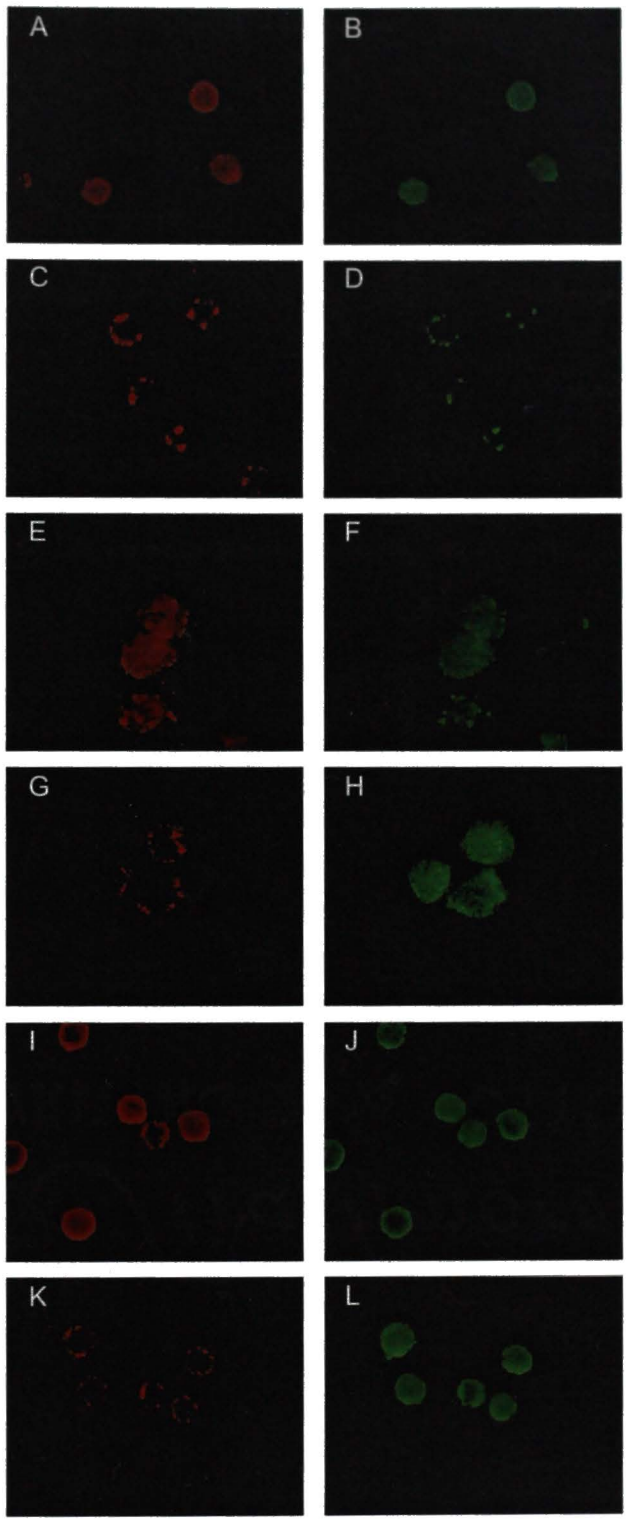

A
B
C
D
E
F
G
H
I
J
K
L

Table I. Percent cocapping of αV and uPAR

Cap	Cocap	Total Cells (#)	Caps (%)	Cocaps (%)
alpha V	uPAR	108	93	86
alpha V	CD 71	65	85	9
alpha V	CD 56	72	94	29

Figure 4 of Chapter III. Cocapping of α_V and uPAR is inhibited by NADG.

Cells were incubated with primary α_V monoclonal antibodies and capped using TRITC-conjugated F(ab')$_2$ secondary antibodies at 37 °C (**A** and **C**). The same cells were examined for uPAR cocapping using BODIPY-uPA (**B** and **D**). The top panels represent cells capped in the presence of NADG (**A** and **B**), while the bottom panels show cells capped in the presence of sucrose (**C** and **D**).

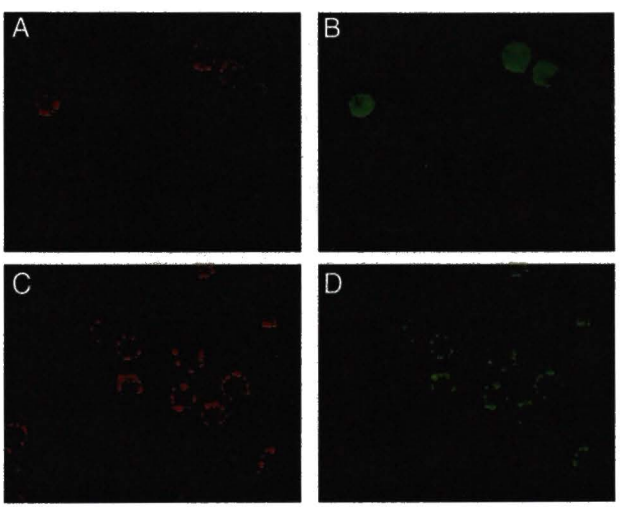

A
B
C
D

Table II. Inhibition of αV and uPAR cocapping by NADG

Saccaride	Total Cells (#)	Caps (%)	Cocaps (%)
NADG	75	88	17
Sucrose	106	80	85

CHAPTER IV

DISCUSSION

The use of NK cells during adoptive immunotherapy has shown therapeutic potential for cancer treatment. While NK cells accumulate within tumors following their adoptive transfer (15), the molecular mechanisms NK cells utilize to achieve tumor infiltration remain elusive. NK cells display significant cytotoxicity against tumor cells; therefore, the problem appears to remain with improving the penetration of NK cells into tumor metastases. The finding that the ECM serves as a potential barrier between NK cells and tumors (22) indicates that there is a need to overcome these hindrances in order to achieve optimal therapeutic results. The recent findings that NK cells express both MMPs and uPA establishes that these cells are equipped with matrix-degrading enzymes needed for dissolution of the ECM (23-27). Nevertheless, the possible relationship these enzymes may have with integrins on the surface of NK cells remains unknown, and the cooperative interaction between them may be an essential feature employed by NK cells for their migration and invasion into cancer metastases. The results presented in this dissertation establish for the first time that, aside from its traditional role as a receptor for uPA and localizing proteolysis to the pericellular surface, uPAR is a signaling receptor and physically associates with integrins on NK cells. In **Chapter II**, the crosslinking of uPAR not only results in activation of MAP kinase signaling but also leads to an increase

in integrin expression on the surface of the NK cell line, YT. **Chapter III** shows that uPAR occupancy with uPA, aside from receptor clustering, also activates MAPK. Furthermore, the physical association between uPAR and integrins is reported in **Chapter III.**

The finding that uPAR crosslinking and uPAR occupancy with uPA both result in the phosphorylation of MEK and MAP kinase (Chapter II) suggests that the GPI-anchored receptor must form an association with a membrane adapter capable of transmitting intracellular signals. In almost all of the studies to date, the most prominent membrane proteins that uPAR has been shown to physically interact with, on cells other than NK cells, are integrins. Moreover, most, if not all, of the signaling pathways that are activated by uPAR are also initiated by integrins (157). Only a few other membrane proteins associate with uPAR, including the α2-MR/LPR (low density lipoprotein receptor-related protein) and caveolin (50, 137). The α2-MR/LPR does not seem to be the major link to uPAR signaling, as its foremost function appears to be internalization of the uPA/uPAR/PAI complex (50). uPAR co-precipitates with caveolin (137); however, it has been well documented that the β subunit of integrins directly associates with caveolin (125). Hence, the interaction of uPAR with caveolin may be a consequence of the coexistence of uPAR within a macromolecular complex containing integrins/caveolin. Therefore, it is highly likely that integrins are the signaling adapters utilized by uPAR in NK cells.

uPAR has been shown to cluster at focal adhesion sites and at the leading edge of migratory cells (158). Similarly, integrins cluster at these sites; therefore, the signaling

that is induced *in vitro* by using crosslinking antibodies (Chapter II), may possibly result in conformational changes in uPAR, either allowing its association with integrins or causing a structural change within the integrin, to activate cell signaling. Likewise, with uPA stimulation, groups have shown that the interaction of uPAR with integrins is promoted by uPA ligation with uPAR (77, 140), again suggesting that uPA alters the conformation of uPAR, exposing an unidentified epitope that recognizes integrins. Correspondingly, uPA stimulation, regardless of proteolytic activity, is enough to activate signaling (Chapter III); hence, the binding of uPA to uPAR alone may either promote uPAR/integrin interactions or induce conformational changes resulting in signaling, a process completely independent of uPA activity.

In vivo, synergism between activation of uPAR-mediated signaling induced by uPA binding and by clustering following adhesion to matrices may exist. Synergy between uPA binding to uPAR and uPAR ligation with vitronectin has been well established, as uPA has been shown to enhance the binding of uPAR to vitronectin (42, 159). As mentioned above, uPA stimulation promotes the physical interaction of uPAR with certain integrins (77, 140). Also, uPA binding to uPAR leads to exposure of a vitronectin-binding domain on uPAR (66), thereby enhancing uPAR-mediated adhesion and possibly increasing uPAR clustering. The binding of uPAR to vitronectin has been reported to activate p130Cas and Rac signaling leading to actin reorganization and increased cell motility (93). Since integrins also bind vitronectin, uPAR and integrins may cluster together at these vitronectin-rich regions of the ECM. Therefore *in vivo*, uPA, which promotes the binding of uPAR to vitronectin, may enhance uPAR-mediated

adhesion and co-clustering with integrins, resulting in uPAR-induced signal transduction. While it appears that uPA promotes uPAR binding to vitronectin and can induce lateral associations of uPAR with certain integrins (42, 77), there have been no reports correlating uPAR antibody-mediated crosslinking with increased uPA secretion. To date, only two groups have investigated the effects of uPAR crosslinking *in vitro* (90-92). One group showed that uPAR clustering leads to activation of JAK1/STAT1 signaling in a human kidney epithelial tumor cell line (92). The other group reported activation of calcium signaling in monocytes and neutrophils following uPAR crosslinking (90, 91). Although these studies did not involve the investigation of uPA secretion following uPAR crosslinking, Sitrin *et al.* found that the magnitude of calcium signaling was related to the quantity of uPAR crosslinking regardless of the state of occupancy with uPA (91). Nevertheless, the finding that uPA promotes uPAR adhesion to vitronectin and to integrins leads to speculation that cells may synergistically increase uPA secretion following uPAR clustering as a strategic mechanism to enhance these adhesive and migratory functions.

The consequence of uPAR crosslinking in NK cells is an increase in integrin surface expression (Chapter II), thus suggesting a role for uPAR in controlling integrin expression and function. This finding is not surprising, as others have shown suPAR to promote cellular adhesion to fibronectin (73). Sitrin *et al.* reported that treatment with antisense uPAR or anti-uPAR monoclonal antibodies reduced monocyte adhesion to certain ECM proteins (44). Another study in uPAR-deficient mice showed impaired neutrophil recruitment to the lungs in response to pulmonary infection, similar to results

seen with cells treated with β_2 integrin blocking antibodies, thereby suggesting that uPAR is a modulator of β_2 integrin function (64). May *et al.* showed that β_2 integrin-mediated adhesion of leukocytes to the endothelium was lost upon removal of surface-bound uPAR (62). Since uPAR and integrins are both found in clusters at sites of cell attachment to the ECM or at leading edges of migrating cells (158), it remains to be determined whether the increased integrin expression *in vitro* by antibody-mediated uPAR crosslinking functions in altering cell adhesion or in providing additional adapters for uPAR signaling or perhaps both.

The increased integrin surface expression as a consequence of uPAR clustering and MAPK signaling shown in Chapter II occurs relatively quickly (within 15 to 30 minutes of crosslinking). Clearly, the increase cannot be a result of transcriptional or translational events; therefore, the integrins probably exist in vesicles within the cytoplasm of NK cells. It has been well documented that integrins are found inside subcellular vesicles of certain leukocytes, and activation by cytokines and inflammatory stimuli leads to immediate vesicle release on the cell surface (118-121). Moreover, MAPK/ERK 1/2 is capable of phosphorylating cytoplasmic substrates, in addition to nuclear transcription factors (129). Therefore, it may be highly likely that the MAPK signaling pathway activated by uPAR crosslinking in NK cells terminates at some cytoplasmic substrate, which allows rapid vesicle release, thus explaining the sudden increase in integrin surface expression. In addition, both the activation of MAPK and the increased integrin expression on the cell surface are inhibited by the MEK inhibitor PD98059 (Chapter II), further supporting the hypothesis that MAP kinase may be

transmitting signals to vesicles prompting their release to the plasma membrane. Furthermore, PD98059 has been shown to inhibit ERK 2 phosphorylation and subsequently block granule exocytosis during target cell recognition in human NK cells (160). Also, PD98059-mediated inhibition of ERK activation was associated with a dose-dependent decrease of CD16-stimulated NK cell degranulation (161). These results linking MAPK signaling with immediate degranulation in NK cells suggests that a similar MAPK-mediated mechanism may result in the release of integrin-containing vesicles.

In Chapter II, uPAR clustering caused activation of MEK/MAPK signaling leading to increased integrin surface expression. Similarly in Chapter III, uPA stimulation initiated the same pathway; however, the functional consequence of uPA-induced signaling in NK cells remains unknown. Since uPA stimulation causes enhanced migration of certain tumor cells (77, 81, 83-85, 147), we suspect such stimulation may also lead to changes that favor NK cell migration. However, additional cellular changes may result from uPA stimulation, such as increased cytokine production, which may have an impact on other immune effector cells, and an increase in the cytotoxicity and killing capacity of NK cells. Nonetheless, more work is needed in this area to establish the physiological significance of uPA-mediated signaling in NK cells.

Also in Chapter III, the physical association of uPAR with integrins is reported for the first time in NK cells. As mentioned, integrins may be the signaling adapter for uPAR, and conformational changes in either receptor may induce uPAR/integrin interactions and initiate intracellular signaling of the uPAR/integrin complex. Since work

presented herein establishes uPAR as a signaling receptor in NK cells, and uPAR is shown to interact with integrins, data imply that integrins may link uPAR with intracellular signaling molecules. However, without inhibitors that disrupt and/or prevent the interaction between uPAR and integrins, we can only speculate. Wei *et al.* were the first to screen a bacteriophage peptide display library for uPAR-binding phages to identify potential peptide inhibitors of uPAR-integrin interactions; this group found that one such peptide, p25, disrupted β_1 integrin-caveolin-uPAR complexes and blocked cell adhesion to vitronectin (137). Another group showed that p25 treatment reduced ERK phosphorylation following its activation by fibronectin and reduced organization of fibrils in human carcinoma cells (73, 94). This peptide has also been reported to decrease tumor cell attachment to vitronectin, but not fibronectin, disrupt uPAR/β_1 co-precipitates and prevent tumor progression (139). Recently a peptide, showing homology to the p25 phage display peptide, has been identified (140). This peptide, M25, is derived from a non-I domain-binding site on the α_M subunit and is capable of disrupting the interaction of uPAR with a subset of β_1 and β_2 integrins, and M25 blocked cell adhesion to fibrinogen, but had no effect on adhesion to fibronectin (140). Interestingly, suPAR was found to bind to immobilized M25, a process inhibited by soluble M25, but not by p25 (140). While there are regions of uPAR that stimulate chemotaxis (67, 69), and cells expressing recombinant β_1 and β_3 integrins form trans interactions with D2/D3 suPAR-coated surfaces (141), the exact region(s) of uPAR that binds to integrins remains obscure. Fazioli *et al.* has mapped the chemotactic site to residues $S_{88}R_{89}S_{90}R_{91}Y_{92}$ in the D1-D2 linker region of uPAR (69), thus implying that this sequence may be an essential

integrin-binding site. However, several recent reports have shown that the D1-D2 linker region of uPAR is not involved in uPAR/integrin interactions (62, 138, 162). Montuori *et al.* found that cleaved forms of uPAR do not interact with β1 integrins, and this group more recently discovered that D1 removal abolishes the association of uPAR with α and β integrin chains and its capability to regulate integrin adhesive functions (138, 162). Also, uPAR-negative cells that were transfected with intact uPAR induced activation of ERK, which was abolished by D1 removal and independent of the presence of SRSRY (162). Furthermore, chymotrypsin-cleaved uPAR, which exposes the SRSRY sequence, is unable to interact with β2 integrins on human leukocytes (62). These results suggest that there is a region on uPAR, other than the D1-D2 linker region that is responsible for forming an association with integrins. The finding that the binding of uPAR to immobilized M25 is not blocked by p25 (140) also leads to speculation that there is more than one interaction site. Furthermore, uPAR-binding sites on integrins may be different for each subunit, as p25 has been shown to inhibit cell adhesion to certain ECM proteins, while having no effect or an increasing effect on the adhesion of cells to other matrix proteins (137, 139, 140). As these sites become identified, strides can be made to develop inhibitors to disrupt the various uPAR/integrin interactions, thereby enhancing our understanding of the significance of these complexes.

Other than protein-protein interactions, lectin-like interactions have been suggested to exist between uPAR and certain integrins (136, 142). Xue *et al.* were the first to show cocapping of uPAR with the $\alpha_M\beta_2$ integrin on neutrophils membranes was inhibited by NADG and D-mannose, but not by fucose, glucose, sorbitol, sucrose and

galactose (142). In Chapter III, data shows that NADG treatment inhibited the cocapping of uPAR with the α_V integrin, whereas sucrose had no effect. Despite these results, NADG did not inhibit the MAP kinase signaling induced by uPAR clustering or following uPA stimulation and had little to no effect on the increase in integrin surface expression induced by uPAR crosslinking. The fact that NADG can prevent cocapping of uPAR with α_V but have no effect on signaling suggests that NADG may prevent unassociated uPA receptors with binding to integrins, while being unable to disrupt those that are already in a complex with integrins. Therefore the signaling that is seen results from activating integrin-bound uPAR, which is already in lateral association with integrins. Furthermore, it is greatly possible that signaling is achieved though the lateral association of uPAR with an integrin other than α_V. While it has been suggested that the $\alpha_M\beta_2$ integrin possesses lectin-like activity, it remains unclear whether NADG is acting on uPAR or integrins or only certain integrins; therefore, some uPAR/integrin complexes may not necessarily be effected by the carbohydrate. The exact identification of these protein-protein and protein-carbohydrate regions and the disruption of between these sites will be crucial steps in expanding knowledge of the functionality of uPAR/integrin complexes and their involvement in migration.

Another possibility in investigating the role integrins play in uPAR signaling and uPAR-mediated changes in cellular adhesion and migration relies on specifically blocking integrin signaling. Certainly there are many kinase inhibitors that block most, if not all, of the pathways initiated by integrins, but inhibitors that are specific for integrins are presently unknown. Inhibiting caveolin expression has been shown to suppress

integrin signaling (125); however, not all integrins require caveolin for signal transduction (150). The same limitations exist for FAK, as FAK is activated by most, but not all, integrins (125). Integrin-linked kinase (ILK) has been largely associated with integrin signaling, but nonetheless also links growth factor receptors to downstream signaling components (163); therefore, inhibiting ILK may inhibit growth factor-related signaling as well as integrin signaling. While many of these findings show promise in inhibiting signaling by certain specific integrins, further investigation is warranted to discover inhibitors specific for integrin signaling alone and confirm uPAR-mediated signaling is acting solely through these integrins.

A model demonstrating the role of uPAR/integrin associations and the cooperative interplay of adhesive and proteolytic components is shown in figure 1 and indicates the possible mechanisms that may be used *in vivo* for NK cell infiltration into tumors. Generally, NK cells must first adhere to the matrix surrounding the tumor, followed by matrix dissolution and migration into the tumor site. As mentioned, the ECM serves as a potential barrier between the tumor and immune effector cells (22). Therefore, to overcome this hindrance, NK cells may employ molecular mechanisms described in this dissertation to enhance their accumulation within tumors. **uPAR clustering**: Adhesion to the matrix mediated by integrins and uPAR/vitronectin interactions probably results in clustering of these matrix-binding receptors at adhesion sites and leads to conformational changes in integrins and uPAR, thus promoting their lateral associations on the surface of NK cells. Whether uPAR clustering is a direct result from its interaction with vitronectin or a result of being linked with integrins as they

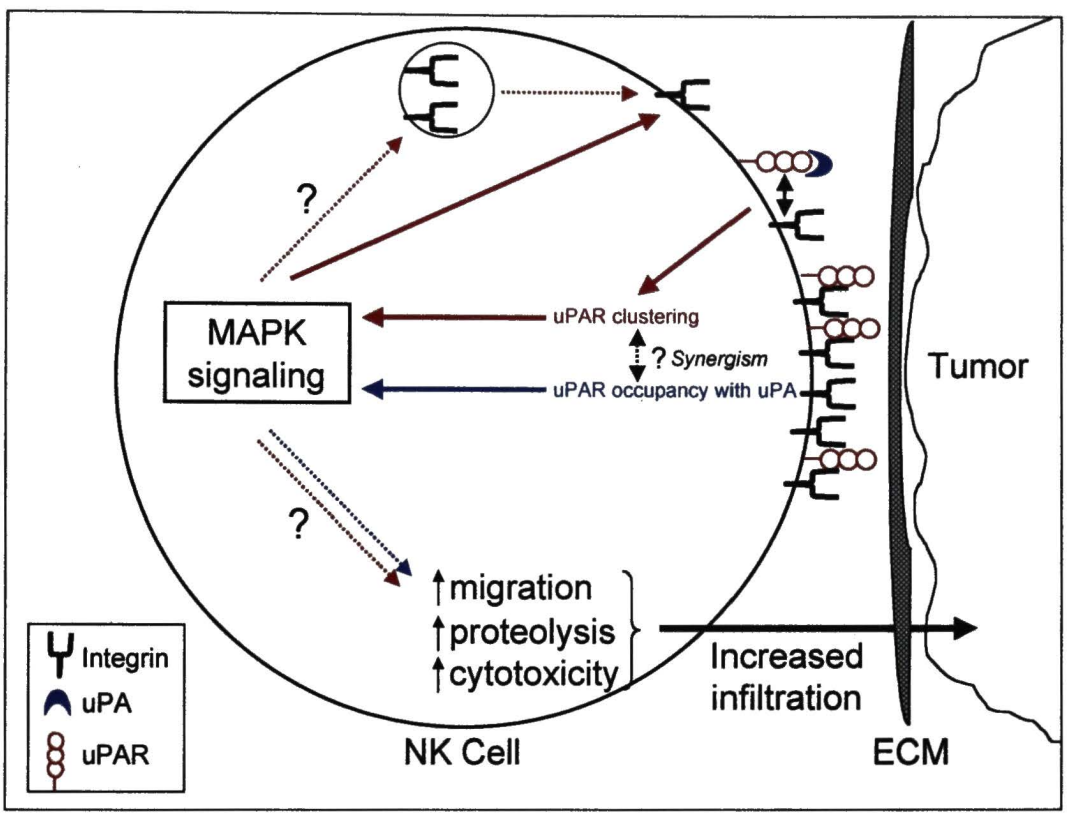
cluster following matrix binding remains elusive. Nevertheless, results presented in Chapter II suggest that clustering alone, independent of uPAR's interaction with vitronectin, causes activation of intracellular signaling. *In vivo*, coclustering of uPAR with integrins may be enhanced by the simultaneous interaction of uPAR with vitronectin and/or uPA binding to uPAR, both of which may lead to conformational changes in uPAR that promotes its lateral association with integrins; however, as mentioned above, this type of synergism remains to be determined. In Chapter II, immediate increases in integrin surface expression occur as a result of MAPK signaling induced by uPAR crosslinking; therefore, this data suggests that MAPK signaling may possibly terminate on a cytoplasmic substrate leading to integrin-containing vesicle release. This increase in integrin surface expression may serve to provide additional adapters for uPAR-mediated signaling or increase the adhesive capabilities of the cell. Following adhesion, NK cells must migrate and invade into the tumor site. Therefore, while uPAR crosslinking increases integrin surface expression as shown in Chapter II, clustering may concurrently lead to increased matrix-degrading capabilities via enhanced expression of uPA and MMPs. This would be an advantageous tactic by NK cells to improve their invasive potential. While the *in vitro* reports regarding uPAR clustering are minimal, showing activation of intracellular signaling (90-92) and granule release of neutrophils (91), the complete functional outcome needs further investigation. Nonetheless, in addition to enhanced adhesion, uPAR clustering may also induce cellular changes leading to increased NK cell migration, invasion or cytotoxicity. **uPA stimulation**: uPA stimulation similarly activates uPAR-mediated MAPK signaling. Likewise, signaling

resulting from uPA binding to uPAR may also lead to increased adhesion and proteolysis of NK cells; therefore, there may be a positive feedback mechanism mediated by uPAR occupancy with uPA leading to additional uPA expression, as well as possible increased expression of other proteases. Since uPA stimulation of various tumor cells has been shown to increase cellular adhesion and migration (77, 81, 83-85, 147), uPA binding to uPAR on NK cells, which has been shown, herein, to induce signaling (Chapter III), probably leads to increase adhesion and motility of NK cells. Moreover, the complex environment *in vivo* leads to the conjecture that NK cells may utilize endogenously produced and secreted uPA, or they may also recruit uPA from neighboring cells. Again, uPA binding to uPAR may also function in promoting the binding of uPAR to vitronectin, thus enhancing uPAR-mediated adhesion and probable co-clustering with integrins. Since uPA has been shown to lead to actin rearrangement and increased motility of other cell types (72, 77, 81, 83-85, 147), the signaling that is activated by uPA binding to uPAR shown in Chapter III may result in actin rearrangement and increased NK cell migration. While most studies have revealed an increase in the migratory and invasive potential of tumor cells following uPA stimulation, other cellular changes may occur in NK cells as a result of the binding of uPA to uPAR, such as increased cytokine production, which may influence other immune effector cells, and an increase in the killing capacity of NK cells, such as increased granule release and cytotoxicity. Therefore, uPAR may be an extraordinarily critical and crucial molecule on NK cells, mediating signaling, following both receptor clustering and occupancy with uPA, through

its lateral interaction with integrins and thereby potentially enhancing the infiltration of NK cells into tumor sites and cytotoxicity of NK cells against tumor cell targets.

The work presented in this dissertation shows that: **1)** uPAR clustering on the surface of NK cells results in activation of MAPK signaling (Chapter II), **2)** MAPK signal transduction induced by uPAR crosslinking leads to increased integrin surface expression (Chapter II), **3)** uPA binding to uPAR causes MAPK signaling and is independent of proteolytic activity (Chapter III), **4)** uPAR is physically associated with the α_V integrin on the surface of NK cells (Chapter III). These results reveal uPAR as a signaling receptor in NK cells and suggest that such signaling is carried out by the physical interaction of uPAR with integrins. Identifying these mechanisms and the process of combining adhesive and proteolytic properties in NK cells may lead to the identification of potential targets for increasing NK cell migration and invasion into tumor metastases. Therefore, increased expression of one or more of these components may lead to enhanced NK cell infiltration into tumors, thereby optimizing this dynamic function and improving their effectiveness during adoptive immunotherapy for cancer treatment.

Figure 1 of Chapter IV. Model for the role of uPAR/integrin interactions in NK cell infiltration into tumors.


?
MAPK signaling
uPAR clustering
? Synergism
uPAR occupancy with uPA
?
migration
proteolysis
cytotoxicity
Increased infiltration
Tumor
Integrin
uPA
uPAR
NK Cell
ECM

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