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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 NK cell function, including the potential for their accumulation within tumor metastases following adoptive transfer. J. Cell. Biochem. 89: 279–288, 2003.

Key words: NK cells; urokinase receptor; MAP kinase; integrins

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) [Herberman et al., 1987], for treatment of both animals bearing cancer metastases as well as in cancer patients [Rosenberg et al., 1985, 1987, 1988; Schoof et al., 1988; Basse et al., 1991a]. We have previously reported that adoptively transferred A-NK cells can accumulate within established cancer metastases in a time and IL-2-dependent manner [Basse et al., 1991a]. Moreover, A-NK cells bind to tumor vasculature and accumulate within established tumor metastases [Basse et al., 1991b]. 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 [Al-Atrash et al., 2001].

uPAR is a glycosylphosphatidylinositol (GPI)-anchored receptor that binds uPA. uPA is a serine protease that cleaves plasminogen to plasmin, contributing to extracellular matrix (ECM) proteolysis, thus promoting cellular migration and invasion (e.g., Al-Atrash et al., 2001). 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 [Estreicher et al., 1990; Myohanen et al., 1993; Pepper et al., 1993; Gyetko et al., 1994; Sitrin et al., 1996].

Although uPAR lacks an intracellular domain, the binding of uPA to uPAR has been shown to activate numerous intracellular signaling

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cascades, including Ras/ERK, JAK/STAT, and calcium signaling pathways [Konakova et al., 1998; Aguirre Ghiso et al., 1999; Dumler et al., 1999]. 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 [Busso et al., 1994; Stahl and Mueller, 1994, 1997; Li et al., 1995; Dumler et al., 1998; Tang et al., 1998; Yebra et al., 1999; Kusch et al., 2000; Nguyen et al., 2000; Jo et al., 2002]. 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 [Sitrin et al., 1999, 2000] and JAK/STAT signal transduction in a human kidney epithelial tumor cell line [Koshelnick et al., 1997]. 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 [Koshelnick et al., 1997; Sitrin et al., 1999; Yebra et al., 1999].

Integrins are a family of cell adhesion receptors that bind to various components of the ECM. Integrins play a role in cell adhesion, stimulating the clustering of various 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 [Xue et al., 1994]. 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 [Xue et al., 1994, 1997; Bohuslav et al., 1995; Sitrin et al., 1996; Aguirre Ghiso et al., 1999; Carriero et al., 1999; Wei et al., 1999; Yebra et al., 1999; van der Pluijm et al., 2001]. Moreover, Bohuslav et al. [1995] have reported the interaction of uPAR, β_2 integrins, and protein tyrosine kinases within a single receptor complex on the surface of human monocytes. 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 [Simon et al., 2000]. Furthermore, complexes of integrins also modulate members of the uPA/uPAR system. Bianchi et al. [1996] reported the induction of uPAR expression following coclustering of CD3 and β_2 integrins on the surface of T cells.

We have previously reported that uPA is expressed by freshly isolated and IL-2 cultured NK cells [Goldfarb et al., 1984; Al-Atrash et al., 2001]. Moreover, we have recently shown that uPA and uPAR are employed by NK cells for their invasion and migration through the ECM [Al-Atrash et al., 2001], either alone or in combination with matrix metalloproteinases (MMPs) [Al-Atrash et al., 2000]. This proteolytic activity leading to ECM 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 [Kuppen et al., 2001]. 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.

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, CA). The α_V-FITC integrin antibody was obtained from Immunotech (Fullerton, CA). Murine isotype IgG_1 controls were purchased from Caltag Laboratories (Burlingame, CA). Phospho- MAPK monoclonal antibody, phospho-MEK, and anti-MEK polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-MAPK polyclonal antibodies were obtained from Upstate Biotechnology (Lake Placid,

NY). Monoclonal mouse anti-uPAR antibodies were purchased from American Diagnostica (Greenwich, CT). Secondary goat anti-mouse-HRP antibodies were purchased from Pierce Chemical (Rockford, IL), and secondary goat anti-rabbit-HRP antibodies were obtained from Sigma (St. Louis, MO).

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 min on ice with continuous vortexing. Lysates were then centrifuged at 1,000*g* for 8 min. The supernatant was saved and stored at −20°C.

uPAR Crosslinking

Crosslinking was performed as described in similar studies by Sitrin et al. [2000]. 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, PA) for 15 min 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 h at 4°C. Cells were washed twice, then warmed to 37°C for 5 min, 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 min and analyzed as described below by flow cytometry. For Western blotting, the crosslinking was performed as above for 0, 1, 5, 15, and 30 min at 37°C. Cells were immediately centrifuged at 5,000 rpm for 30 s 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, CA) and U0126 (Cell Signaling Technology) were incubated with the cells at the concentration of 50 μM for 30 min 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 5×10^5 cells/tube in 0.1% PBS–BSA. The cells were incubated with the indicated monoclonal antibodies for 1 h 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, OR) for 1 h 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 h 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 1 h. The membranes were incubated in the indicated primary antibodies at 1:1,000 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 min, 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, NJ). In some cases, membranes were stripped for 30 min 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 [Estreicher et al., 1990; Sitrin et al., 1996]. Moreover, uPAR co-localizes with integrins at cell-substratum sites as shown by immunofluoresence and confocal microscopy [Sitrin et al., 1996; Xue et al., 1997; van der Pluijm et al., 2001]. 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 [Maenpaa et al., 1993; Bohuslav et al., 1995; Carriero et al., 1999]; 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 min. 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,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 min and threefold higher at 30 min of uPAR crosslinking for α_M (Fig. 2A). For α_V, the mean fluorescence intensity was 4.7-fold higher at both 15 and 30 min of uPAR crosslinking (Fig. 2B). The mean fluorescence intensity for β_2 was 1.5 and 1.7-fold higher at 15 and 30 min 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 min 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,

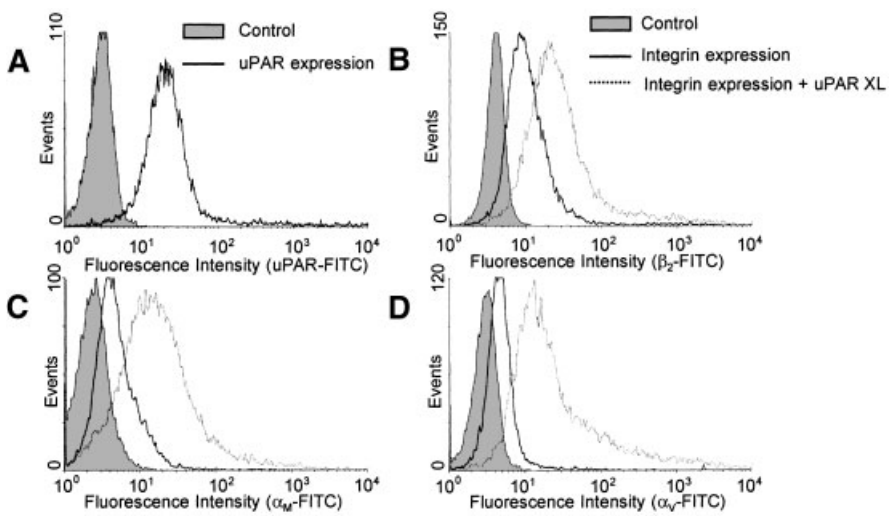

Fig. 1. uPAR crosslinking increases integrin expression. Control peaks represent cells alone without antibody treatment. **A**: YT cells (5×10^5 cells/tube) were incubated with uPAR antibody to show uPAR expression. **B–D**: YT cells were incubated with uPAR antibody for 1 h, followed by addition of crosslinking antibody for 30 min 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.

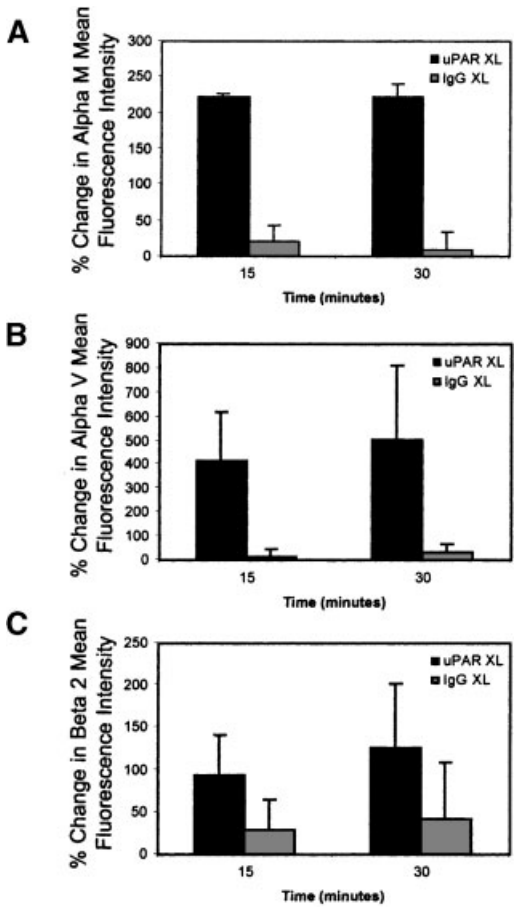

Fig. 2. Percent change in integrin mean fluorescence intensity upon uPAR clustering. YT cells were incubated with uPAR antibody for 1 h, followed by addition of crosslinking antibody for 15 and 30 min at 37°C. Changes in integrin expression were evaluated using flow cytometry. uPAR crosslinking resulted in a 2.6 and 3-fold increase in the mean fluorescence intensity of α_M at 15 and 30 min, respectively (**A**). The percent change in mean fluorescence intensity of α_V was 4.7-fold higher at both 15 and 30 min of uPAR crosslinking (**B**) and 1.5 and 1.7-fold higher for β_2 at 15 and 30 min of uPAR crosslinking, respectively (**C**). All experiments were done at least three times. Experimental significance was determined using a paired *t*-test, $P < 0.05$.

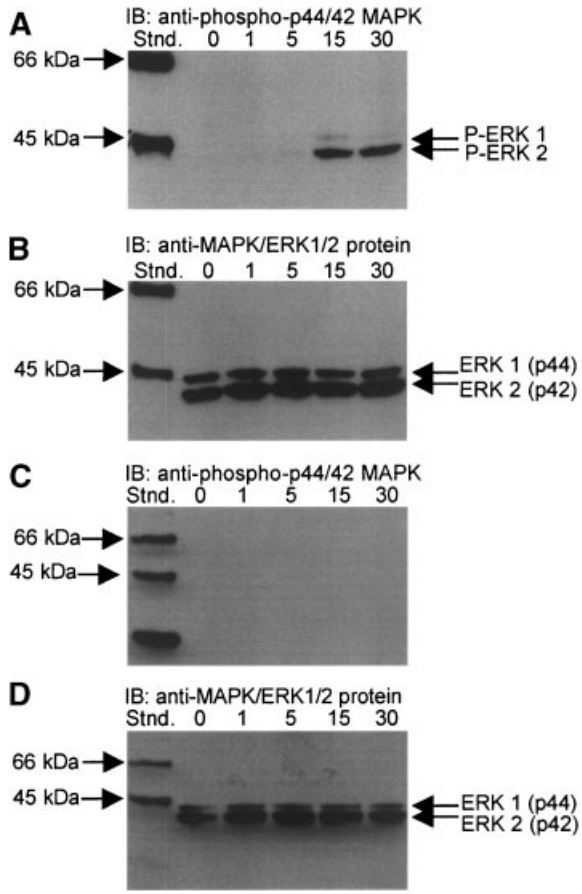

Fig. 3. Activation of MAPK/ERK1/2 by uPAR clustering. **A**: Cells were incubated with uPAR antibody for 1 h, followed by incubation with crosslinking antibody for the indicated times in minutes. Cell lysates were loaded on 10% SDS–PAGE gels and immunoblotted with anti-phospho-p44/42 MAPK antibody. **B**: 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.

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,D).

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 min. Crosslinking of uPAR was carried out for the indicated times. For samples with the MEK inhibitors added, uPAR was crosslinked for 30 min. 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

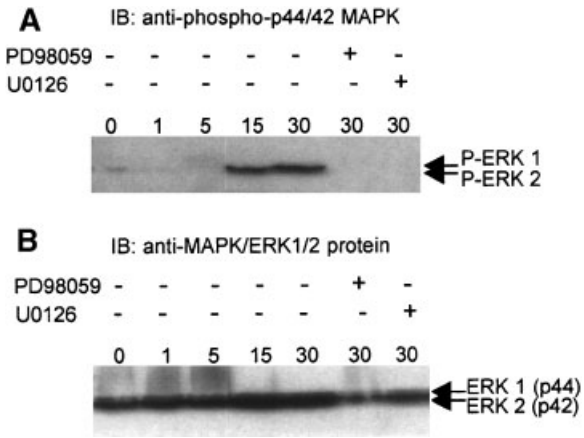

Fig. 4. PD98059 and U0126 inhibit the phosphorylation of MAPK induced by uPAR clustering. **A**: YT cells were incubated with uPAR antibody for 1 h, followed by incubation with crosslinking antibody for the indicated times in minutes (**lanes 1–5**). In **lanes 6–7**, YT cells were incubated with uPAR antibody for 1 h and then pretreated with 50 μM of either PD98059 (**lane 6**) or U0126 (**lane 7**) MEK inhibitor for 30 min prior to crosslinking for 30 min. 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.

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 min 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).

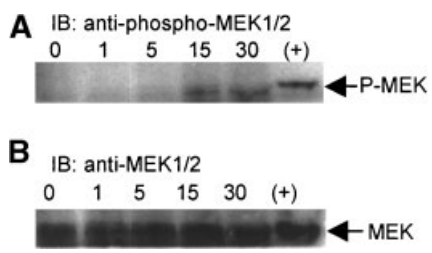

Fig. 5. Activation of MEK by uPAR clustering. **A**: YT cells were incubated with uPAR antibody for 1 h, followed by incubation with crosslinking antibody for the indicated time periods in minutes. Cell lysates were immunoblotted with anti-phospho-MEK1/2 (Ser217/221) antibody. NIH-3T3 total cell extracts 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.

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 min 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 min 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 twofold 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 and 1.5-fold less, respectively, following uPAR crosslinking in the presence of PD98059 (Fig. 7B,C). 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 ECM 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 [Busso et al., 1994; Stahl and Mueller, 1994, 1997; Li et al., 1995; Dumler et al., 1998; Tang et al., 1998; Yebra et al., 1999; Kusch et al., 2000; Nguyen et al., 2000; Jo et al., 2002] and receptor clustering [Koshelnick et al., 1997; Sitrin et al., 1999, 2000]. We have previously documented that IL-2 activated NK cells can accumulate within tumor metastases following their adoptive transfer [Basse et al.,

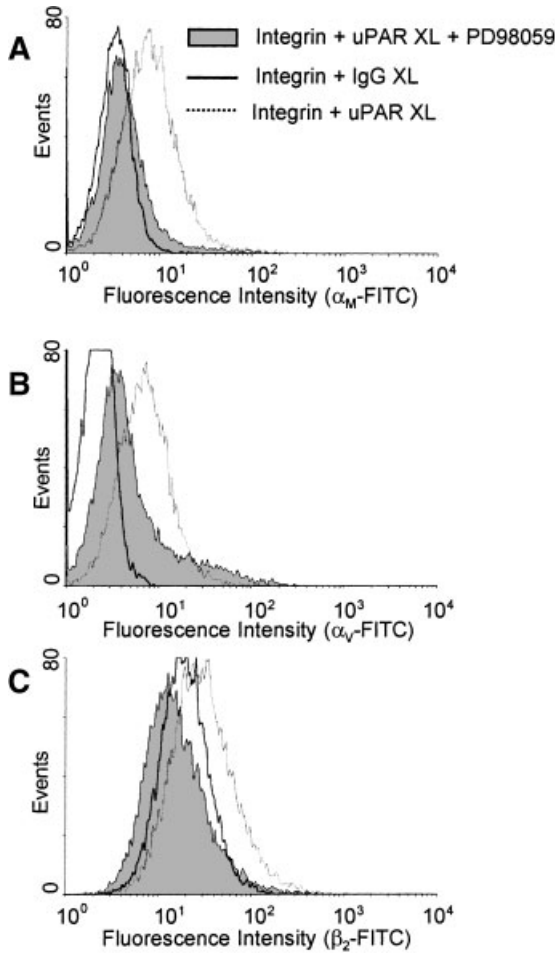

Fig. 6. PD98059 inhibits the increased integrin expression induced by uPAR crosslinking. **A**–**C**: YT cells were incubated at a concentration of 5×10^5 cells per tube with uPAR antibody for 1 h and pretreated with 50 μM of the MEK-specific inhibitor PD98059 at 37°C for 30 min prior to crosslinking uPAR for 30 min. 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.

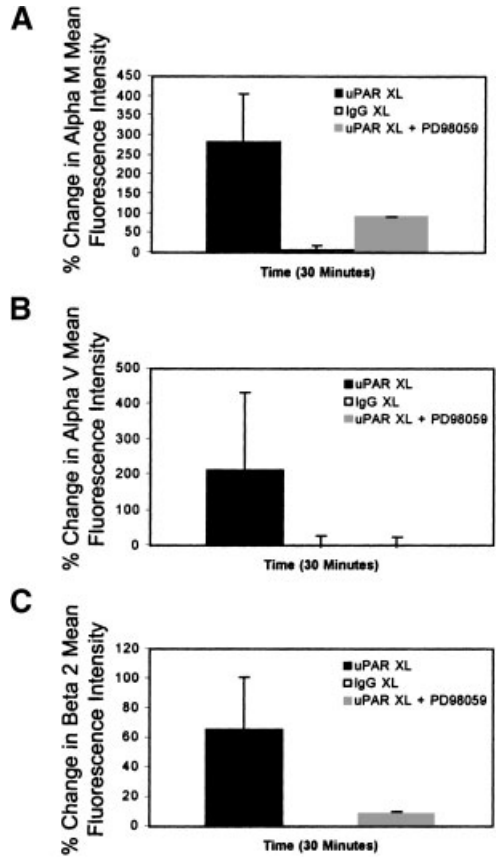

Fig. 7. PD98059 inhibits the increased integrin mean fluorescence intensity following uPAR crosslinking. YT cells were incubated with uPAR antibody for 1 h and pretreated with 50 μM of PD98059 at 37°C for 30 min prior to crosslinking uPAR for 30 min. Changes in integrin expression were evaluated using 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, 3.5, 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, $P < 0.05$.

1991a,b]. 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 [Al-Atrash et al., 2000, 2001]. 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 [Chapman et al., 1999]. Similarly, others have demonstrated uPAR polarization to the leading edge of migrating monocytes and endothelial cells [Estreicher et al., 1990; Myohanen et al., 1993; Pepper et al., 1993; Gyetko et al., 1994; Sitrin et al., 1996]. 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 [Xue et al., 1994, 1997; Bohuslav et al., 1995; Sitrin et al., 1996; Yebra et al., 1996; Carriero et al., 1999; Wei et al., 1999; van der Pluijm et al., 2001]. 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 [Basse et al., 1991a,b; Nannmark et al., 1995].

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 [Ossowski and Aguirre-Ghiso, 2000]. 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. [1996] in which a soluble form of uPAR induced chemotaxis in cells lacking endogenous uPAR. 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 [Konakova et al., 1998; Aguirre Ghiso et al., 1999; Nguyen et al., 2000; Jo et al., 2002]. 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 (Gellert et al., Submitted). In tumor cells, the binding of uPA to uPAR has been correlated with increased cell adhesion and migration [Aguirre Ghiso et al., 1999; Nguyen et al., 2000; Jo et al., 2002]; 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 ECM surrounding certain tumors provides a barrier for the tumor cells against immune cells, including NK cells, during immunotherapy [Kuppen et al., 2001]. Our studies suggest that NK cells integrate aspects of adhesion and ECM 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.

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