Integrin-dependent Induction of Functional Urokinase Receptors in Primary T Lymphocytes

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Abstract

In order to reach the sites of inflammation, lymphocytes leave the bloodstream and migrate into peripheral tissues, in a process involving integrin-mediated adhesion to the vascular endothelium, followed by transmigration across the endothelial barrier and through the underlying interstitial matrix. We have investigated the role of the plasminogen activator/plasmin system in normal T cell migration. Receptors for urokinase plasminogen activator (uPAR) were not expressed in resting T lymphocytes, but could be efficiently induced at the mRNA and protein level by co-clustering of the antigen receptor complex and β1 or β2 integrins, through a signalling pathway involving both protein kinase C activation and an increase in intracellular cyclic AMP. Catalytic activation of plasminogen by uPAR-expressing T cells promoted their migration through an extracellular matrix in vitro. Plasmin-induced invasion was inhibited by plasmin-and urokinase inhibitors and by anti-uPAR antibodies. Finally, cytfluorimetric and immunohistochemical analysis of primary human tumor specimens showed the presence of uPAR positive infiltrating T cells in vivo. Collectively, these findings suggest that plasminogen activation may play a role in lymphocyte migration in vivo, and that integrin-dependent expression of membrane-associated endopeptidases could represent an additional step in the regulated process of leukocyte transmigration. (J. Clin. Invest. 1996, 98:1133–1141.) Key words: plasminogen activators • cell adhesion molecules • cell movement • neoplasms • cyclic AMP

Introduction

Extravasation and migration into inflammatory or neoplastic foci in peripheral tissues are instrumental to the ability of T lymphocytes to perform antigen-specific effector functions. Transendothelial migration of lymphocytes is currently viewed as a step-wise process that involves initial, selectin-mediated rolling along the luminal side of postcapillary venules, followed by integrin-mediated firm adhesion to the endothelium and, finally, chemokine-driven transmigration across the endothelial cell monolayer (1, 2). Integrin engagement is dependent on prior activation of the T cell, which causes a shift in the avidity of the integrins for their ligands (3). Transmigrated lymphocytes need to interact dynamically with the extracellular matrix in order to reach their final destination, but how this process is regulated at the molecular level has not yet been defined. The plasminogen activation system has been shown to play a prominent role in many physiological and pathological processes involving cell migration and infiltration in the surrounding tissues which require the remodelling of the extracellular matrix, such as trophoblast implantation, angiogenesis, wound healing and tumor invasion (4–6, and references therein). Urokinase-type plasminogen activator (uPA)1 converts plasminogen to the active form plasmin, a serine protease with broad specificity, which activates a cascade of proteolytic events, leading to fibronectin, laminin, and collagen degradation. Receptors for urokinase (uPAR) localize uPA at the cell surface (7–9). Binding of uPA to its receptor, not only increases the rate of enzymatic activation of plasmin, but also makes cell-associated proteolysis a focal and spatially oriented process, which may be more functional to cell migration (10). uPAR has been identified as the monocye activation antigen Mo-3 (11) and is expressed in addition by several unstimulated, circulating hematopoietic cells, such as granulocytes and natural killer cells, as well as by in vitro activated T cells (12–14). However, the functional significance of plasminogen activation for T cell function has not been investigated. In this work we asked whether uPAR could be induced in normal T cells by physiological stimuli associated with the activation/transmigration process and whether uPAR expression was functionally important for T cell migration. Co-clustering of the antigen receptor complex and β1 or β2 integrins by the use of specific mAbs was found to be one of the most efficient stimuli for uPAR membrane expression. The analysis of the second messengers involved in integrin-dependent expression of uPAR pointed to a major contribution, together with protein kinase C (PKC) activation, of a CAM-dependent pathway, as co-clustering of the activation and adhesion receptors induced persistent elevation of [cAMP] and uPAR expression under these conditions could be abrogated by selective adenylyl cyclase inhibitors. uPAR expressing T cells utilized the receptor to actively in-

1. Abbreviations used in this paper: AC, adenylyl cyclase; BIM, bisindolylmaleimide; DAG, diacylglycerol; EACA, epsilon-aminocaproic acid; FSK, forskolin; PI, phosphatidylinositol; PKC, protein kinase C; TIL, tumor infiltrating lymphocytes; uPA, urokinase-type plasminogen activator; uPAR, receptors for urokinase.
the presence of infiltrating T lymphocytes indicated that uPAR tochemical analysis of human tumor specimens characterized by vade matrigel-based matrices in vitro, when the appropriate co-

Methods

Chemicals and antibodies. PMA, forskolin, epsilon-aminocaproic acid (EACA), amiloride, plasmin-free plasminogen, collagenase, jalu-

Northern blot analysis. Total RNA was extracted from resting and activated T cells with the RNAzol B kit (Biotech Lab., Houston, TX) following manufacturer’s instruction. 15 μg of total RNA were run on 1.5% denaturing agarose gels and blotted on positively charged membranes (Hybond N°; Amersham, Buckinghamshire, UK) by capillary transfer. Probes (uPAR full length cDNA) were la-

Cell isolation and cultures. T lymphocytes were purified from buffy coats obtained from the San Raffaele Hospital Blood Center (Milan, Italy). PBMC were isolated by density gradient centrifugation on Ficoll Hypaque (Nycomed Pharma AS, Oslo, Norway). Monocytes were depleted by two rounds of plastic adherence for 1 h at 37°C followed by a passage on nylon wool columns at 37°C. NK cells and resid-

Matrigel invasion assay. The ability of activated T lymphocytes to traverse a basement membrane substrate in vitro was assayed using a Matrigel-coated (Collaborative Research Biomedical Products, Bed-

Immunohistochemistry. 4 μm cryostat sections of human breast, pancreatic, and lung tumors were fixed in acetone/chloroform, 5 min at 4°C, and immediately air-dried. Sections were incubated several hours at 4°C in PBS with 0.1% Tween (PBT) and 5% BSA. Sections

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from eight different tumor samples were analyzed. The sections were blocked 20 min at room temperature (RT) in PBS 20% normal swine serum, followed by an anti–human CD3 polyclonal Ab (1:100 in PBT, 5% BSA). Sections were washed three times in PBT at RT and incubated with the secondary Ab (FITC-swine anti-rabbit polyclonal, 1:25) for 30 min at RT. After extensive washing, sections were blocked for 20 min at RT in PBS with 10% normal goat serum, followed by 60 min at RT with the anti-uPAR mAb R2 and R4 (5 μg/ml each). Sections were washed three times in PBT and incubated 30 min at RT with the TRITC-conjugated anti-IgG1 and anti-kappa chain antibodies (1:100). After washing in PBT, sections were mounted in 50% glycerol and examined under a Zeiss microscope (Jena, Germany).

Results

**uPAR expression on activated lymphocytes.** Highly enriched T cells (> 95% CD3<sup>+</sup>) were isolated from the peripheral blood of healthy donors. Unstimulated T cells were negative for surface uPAR expression for several days after isolation, as shown by FACS® analysis (Fig. 1A). Immobilized monoclonal antibodies were used to mimic natural ligands, to explore the role of different cell surface receptors in the induction of uPAR. Cross-linking of the antigen receptor with anti-CD3 antibody induced low but detectable levels of uPAR on a large fraction of the population (Fig. 1B). While ligation of the LFA-1 receptor alone had no effect on uPAR induction at any of the time-points examined (Fig. 1C), simultaneous clustering of the CD3 receptor with the integrin receptor LFA-1 had a consistent synergistic effect in inducing uPAR homogeneously throughout the T cell population (Fig. 1D). uPAR expression steadily increased with time and reached a plateau at 48 to 72 h following the initial stimulus (Fig. 2). Similar results were obtained using immobilized anti–LFA-1 antibody combined to
soluble anti-CD3 antibody (not shown), suggesting that antibody-mediated co-capping of the two receptors is not strictly required for the synergistic induction of uPAR expression. Antibodies directed against integrins of the \( \beta 1 \) family, such as VLA-4 and VLA-5, cooperated with anti-CD3 in stimulating uPAR expression (Fig. 1 F, Fig. 2 and data not shown), although less efficiently than antibodies to LFA-1. Interestingly, simultaneous cross-linking of LFA-1 and VLA-4 with the CD3 complex did not further increase the overall surface density of uPAR, but resulted in long-term persistence (\( > 8 \) d) of its expression in T cells (Fig. 2).

The increase in functional uPAR in anti-CD3 plus anti-LFA-1 stimulated T cells was also tested by direct binding experiments with iodinated ATF, the amino-terminal fragment of uPA deprived of protease activity but including the functional uPAR-binding site (18). Comparison of unstimulated and antibody-stimulated cells, at the peak of surface uPAR expression, showed that the number of ATF-binding sites was increased more than 10-fold (Fig. 3). Analysis of the data indicated that the induced receptors display high affinity, with 50% occupancy in stimulated lymphocytes obtained at 2 nM ATF, comparable to that previously reported in many other cell lines (19). Scatchard analysis confirmed these data (not shown).

**Second messengers involved in the co-stimulatory activity of \( \beta 2 \) integrins.** Signaling through the T cell antigen receptor complex results in the activation of the phosphatidylinositol (PI) pathway, which leads to generation of diacylglycerol (DAG) and protein kinase C activation (20, 21). Clustering of LFA-1 has also been reported to be an efficient stimulus for PI hydrolysis and DAG generation (22, 23). The importance of this pathway in the induction of uPAR in our model was shown by the fact that phorbol esters (PMA, 100 nM), as previously reported (14), were sufficient to induce T cell surface uPAR expression (Figs. 1 G and 4). This effect was completely blocked by treatment with the selective PKC inhibitor BIM (Fig. 4). Addition of BIM also blocked uPAR expression induced by simultaneous cross-linking of CD3 and LFA-1, indicating a role for PKC activation in this pathway of uPAR induction. Cross-linking of LFA-1 synergized with PMA in stimulating uPAR expression, suggesting the existence of a complementary activation pathway (Fig. 1).

We have previously reported that simultaneous clustering of the CD3 and the LFA-1 receptors induces also a gradual and persistent elevation of the intracellular concentration of cAMP (24). We have therefore studied the role of the increased cAMP concentration in the induction of uPAR, by stimulating uPAR expression in the presence of the specific adenylyl cyclase (AC) inhibitor SQ22536 (100 \( \mu M \)). Under these conditions, the effect of anti-CD3 alone on uPAR stimulation was abolished and that of anti-CD3 plus anti-LFA-1 mAbs strongly reduced (Fig. 4). In the latter case, the further addition of BIM decreased uPAR expression to background levels (not shown). Activation of AC alone was inefficient in stimulating uPAR expression, as treatment of T cells with forskolin (FSK), which directly activates AC, did not induce significant expression of uPAR (not shown). FSK had an additive effect on uPAR induction by PMA treatment; addition of SQ22536, to block adenylyl cyclase activation by FSK, reduced uPAR expression to levels seen with PMA treatment alone, pointing to the fact that the two pathways of induction may be, at least in part, independent (Fig. 4). The inhibitor SQ22536 had no effect on uPAR induction by PMA (Fig. 4).

**uPAR expression is modulated at the mRNA level.** To test...
the influence of selected pathways of cell activation on uPAR mRNA levels, Northern blot analysis was performed on total RNA extracted from purified T cells, stimulated under various conditions. Unstimulated T cells displayed low but detectable baseline levels of uPAR mRNA (Fig. 5A). These were not due to contamination by cells of the myelomonocytic lineage, as the same blot was negative when hybridized with a CD14 probe (not shown). Co-stimulation via CD3 and LFA-1 receptors induced a marked increase in uPAR mRNA levels, which declined after 12 h, while stimulation with anti-CD3 alone was not as efficient in increasing uPAR mRNA. The increases in uPAR mRNA were strongly impaired by treatment with the PKC or the AC inhibitors, BIM and SQ22536 (Fig. 5B). Activation of AC with forskolin was cooperative with the effect of PMA also at the mRNA level, resulting in the strongest increase in uPAR mRNA (not shown).

The plasminogen activation system is functional in T lymphocyte migration. To study whether uPAR plays a role in primary T cell migration across a basal lamina, purified T cells were activated by cross-linking of the CD3 and of the LFA-1 receptors (empty bars) were labeled with $^{51}$Cr and tested for their ability to invade through a Matrigel layer, in a double-chamber assay. Invading cells were recovered from the bottom chamber and from the Matrigel layer and are expressed as percentage of cells on the total cell input (mean±SD of eight experiments). Unstimulated T cells (black bars) showed consistently low levels of invasion. The assay was also performed in the presence of Matrigel alone (M) or following addition of plasminogen (100 µM, M + P) alone or plasminogen plus urokinase (0.05 nM, M + P + U). (B) T cells activated by crosslinking of CD3 and LFA-1 were tested in the Matrigel invasion assay, in the presence of a plasmin inhibitor (Aprotinin), a urokinase inhibitor (Amiloride) or a blocking or nonblocking antibody against u-PAR.
affect the levels of T cell migration. Plasminogen-driven invasion was completely abrogated by aprotinin (10 μg/ml), a serine protease inhibitor (Fig. 6B). Strong but partial inhibition was observed with amiloride (100 μg/ml), which blocks uPA activity, or in the presence of an antibody directed against the uPA binding site of uPAR (R3, 5 μg/ml). A nonblocking anti-uPAR antibody was ineffective (R4, Fig. 6B).

**uPAR expression on tissue-infiltrating T lymphocytes in vivo.**

To study whether in vivo activated lymphocytes express uPAR, T cells were isolated from fresh human renal and ovarian carcinoma specimens by partial enzymatic digestion, followed by separation on a density gradient. The mononuclear population so obtained was analyzed for uPAR expression by surface immunofluorescence and cytofluorimetric analysis. A variable fraction (21–47%) of the tumor infiltrating CD3⁺ T lymphocytes expressed uPAR on their surface, as detected by cytofluorimetry (Fig. 7B–D), while fewer than 2% of T cells isolated from peripheral blood were uPAR⁺ (Fig. 7A). To confirm this finding in vivo, frozen sections of human tumor samples were analyzed by double immunofluorescence staining. uPAR was detected with a combination of two monoclonal antibodies against nonoverlapping epitopes of the receptor (R2 and R3), followed by detection with a TRITC-conjugated secondary antibody. T cells were identified by staining with an anti-CD3 rabbit polyclonal antiserum, followed by a FITC-conjugated swine anti-rabbit polyclonal. Mononuclear infiltrates were visible in all specimens examined (10 cases); in all cases, T cells were clearly detected by surface staining with the anti-CD3 antiserum. uPAR expression was detected in most cases, but showed variable degrees of intensity, even among T cells within the same infiltrate (Fig. 8). In several cases, uPAR positive, CD3 negative cells were also visible, possibly representing macrophages, tumor cells or vascular endothelium lining capillaries or small vessels (Fig. 8). Specificity of the staining was confirmed by omission of the anti-uPAR or the anti-CD3 antibodies, or incubation with an irrelevant control antibody. Cross-reactivity of the secondary antibodies was ruled out by incubating the anti-CD3 stained sections with the anti–mouse secondary antibody, and the anti-uPAR stained sections with the anti–rabbit antiserum. Under all these conditions no specific staining was visible (not shown).

**Discussion**

During their life span, T lymphocytes need to leave the bloodstream multiple times and extravasate into perivascular tissues to perform immune surveillance and reach the sites of the inflammatory response. T cell transmigration requires an initial interaction with the endothelial lining of the blood vessel, which appears to be mediated mostly by C-type lectins, followed by strengthening of the adhesion, operated by binding of lymphocyte adhesion receptors of the β1 or β2 integrin subfam-
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For integrin-mediated adhesion to occur, the integrin receptors themselves have to undergo a change in their avidity for the ligand, which is brought on by a variety of stimuli, including antigen recognition or chemokine-induced T cell activation (3, 25, 26). T cell integrins may also play a central role in the subsequent transmigration through the basement membrane into the interstitial matrix, though the molecular mechanisms that regulate these phenomena are still poorly defined. Integrins are not only responsible for the attachment to ECM proteins, but are also involved in transducing signals from the extracellular environment, initiating the intracellular cascade of metabolic events necessary for the invasion process (27).

The aim of the present study was to assess the inducibility and the functional role of uPAR in promoting invasion of the extracellular matrix by T lymphocytes, activated by stimuli which are likely to be associated with the interaction between T cells and endothelial cells or ECM, that occurs during the transmigration process. Our results show that co-clustering of CD3 and of the integrin αLβ2 (LFA-1) with specific antibodies is a very efficient stimulus for high affinity uPAR induction on T cells, while cross-linking of the antigen receptor complex (CD3) alone induces low levels of uPAR. The increased cell surface expression is paralleled by an increase in uPAR mRNA, suggesting the existence of transcriptional and/or posttranscriptional mechanisms for the control of uPAR levels by membrane receptors involved in cell–cell and cell–extracellular matrix adhesion. Other integrin receptors present on lymphocytes, such as α4β1 (VLA-4) or α5β1 (VLA-5), were also capable of synergizing with signalling through the CD3 complex in inducing uPAR. Although β1 integrin costimulation did not induce uPAR expression levels higher than those obtained by co-clustering of CD3 and β2 integrins on T cells, it delayed significantly the disappearance of the receptor in stimulated cells (Fig. 2). These data are in agreement with the studies showing that the engagement of integrins is capable of modifying gene expression, by acting both at the transcriptional and posttranscriptional level. Interestingly, among the genes induced upon stimulation of integrin receptors are several proteases: stimulation of α5β1 on fibroblasts induces stromelysin and collagenase (28), engagement of αVβ3 stimulates expression of a 72-kD gelatinase in a melanoma cell line (29), antibodies against α3β1 induce a 92-kD type IV collagenase in keratinocytes (30). In T lymphocytes, adhesion to VCAM1 (presumably through its counter receptor α4β1) induces secretion of the 72-kD gelatinase, an event that was

![Image](image_url)
shown to facilitate T cell invasion through a three-dimensional matrix (31).

The intracellular pathways that mediate integrin signaling are largely unknown. We have analyzed the intracellular messengers responsible for uPAR induction, with the use of specific modulators of known metabolic pathways. In agreement with data in the literature (14), our experiments confirm that activation of PKC by phorbol esters (PMA) is sufficient to induce uPAR expression. A selective PKC inhibitor (BIM) abolished completely not only uPAR induction by PMA, but also uPAR expression following CD3 stimulation, providing evidence for a central role for PKC activation in uPAR induction through CD3. Phosphatidylinositol hydrolysis and production of 1,2-diacylglycerol (DAG) is an early event following T cell antigen receptor stimulation (20). The activation of PKC by DAG is an important event in inducing the synthesis of T cell nuclear transcription factor (NF-AT) and regulating the activity of transcription factors of the AP-1 and NF-kB family (32–34). Though the transcription factors possibly involved in uPAR induction in T cells have not been characterized, it is interesting to note the existence, in the uPAR promoter, of consensus binding sites for AP-1 and NF-kB factors (35).

It has been reported that a cAMP-dependent pathway mediates uPAR expression in cultured endothelial cells (36). Since cross-linking of the CD3 receptor on lymphocytes increases the intracellular concentration of cAMP and the simultaneous clustering of CD3 and LFA-1 is even a more potent agonist in stimulating cAMP increase (24), we tested whether variations in intracellular cAMP contributed to uPAR induction also in T lymphocytes. The increased cAMP concentration induced by direct activation of adenyl cyclase (AC) by FSK synergized with PKC activation in inducing uPAR expression, though treatment with FSK alone had no effect on uPAR induction in T cells (not shown), in contrast with what described for endothelial cells. Addition of a specific AC inhibitor (SQ22536) abolished the synergistic effect of FSK treatment, suggesting that the two pathways for uPAR induction may be at least partially independent. The AC inhibitor also abolished uPAR expression induced in T cells by cross-linking of CD3 alone and greatly reduced the effect of CD3 and LFA-1 co-clustering, suggesting that an increase in cAMP is important in signalling through this pathway.

uPAR expressed on activated T cells appears to be functional in promoting T cell migration: invasion of T cells in vitro through a basement membrane equivalent (Matrigel) was enhanced by the addition of plasminogen, the substrate for urokinase, and impaired by treatment with a uPAR inhibitor, amiloride (37), or by addition to the system of a blocking antibody against uPAR. Treatment with a serine protease inhibitor, aprotinin, also blocked plasminogen-dependent migration. A small fraction of activated T cells (16±3%) migrated also in the absence of added plasminogen: this fraction could not be reduced by addition of aprotinin to the system (not shown), suggesting that other proteolytic systems are also likely to play a role in lymphocyte migration. Activated T cells are known to express, in addition to the metalloproteinases (72 and 92 kD), active against collagen types IV and V, an array of enzymes potentially active against components of the ECM, such as heparanase, an endoglycosidase which degrades proteoglycans of the subendothelial basement membrane, cathepsin D and elastase (31, 38–41). Though the role of these proteases in lymphocyte biology has not been established, the outcome of T cell migration is possibly dictated by the concerted action of all these degradative pathways. Interestingly, addition of urokinase had no influence on the extent of lymphocyte migration. This could be due to the fact that anti-CD3 plus anti-LFA-1–stimulated, but not resting, T cells secrete small amounts of urokinase, as demonstrated by the activity of T cell supernatants in a zymography assay (not shown). Consistent with this result, we observed low but detectable levels of uPA mRNA in activated T cells by Northern blot analysis (not shown).

To understand the functional relevance of the expression of uPAR by T lymphocytes in vivo, we asked whether infiltrating T cells in human tissues expressed uPAR on their plasma membrane. Immunofluorescence analysis of lymphocyte infiltrates in tumor sections confirmed that a significant fraction of T cells present in the context of the extravascular tissues expressed uPAR on their surface, though with different levels of intensity. These data are in agreement with the analysis of infiltrating T lymphocytes, isolated ex vivo from human tumors, which demonstrated the presence of a variable fraction of uPAR positive, CD3 positive cells. The previously undescribed presence of uPAR on T lymphocytes in vivo is in agreement with a possible role for plasminogen activation in processes that require T cell migration.

Many reports have underlined the importance of the uPA system in promoting experimental tumor metastasis in vitro and in vivo in animal models, and have suggested the possibility of interfering with the metastatic process by inhibiting uPA activity (42–48). uPA bound to the cell surface of the T cell leukemia Jurkat has been shown to facilitate its invasion into fibrin matrices in vitro (49). Studies focused on the involvement of the plasminogen activation system in primary human T leukemias and lymphomas will be necessary to attest the importance of uPA in tumor T cell invasion of solid tissues. Finally, recent evidence indicates that uPAR is part of a molecular complex capable of mediating adhesion to matrix components (50) and transmembrane signalling (15), thus suggesting the existence of strong co-operative interactions amongst receptors involved in migration, adhesion, and matrix degradation. Our data are consistent with the notion that the stromal component participates in establishing the “invasive phenotype” of tumors. It has been shown that in human colon cancer that uPA is produced by stromal fibroblasts, whereas uPAR is present on tumor cells (51). In breast tumors, in addition to cancer cells, tumor-infiltrating macrophages and endothelial cells express uPAR (52, 53). Our results show that uPAR-positive T lymphocytes are also present in human tumors and may participate in the establishment of the overall invasive phenotype. Confirmation in this context of the pathogenic role of uPA activity, together with the design of selective inhibitors, might be useful for the development of novel therapeutic approaches to T cell–mediated diseases.

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