CYTOKERATIN 8 FUNCTIONS AS A MAJOR PLASMINOGEN RECEPTOR IN SELECT EPITHELIAL AND CARCINOMA CELLS

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1. ABSTRACT

Cytokeratin 8 (K8) is a member of the intermediate filament (IF) gene family expressed by simple epithelial cells and by some carcinoma cells. The majority of the cellular K8 is assembled with its partner, K18, into highly insoluble 10 nm filaments that extend from the nucleus to the internal leaflet of the plasma membrane. At desmosomes and hemidesmosomes, K8, K18, and other IF proteins are bridged to proteins with transmembrane domains by a family of proteins called plakins. K8 does not have a signal peptide or a well-defined transmembrane domain; however, there is substantial evidence that this protein is available to bind plasminogen and K8-specific antibodies on the surfaces of certain epithelial cells in culture, including hepatocytes, hepatocellular carcinoma cells, and various breast cancer cell lines. This may reflect a novel mechanism of protein penetration through the plasma membrane or binding of secreted K8 to other cell-surface molecules. Cancer cells are known to secrete K8-containing protein complexes *in vitro* and *in vivo*. These complexes bind plasminogen as well. The plasminogen-binding activity of K8 is unique amongst IF proteins, probably because its sequence includes a carboxyl-terminal Lys residue. However, a K8 mutant that lacks the C-terminal Lys still binds plasminogen, albeit with decreased affinity. K18 does not bind plasminogen; however, K8 and K18 bind tissue-type plasminogen activator (tPA) equivalently. tPA-binding to K18 may be important in the mechanism whereby K8-K18 complexes promote plasminogen activation by tPA. Numerous studies have demonstrated correlations between high levels of K8 expression and increased migration and invasion of certain cancer cells. These correlations are most easily explained by the function of IF proteins in determining the rigidity of the cytoskeleton; however, the function of cell-surface K8 as a plasminogen receptor merits consideration. We have demonstrated that certain aggressive breast cancer cell lines, which have highly activated endogenous urokinase type-plasminogen activator (uPA)-uPA receptor (uPAR) systems, do not express high levels of cell-surface K8. The membrane macromolecule that is responsible for plasminogen-binding and for supporting activation of plasminogen by uPA on the surfaces of these cell types remains to be determined. This review focuses on the function of K8 as a plasminogen receptor and its potential role in cancer.

2. INTRODUCTION

Plasminogen receptors have emerged as a family of structurally diverse plasma membrane macromolecules with similar functions. Each receptor binds plasminogen or plasmin with similar affinity (K_D of 0.1-2.0 micromolar) (1). Localization of plasminogen to the cell surface promotes plasminogen activation by uPA and tPA, by dramatically decreasing the K_M values for these reactions (2,3). The decrease in K_M is consistent with a model in which the cell surface functions as a scaffold, bringing the enzyme (tPA and uPA) and substrate (plasminogen) into close juxtaposition. Because of the high density of plasminogen receptors in many cell types, these receptors may concentrate plasmin at the cell surface and thereby facilitate localized degradation of specific plasmin substrates. Furthermore, receptor-associated plasmin is protected from its major inhibitors, alpha$_2$-antiplasmin and alpha$_2$-macroglobulin (4,5). For the reaction of plasmin with alpha$_2$-antiplasmin, plasminogen receptors are inhibi-

tory based on their ability to compete for the plasmin kringle domains. The resistance of receptor-associated plasmin towards alpha$_2$-macroglobulin is probably based on the fact that this very large proteinase inhibitor has poor access to the cell surface.

As many as twenty years ago, a model emerged, based on *in vitro* experimentation, in which events that require cell migration and tissue-remodeling are supported by a cell-surface proteinase cascade (6). Although various versions of the cascade may exist, typically uPAR-associated uPA activates cell-associated plasminogen. The plasmin then directly degrades glycoprotein components of the extracellular matrix or activates matrix metalloproteinases (MMPs), allowing cellular penetration of basement membranes and other tissue boundaries. More recently, *in vivo* experiments using transgenic and gene knock-out mice have supported the function of at least components of cell-surface proteinase cascade in processes such as vascular remodeling, aneurysm formation, wound healing, liver regeneration, inflammation, and cancer invasion/metastasis (7-13). In some cases, the major function of plasmin and its activators may involve fibrin degradation; however, there is considerable evidence that other plasmin substrates, including MMPs and extracellular matrix proteins, are physiologically significant targets *in vivo* as well (8,9,13).

The importance of most plasminogen receptors in the function of the cell-surface proteinase cascade has not been addressed by experiments in transgenic mice. Thus, the role of these receptors *in vivo* remains incompletely understood. At least one plasminogen receptor, annexin II, has been implicated as playing an important role in the hemorrhagic diathesis that frequently complicates acute promyelocytic leukemia *in vivo* in humans (14). Because leukemia cells may be isolated in significant numbers from the blood and studied *in vitro*, these cancer cells may represent an important system for further analysis of plasminogen receptors. Our laboratory has a specific interest in the role of plasminogen receptors in cancer progression. The central location of plasminogen receptors in the cell-surface proteinase cascade suggests that these receptors may play a pivotal role in cancer invasion and metastasis. Furthermore, plasminogen receptors may represent a target for rational drug design in cancer chemotherapy.

In this review, we will discuss published results and present some new studies in which we identify cell-surface K8 as a plasminogen receptor on hepatocytes, hepatocellular carcinoma cells, and on certain breast cancer cells. K8 is a member of the IF gene family, which polymerizes with K18 to form an important component of the cytoskeleton in simple epithelial cells and many carcinoma cells (15). The sequence of K8 does not include a signal peptide or a series of hydrophobic amino acids of sufficient length to form a clearly independent transmembrane domain. Nevertheless, since our initial discovery of K8 on the surfaces of rat hepatocytes and cancer cells (16), many additional studies have been published supporting the hypothesis that not only K8 but

also other cytokeratins are present on the cell surface. An exciting collection of activities have been attributed to cell-surface cytokeratins, as will be described. We hypothesize that the subcellular location of plasminogen receptors, in various domains of the plasma membrane, may influence their function in supporting plasminogen activation by uPA or tPA. This hypothesis will be discussed in relation to K8. Finally, we will discuss the possible contribution of plasminogen receptor function to the known correlation of high K8 expression levels with increased migration and invasion of certain types of cancer.

3. INTERMEDIATE FILAMENTS AND THE CYTOSKELETON

The cytoskeleton in higher eukaryotes includes three major components: actin microfilaments that have a characteristic diameter of 6 nm, microtubules, which have a larger diameter (23 nm), and 10 nm IFs. Of the three networks, IFs evolved last in the transition from prokaryotic cells and are lacking in lower eukaryotes (17). There is substantial overlap in the function of the various cytoskeletal networks. In a somewhat simplified model, the actin cytoskeleton controls cell migration, polarity, and contractility. Microtubules regulate the intracellular distribution and trafficking of organelles and vesicles and IFs function to impart mechanical strength to the cell, which may be particularly important when the function of the cell includes withstanding external forces (16-18).

IFs are polymers of heterodimers that are intertwined to form a coiled-coil structure (for review of IF structure and expression, see ref. 16). The IFs include as many as thirty cytokeratins, expressed mostly by epithelial cells, and other proteins such as glial fibrillary acidic protein, desmin, vimentin, and peripherin. The basic IF structure includes a central alpha-helical rod region flanked on either side by non-helical domains. The alpha-helical rod is interrupted by three non-alpha-helical linker regions. IF heterodimers are organized in an antiparallel manner to form protofilaments. The intact 10 nm filament consists of sixteen linear chains of IF monomers. Importantly, IF polymers form spontaneously *in vitro* and apparently do not require accessory proteins for assembly (19). Nevertheless, filament polymer structure and distribution within the cell may be regulated by cell-signaling pathways that alter cytokeratin serine-phosphorylation *in vivo* (20,21). The particular IF proteins that are expressed by a given cell is tightly controlled. For example, well-characterized changes in IF protein expression occur in the epidermis, as cells migrate from the basal layer to the stratum corneum (16). K8 and its usual partner, K18, are expressed by many simple epithelial cells and carcinoma cells (22). Determining how IF proteins differentially regulate cytoskeletal structure is an important question under active investigation.

IF proteins do not include signal peptides or characterized transmembrane domains; however, IFs attach to the internal leaflet of the plasma membrane, principally in association with desmosomes and hemidesmosomes (17). Proteins in the plakin family, including desmoplakin,
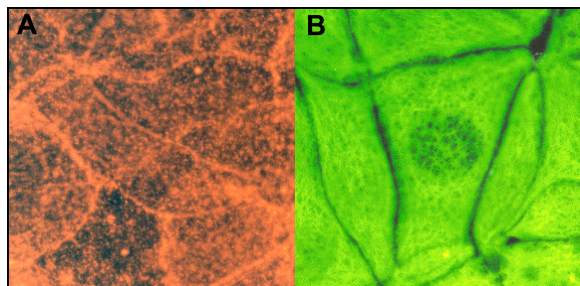
Figure 1. Immunofluorescent staining of cell-surface and cytoplasmic cytokeratin 8. MCF-7 breast cancer cells were subjected to indirect immunofluorescence with the cytokeratin 8-specific antibody, 1E8. In panel A, living, nonpermabilized cells were incubated with antibody 1E8. The cells were then fixed and antibody was detected with Texas red-conjugated secondary antibody. In panel B, cells were fixed with paraformaldehyde and permeabilized with 0.02% saponin, prior to incubation with antibody 1E8. These cells display the characteristic network of cytoplasmic intermediate filaments.

bullous pemphigoid antigen 1e, and plectin, bridge IFs to plasma membrane proteins with true transmembrane domains. In the case of plectin, a bridge is formed between IF proteins and an integrin, $alpha_6 beta_4$ (23).

4. CYTOKERATIN 8 AS A PLASMINOGEN RECEPTOR

We began our research in the area of plasminogen receptors at a time when the molecular diversity in the plasminogen receptor family was not fully appreciated. At first, we characterized the interaction of plasminogen with rat hepatocytes in primary culture. Hepatocytes could be acquired in large quantities and expressed a very high binding-capacity for plasminogen ($B_{max} > 10^7$/cell) (24,25). The K_D for plasminogen binding to receptors on hepatocytes was similar to the values reported with other cell types. At the same time, other laboratories had major efforts underway to characterize plasminogen receptors in endothelial cells, macrophages, and inflammatory cells. Thus, our work with hepatocytes was somewhat unique in that is focused on a simple epithelial cell.

To purify proteins responsible for plasminogen-binding to hepatocytes, we isolated cells from rat livers by collagenase perfusion (16). Plasma membranes were prepared and these were compared to whole cell extracts by plasminogen ligand-blotting. In this method, proteins are subjected to SDS-PAGE, electrotransferred to polyvinylidene difluoride (PVDF) membranes, and then incubated with ^{125}I-plasminogen. Because proteins are exposed to SDS prior to immobilization on PVDF, ligand-blotting can only be trusted to detect binding interactions that do not depend on higher-order (tertiary or quaternary) protein structure. A single major ^{125}I-plasminogen-binding species was detected in the plasma membrane fraction. This species migrated with an apparent mass of 59-kDa (16). Interestingly, the same band was a very minor plasminogen-binding species in whole-cell extracts.

Our first hint as to the nature of the 59-kDa band was its highly insoluble nature. Sodium deoxycholate

completely failed to solubilize the protein and, in fact, positive identification by sequence analysis required initial dissolving of the protein in 6 M urea. The partial sequences obtained were 100% identical with amino acids 12-21 and 263-272 of K8. Given the mass and biochemical properties of the purified protein and the sequence identities, we hypothesized that the 59-kDa protein, from hepatocyte plasma membranes, was full-length K8. This cytokeratin is unique in that it has a C-terminal Lys residue, which is typically required for plasminogen-binding activity (1,26). Furthermore, as will be described below, an antibody raised against the C-terminus of K8 bound to the 59-kDa band in immunoblots and recognized an equivalent pattern of epitopes on the surfaces of breast cancer cells, by immunofluorescence microscopy, compared with the well-characterized K8-specific antibody, AB6.01. Nevertheless, at this point, we still cannot be certain that the protein identified as a plasminogen receptor in hepatocyte plasma membranes was indeed full-length K8 and not a modified form of the protein. Because K8 is known to associate directly or indirectly with proteins and lipids in the internal leaflet of the plasma membrane (17,27), our next goal was to determine whether K8 is also associated with the external leaflet of the plasma membrane. To address this question, we performed immunofluorescence microscopy and immunoelectron microscopy experiments. Biochemical approaches were considered less desirable because it is known that cells in culture secrete K8-containing fragments (28,29), which may make interpretation of the results obtained using biochemical assays difficult.

The antibodies we chose for our imaging experiments included AB6.01, which had been originally characterized by Donald et al (30) as recognizing a K8-like epitope expressed on the surfaces of malignant epithelial cells. Cell-surface localization of K8, K18, and K19, in epithelial cells, was also demonstrated by Godfroid et al. (31). Interestingly, both groups evaluated multiple cell lines and identified MCF-7 cells as particularly rich in cell-surface K8. Other investigators reported that they labeled members of the cytokeratin family using cell-surface radioiodination methods (32). Nevertheless, the significance of cell-surface cytokeratins remained in question (33). We also performed imaging experiments with monoclonal antibodies M20 and PKC-26, which are commercially available (Sigma), and monoclonal antibody 1E8, which we raised in our laboratory (see below). In our immunofluorescence microscopy studies, hepatocytes, hepatocellular carcinoma cells, and breast cancer cells were cultured on coverslips, washed, and incubated with primary antibody, followed by fluorophore-conjugated secondary antibody, before fixation (26). In some experiments, cells were fixed prior to incubation with secondary antibody. As shown in Figure 1, the punctate pattern of immunofluorescence, observed in non-permeabilized cells (panel A), was entirely different from the diffuse, filamentous, nucleus-sparing pattern observed when the cells were permeabilized (panel B). Notice that the pattern and intensity of cell-surface immunofluorescence is equivalent in all of the cells in the photomicrograph shown in Figure 1A. This is important because it suggests that the detected K8 does not derive from a subpopulation of cells that is injured, undergoing cell death, or in the process of mitosis.
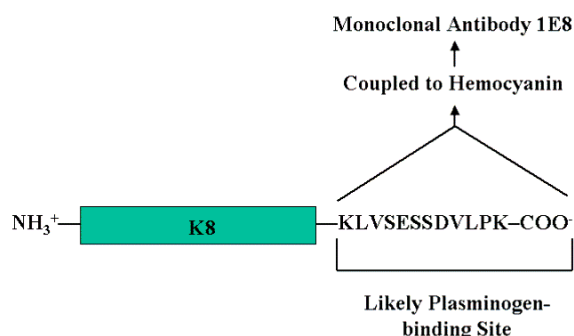
Monoclonal Antibody 1E8

↑

Coupled to Hemocyanin

↑

NH_3^+— [K8] —KLVSESSDVLPK–COO⁻

Likely Plasminogen-
binding Site

Figure 2. The immunogen for antibody 1E8. A synthetic peptide corresponding to the C-terminal twelve amino acids of K8 was coupled to hemocyanin via an N-terminal Cys residue (not shown). Notice that the K8 sequence includes a C-terminal Lys residue and a second Lys residue eleven amino acids upstream from the C-terminus.
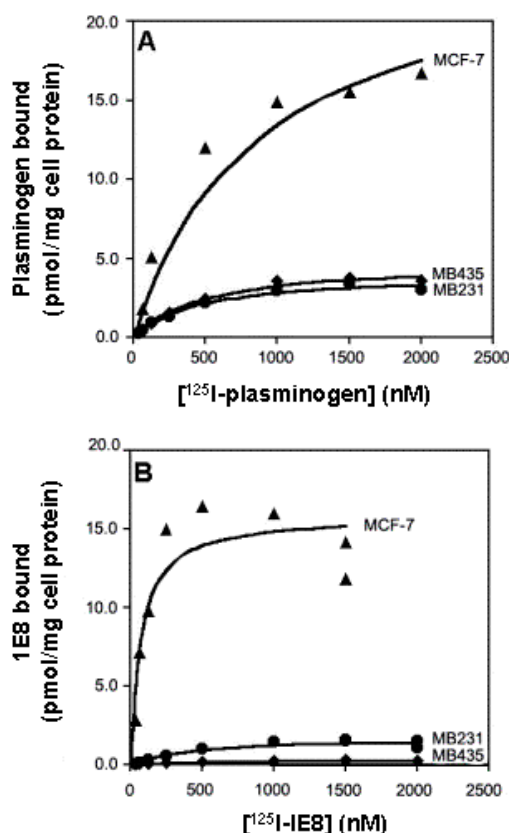

Figure 3. Specific binding of plasminogen and antibody 1E8 to breast cancer cells. Monolayer cultures were incubated with increasing concentrations of radioiodinated plasminogen (panel A) or radioiodinated antibody 1E8 (panel B) in the presence or absence of a 50-fold molar excess of the same unlabeled ligand for 4 hours at 4° C. The binding buffer was Earle's Balanced Salt Solution with 10 mM HEPES and 10 mg/ml bovine serum albumin, pH 7.4. After washing the cells three times, cell-associated radioactivity was recovered in 1.0% SDS, 0.1 M NaOH and measured in a gamma counter. Specific binding was determined as the difference between the radioactivity measured in the presence and absence of competing ligand.

We subsequently performed immunoelectron microscopy studies, which provided strong confirmatory evidence that K8 is present on the surfaces of the various epithelial cells under investigation (16). Further confirmation and extension of our observations regarding cell-surface K8 were provided by other laboratories. Wells et al. (34) demonstrated that K18-specific antibody binds to the external surfaces in perfused rat livers. These investigators also identified cell-surface K18 as a receptor for thrombin-antithrombin III complex. Ditzel et al. (35) prepared an antibody and a recombinant Fab fragment of this antibody that recognize epitopes, selectively on malignant cells, that are apparently modified forms of K8 or K18. Finally, Schmaier and colleagues (36,37) identified K1 on endothelial cell surfaces and demonstrated that K1 may function as a receptor for high and low molecular weight kininogen. Interestingly, cell-surface K1 co-localizes with uPAR in endothelial cells (37). uPAR may also bind kininogens. K8 and K18 may provide cell-surface binding sites for annexin-1 (38). Thus, the cytokeratins emerge as a family of proteins that express a wide spectrum of potentially physiologically significant functions when exposed on external cell surfaces.

To determine whether cell-surface K8 is functional as a plasminogen receptor, we raised monoclonal antibody 1E8 against the C-terminal 12 amino acids of K8, which we considered the most likely plasminogen-binding site in K8 (Figure 2). Fab fragments of this antibody blocked over 80% of the plasminogen binding to MCF-7 cells (39). Significant inhibition of plasminogen-binding (∃50%) was also observed in experiments with BT20 and MDA-MB-157 breast cancer cells (39). Thus, K8 is responsible for the majority of the cell-surface plasminogen-binding sites in a number of breast cancer cell lines. However, the total plasminogen-binding capacity and the level of cell-surface K8 expression may not correlate with the invasive capacity of different breast cancer cells. MCF-7 cells are minimally invasive and do not metastasize in animal model systems, whereas MDA-MB-231 and MDA-MB-435 cells are highly invasive *in vitro* and metastasize *in vivo* (40,41). There are also substantial differences in the rates of cell growth of these cell lines, which may be related to the baseline level of extracellular signal-regulated kinase (ERK/MAP kinase) phosphorylation and the activity of the endogenous uPA-uPAR system (41-43).

Figure 3A shows the results of experiments in which we compared specific binding of ^{125}I-plasminogen to MCF-7, MDA-MB-231, and MDA-MB-435 cells. Each of these three cell types bound plasminogen with similar affinity (K_D = 0.5-0.9 micromolar); however the B_{max} for plasminogen-binding to MCF-7 cells (25 pmol/mg cell protein) was substantially higher than the B_{max} for plasminogen-binding to MDA-MB-231 cells (2 pmol/mg cell protein) or MDA-MB-435 cells (3 pmol/mg cell protein). Interestingly, the B_{max} for plasminogen-binding correlated with the B_{max} for specific-binding of ^{125}I-1E8. As shown in Figure 3B, MCF-7 cells bound 16 pmol of 1E8/mg cell protein, whereas MDA-MB-231 and MDA-MB-435 cells demonstrated little or no specific binding of this antibody. Thus, the more aggressive breast cancer cell

lines (MDA-MB-231 and MDA-MB-435) bind less plasminogen and have little or no cell-surface K8, compared with the better differentiated MCF-7 cell line. We hypothesize that the difference in cell-surface K8 expression in the various cell types may be related to the internal cytoskeleton. MCF-7 cells are polarized with abundant IF proteins, significant cell-cell adhesions, intracytoplasmic plaques resembling desmosomes, and a tendency to form gland-like structures (44). By contrast, MDA-MB-231 and MDA-MB-435 cells demonstrate limited cell-cell interactions in monolayer culture. Penetration of K8, to the outer surface of the cell, may be related to the degree to which well-formed IF fibrils connect to desmosome and hemidesmosome-like structures.

In our experiments to determine whether K8-specific antibodies block plasminogen-binding to cells in culture, we utilized papain-derived Fab fragments (39). This is because a C-terminal Lys residue is conserved in immunoglobulin gamma chain subclasses, which may allow antibodies to bind plasminogen in solution and thereby indirectly inhibit plasminogen-binding to cell surface receptors (39,45). Furthermore, immunoglobulins are readily modified by plasmin to generate products that also may bind plasminogen in solution (46). In practice, plasminogen-binding to immunoglobulins is observed primarily with monoclonal antibodies and not polyclonal antibodies, perhaps due to the activity of carboxypeptidases in the plasma (47).

To determine whether K8 supports plasminogen activation by tPA, on the cell surface, we examined the effects of 1E8 Fab on plasminogen activation in MCF-7 cell cultures (39). In the absence of 1E8 Fab, activation of cell-associated plasminogen by tPA was greatly accelerated. The increase in reaction velocity was attributed to a 2000-fold decrease in the K_M. When the MCF-7 cells were pre-treated with 1E8 Fab, plasminogen activation by tPA was inhibited by at least 80%. Thus, K8 was responsible for promoting plasminogen activation by tPA on the MCF-7 cell surface; however, our initial analysis did not allow us to determine whether facilitated plasminogen activation required plasminogen-binding, tPA-binding, or both.

An important question that remains to be answered is whether K8 supports plasminogen activation by uPAR-associated uPA. Busso et al. (21) identified uPAR as part of a macromolecular complex that includes K8, K18, and a kinase that was apparently protein kinase C. The mechanism of association of uPAR with K8 within the complex was not determined; however, it is intriguing to hypothesize that the uPAR-associated K8 was present on the cell surface. In our preliminary experiments, 1E8 was ineffective at inhibiting plasminogen activation by MCF-7 cell-associated uPA; however, MCF-7 cells express very low levels of cell-surface uPAR (4,000 copies/cell) (42), compared with the number of plasminogen receptors. Thus, the relationship between K8 and the uPA-uPAR system merits further attention. In a more general sense, the ability to support plasminogen activation by uPA,

tPA, or both may be an important characteristic that distinguishes between various members of the plasminogen receptor functional family. For example, MDA-MB-231 cells express nearly 70,000 copies of cell-surface uPAR/cell (43). Although MDA-MB-231 cells demonstrate a low level of plasminogen binding and little or no cell-surface K8, the molecules that are responsible for plasminogen-binding to this cell type may have an optimal structure and subcellular localization to support uPA-mediated plasminogen activation.

To further explore the biochemistry of the plasminogen-K8 interaction, we prepared constructs that encode glutathione-S-transferase (GST) fusion proteins with 174 amino acids from the C-terminus of K8 (CK8f) or 134 amino acids from the C-terminus of K18 (CK18f) (48). We also prepared a GST-fusion protein with 134 amino acids from the C-terminus of K8 except that the C-terminal Lys was mutated to glutamine (CK8f$_{K483Q}$). As anticipated, CK8f bound plasminogen whereas CK18f did not. Interestingly, CK8f$_{K483Q}$ also abound plasminogen, although the K_D was increased from 0.4 micromolar to 1.5 micromolar. This result suggests that the plasminogen-binding site in K8 is more complex than the simple C-terminal Lys residue. Importantly, tPA bound to all three fusion proteins. In control experiments, tPA did not bind to an irrelevant GST-fusion protein (GST-receptor-associated protein) or to albumin. Plasminogen activation by tPA was supported by CK8f and, to a lesser degree, by CK8f$_{K483Q}$. CK18f failed to promote plasminogen activation, indicating that plasminogen binding is essential. tPA and plasminogen cross-competed for binding to CK8f; however, plasminogen did not inhibit tPA-binding to CK18f.

From these *in vitro* experiments, an interesting model emerges in which K8 and K18 function in a complementary manner to promote plasminogen activation by tPA. Although we have no direct evidence that both tPA and plasminogen must be cytokeratin-associated to promote plasminogen activation, close positioning of enzyme (tPA) and substrate (plasminogen) on adjoining K8 and K18 monomers may explain the substantial decrease in the K_M for plasminogen activation. In the absence of K18, closely-immobilized K8 monomers may also promote plasminogen activation (29). In this case, plasminogen and tPA are probably bound to adjacent K8 monomers but not the same K8 molecule.

There is substantial evidence that K8 expression in cancer cells may be linked to an aggressive phenotype. When mouse L cells, a fibroblast cell line, are co-transfected to express K8 and K18, these cells demonstrate increased migration and invasion of extracellular matrix-coated filters (49). Aberrant expression of K8 in squamous cell carcinoma and transitional cell carcinoma is greatest in cells near the invasion front (50,51). In H-Ras-transformed epidermal cells, increased levels of K8 expression may be linked to an increase in the malignancy of the cells (52).
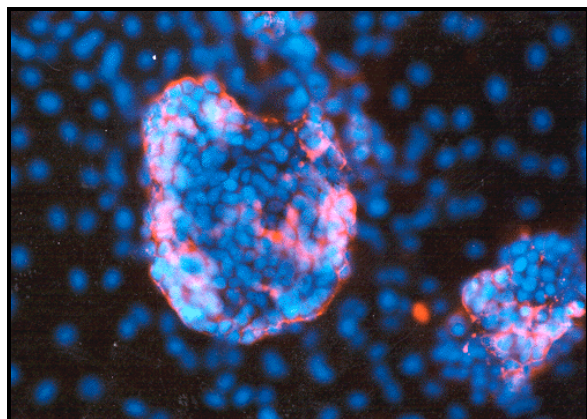
Figure 4. Cell-surface cytokeratin 8 in co-cultures of breast cancer cells and fibroblasts. Human fibroblasts were grown to near confluence, after which MCF-7 breast cancer cells were added to the culture. MCF-7 cells grew as aggregates above the monolayer of fibroblasts. Living, nonpermeabilized cells were then incubated with the cytokeratin 8 specific antibody, 1E8. After washing to remove unbound 1E8, the cells were fixed and permeabilized, and Texas red-conjugated secondary antibody was added. In addition, the fluorescent nuclear stain, DAPI, was added. Cell-surface cytokeratin 8 staining is clearly apparent in the MCF-7 cells, particularly at the edges of the cell clusters. No cell-surface cytokeratin 8 was detected in association with the fibroblasts.

Similarly, aberrant K8 expression is associated with de-differentiation and a more malignant phenotype in keratinocytes and melanoma cells (53,54). All of these results may reflect the contribution of K8, K18 and other IFs to the structure and rigidity of the cytoskeleton; however, a role for cell-surface K8 in promoting proteinase activation cannot be ruled out.

5. MODELS FOR THE ACCUMULATION OF CYTOKERATIN ON THE CELL SURFACE

K8 is secreted in the form of variably-sized molecular complexes by epithelial cancer cells in culture (29,55,56). The K8 in these complexes may be partially proteolyzed (56). Nevertheless, we have shown that breast cancer cell-released K8-containing complexes bind plasminogen and promote plasminogen activation by tPA (29). K8 is also a prominent component of tissue polypeptide antigen, a plasma marker of cancer in patients (57). Thus, K8 is apparently released by cancer cells *in vivo*. It is not clear whether K8 is released by a subpopulation of cells, such as those that are undergoing cell death, or by all cancer cells, as part of their normal physiology. However, once K8-containing complexes have been released, they are available to interact with potential binding sites on the cell surface and thus become peripheral membrane proteins. This represents a first possible mechanism whereby K8 may accumulate on the cell surface.

An alternative explanation for why K8 is detected on the cell-surface is that the K8 penetrates the plasma membrane. There is no signal peptide or a clear-cut sequence of hydrophobic amino acids of sufficient length to form a definite independent plasma membrane-penetrating $\forall$-helix. However, the sequence gglgggyggasgmggi (aa 48-63) deserves consideration. The effectiveness of this sequence in penetrating the plasma membrane may be enhanced if it is part of a protein complex and thus behaves like a multipass transmembrane protein. In such cases, a complete unbent $\forall$-helix is not necessary. A similar hypothesis was proposed by Mahdi et al (37) to explain the presence of K1 on endothelial cell surfaces.

Finally, Asch et al. (58) demonstrated that K8 binds covalently to plasma membrane lipids. It is possible that these lipids may translocate to the external surface of the plasma membrane, carrying K8. Distinguishing between these three models is not simple and it should be emphasized that the three proposals regarding how K8 may accumulate on cell surfaces are not mutually exclusive. Figure 4 shows an experiment in which we attempted to demonstrate that the first mechanism is operative in cell culture. MCF-7 cells were co-cultured with human embryonic fibroblasts for 48 h. The difference in the morphology of these cells made them readily distinguishable. The MCF-7 cells formed tight intercellular adhesions and gland-like structures whereas the fibroblasts were spindle-shaped and free-standing. To mark the location of the cells, we used the blue-fluorescing nuclear stain, DAPI (4', 6-diamidine-2'-phenylindole,dihydrochloride). Unfixed and unpermeabilized cells were incubated with antibody 1E8 and then with Texas Red-conjugated rabbit anti-mouse IgG. K8 was clearly detected on the surfaces of the MCF-7 cells; however, K8 was not detected on the fibroblast surfaces. Thus, K8-containing complexes, that are secreted by MCF-7 cells, do not bind indiscriminately to cell surfaces. While it is possible that MCF-7 cells express a specific cell-surface K8-binding protein, that is not expressed by fibroblasts, we consider this possibility unlikely, especially given the fact that we have detected over 10^6 1E8-binding sites on each MCF-7 cell.

6. PLASMINOGEN RECEPTORS IN CANCER – FUTURE DIRECTIONS

The importance of identifying plasminogen receptors in various cell types is supported by a growing body of results from animal experimentation. Furthermore, the fact that a growing number of proteins are recognized for their ability to function as cell-surface plasminogen receptors does not diminish the importance of understanding this activity. In epithelial cell carcinomas and in other cancers that aberrantly express K8, the function of K8 as a plasminogen receptor merits attention. However, the function of other plasminogen receptors, including those described in adjoining reviews, should not be ignored. Furthermore, it is quite possible that the most prevalent plasminogen receptor on the cell-surface may not be the most important. Instead, the association of plasminogen receptors with specific plasma membrane domains may promote or limit interactions of cell-associated plasmin-(ogen) with other pivotal plasma membrane proteins, including uPAR and potential plasmin substrates. This is an important topic for future consideration.

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