Plasminogen-Dependent Matriptase Activation Accelerates Plasmin Generation by Differentiating Primary Human Keratinocytes

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Pericellular plasmin generation, an important pathophysiological process, can be initiated and accelerated by the autoactivation of the type 2 transmembrane serine protease matriptase and subsequent activation of urokinase plasminogen activator. The link between matriptase and plasminogen was initially thought to be one-directional: from matriptase, through plasminogen activator, to plasminogen. However, in the current study, we now show that primary human keratinocytes that are undergoing calcium-induced differentiation can rapidly activate matriptase in response to serum treatment via a mechanism dependent on intracellular calcium, protein kinase C, and phosphatidylinositol 3-kinases-based signaling. The serum factor, responsible for the induction of matriptase zymogen activation, was shown to be plasminogen. A sub-pM concentration of plasminogen (but not plasmin) acting at the cell surface is sufficient to induce matriptase activation, suggesting high potency and specificity of the induction. After matriptase zymogen activation, a proportion of active matriptase is shed into extracellular milieu and returns to the cell surface to accelerate plasmin generation. The ability of plasminogen to induce matriptase zymogen activation and the subsequent acceleration of plasmin generation by active matriptase reveals a feed-forward mechanism that allows differentiating human keratinocytes to rapidly and robustly activate pericellular proteolysis.

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INTRODUCTION

During the healing of cutaneous wounds, one step that is critical for the keratinocytes role in epithelialization is the precise induction of the plasminogen activator (PA)/plasmin system, which commences with the activation of PA and leads to the generation of plasmin from its zymogen, plasminogen. Plasmin is a potent serine protease that is not only required for dissolving fibrin clots and the removal of necrotic tissue along the path of keratinocyte migration during wound healing, but is also involved in migration of keratinocytes and inflammatory cells, through the activation and release of growth factors (Koolwijk et al., 1996; Rifkin et al., 1997), the activation of matrix metalloproteases (Netzel-Arnett et al., 2006), angiogenesis (Chapman, 1997), and collagen remodeling (Li et al., 2000; Singer and Clark, 1999). The regulation of the PA/plasmin system, therefore, serves multiple roles critical for each and every step of the healing process, and any disruption in this tightly controlled system may lead to aberrant healing, such as in chronic wounds. By restricting the expression of urokinase PA (uPA, the major PA in keratinocytes) and its membrane receptor, uPAR, to only the keratinocytes in the outgrowing epithelial sheet in the healing wound (Gissler et al., 1993; Justus et al., 1987; Kramer et al., 1995; Romer et al., 1991, 1994), plasminogen activation is temporally and spatially targeted to respond to tissue damage and participate in the subsequent repair processes. The initiation of the PA/plasmin system is believed to rely on the very low intrinsic enzymatic activity of pro-uPA to generate an initially undetectable amount of plasmin from plasminogen (Ellis and Dano, 1993). Because plasmin is itself a potent activator of pro-uPA (the zymogen of uPA), the small amount of newly generated plasmin can covert pro-uPA into active uPA, thereby initiating a self-amplifying process named reciprocal zymogen activation. As a result of this process, a burst plasmin generation follows a lag phase because of the need for the accumulation of sufficient plasmin for detection.

Although generation of plasmin by reciprocal zymogen activation is an important mechanism, the presence of active uPA in the urine of plasminogen ($Plg^{-/-}$) gene-inactivated mice (Bugge et al., 1995) demonstrates that alternative mechanisms for the initiation of the system must be present. By examining the distinct patterns of

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Abbreviations: 6-AHA, 6-aminohexanoic acid; Ca^{2+}, calcium; PA, plasminogen activator; uPA, urokinase plasminogen activator

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plasminogen activation in two monocytic cell lines, a recent study revealed that the type 2 transmembrane serine protease matriptase can generate active uPA through a plasmin-independent mechanism (Kilpatrick et al., 2006). Matriptase is expressed by virtually all types of epithelial cells and contributes to the maintenance of epithelial integrity and function (List et al., 2009; Oberst et al., 2001). However, it is the skin, including the epidermis and hair follicles, that is the organ system most affected in patients with matriptase mutations, including those resulting in the complete loss of the enzyme, resulting in an ichthyosis with hypotrichosis syndrome (Alef et al., 2009; Avrahami et al., 2008; Basel-Vanagaite et al., 2007). The manifestation of the defect in the skin rather than in the other epithelia of the body may relate to the fact that matriptase zymogen activation seems to be significantly higher in certain areas of the skin compared with the rest of the body. Matriptase zymogen activation is observed at the highest levels in the basaloid cells of the sebaceous gland, the matrix cells in the hair bulb, and the cells in the outer root sheath of the hair follicles, including the bulge area, which contain the stem cells (Wu et al., 2013). Modest levels of activated matriptase can also be detected in the basal keratinocytes in slightly less than half of the human skin specimen examined (Chen et al., 2013a). In contrast, activated matriptase is not detectable in prostate and kidney, both of which express high levels of matriptase (Wang et al., 2009). The pattern of matriptase expression during the process of epidermal differentiation in organotypic skin culture and in vivo in human skin indicates that matriptase contributes to human skin through the regulation of proliferation and early differentiation of human keratinocytes (Chen et al., 2013a). Matriptase may regulate skin function via activation of uPA, hepatocyte growth factor, and protease activated receptor-2 (Lee et al., 2000; Takeuchi et al., 2000).

Matriptase is itself synthesized as a single-chain zymogen and acquires full enzymatic activity only after conversion into a two-chain active protease via autoactivation (Oberst et al., 2003), a process that can be observed as an early event associated with epidermal differentiation and regeneration in organotypic skin culture systems (Chen et al., 2010, 2013a). Given the role of matriptase in plasmin generation through activation of uPAR-bound pro-uPA, it seems likely that a matriptase-uPA-plasmin pericellular protease cascade might be present in keratinocytes, and that the initiation of matriptase zymogen activation could, therefore, represent an important mechanism for the control of plasmin generation in processes such as wound healing. The induction of matriptase activation in keratinocytes is, however, poorly understood, and so a search for keratinocyte-selective extracellular stimuli for matriptase zymogen activation should provide valuable insights into the control of pericellular plasmin generation. In the current study, we have discovered that rapid matriptase zymogen activation can be induced by plasminogen in calcium-induced differentiating primary human keratinocytes, leading to accelerated pericellular plasmin generation.

RESULTS

Calcium ions and serum collaborate to stimulate matriptase activation in primary human keratinocytes

The discovery that plasminogen can act as an initiator of plasmin generation through stimulating matriptase zymogen activation in human primary keratinocytes began with the observation that a combination of calcium (Ca^{2+}) and serum stimulates keratinocytes to convert the 70-kDa matriptase zymogen into the 120-kDa activated matriptase complex with hepatocyte growth factor activator inhibitor-1 (Figure 1a and b). Zymogen activation was, however, found to occur at very low levels when the cells were grown in low (0.0 9 mM) Ca^{2+} medium supplemented with up to 2% fetal bovine serum alone, or in fetal bovine serum-free medium supplemented with Ca^{2+} at up to a concentration of 1.5 mM. We then examined if the sequence of Ca^{2+} and serum exposure was important, and found that the induction of matriptase activation was observed if the cells were pretreated with Ca^{2+}, followed by human serum exposure, whereas the inverse treatment with serum exposure first followed by Ca^{2+} failed to stimulate matriptase activation (Figure 1c). These data suggest that Ca^{2+} and serum play distinct, but coordinated roles in the stimulation of matriptase zymogen activation. This distinction was further highlighted by observed differences in the exposure time requirement for the induction of matriptase activation by Ca^{2+} and serum. After pretreatment of the cells with Ca^{2+} (0.2 mM) overnight (16 hours), matriptase activation was induced by exposure to serum for as little as 30 minutes with the level of matriptase-hepatocyte growth factor activator inhibitor-1 complex reaching a plateau approximately 1 hour after the start of serum exposure (Figure 1d). In fact, to induce significant matriptase activation by serum exposure, the cells must be pretreated with Ca^{2+} for at least 8–16 hours (Figure 1e). These data suggest that primary human keratinocytes are only able to rapidly activate matriptase in response to serum after the cells have been primed by Ca^{2+} exposure. Given that Ca^{2+} induces keratinocyte differentiation, the requirement for Ca^{2+} priming in serum-induced matriptase activation suggests that differentiating, but not proliferating, keratinocytes can initiate this matriptase-mediated cell surface proteolytic cascade in response to serum. The notion that matriptase activation is induced in differentiating keratinocytes by serum is further supported by the induced matriptase activation in keratinocytes treated with 1,25-dihydroxvitamin D, although to a lesser extent (data not shown).

The time required for Ca^{2+} priming suggested that de novo protein synthesis might be involved in Ca^{2+} priming in contrast to serum-induced matriptase activation where the rapidity of the effect probably precludes that. We tested the effect of both the RNA synthesis inhibitor actinomycin D (Figure 2a) and protein synthesis inhibitor cycloheximide (Figure 2b) and found that both are able to inhibit matriptase activation induced by Ca^{2+} and followed by human serum in a dose-dependent manner, confirming the importance of de novo protein synthesis.

Extracellular Ca^{2+} induces many cellular and molecular events, including increasing the intracellular Ca^{2+} concentration (Sharpe et al., 1989; Yang et al., 2003), and the activation of protein kinase C and phosphatidylinositol 3-kinases