RESEARCH ARTICLE

Coagulation factor IX regulates cell migration and adhesion in vitro

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Abstract

Coagulation factor IX is thought to circulate in the blood as an inactive zymogen before being activated in the coagulation process. The effect of coagulation factor IX on cells is poorly understood. This study aimed to evaluate the effects of intact coagulation factor IX and its cleavage fragments on cell behavior. A431 cells (derived from human squamous cell carcinoma), Pro5 cells (derived from mouse embryonic endothelial cells), Cos7 cells, and human umbilical vein endothelial cells were utilized in this study. The effects of coagulation factor IX and its cleavage fragments on cell behavior were investigated in several types of experiments, including wound-healing assays and modified Boyden chamber assays. The effect of coagulation factor IX depended on its processing; full-length coagulation factor IX suppressed cell migration, increased adhesion to matrix, and enhanced intercellular adhesion. In contrast, activated coagulation factor IX enhanced cell migration, suppressed adhesion to matrix, and inhibited intercellular adhesion. An activation peptide that is removed during the coagulation process was found to be responsible for the activity of full-length coagulation factor IX, and the activity of activated coagulation factor IX was localized to an EGF domain of the coagulation factor IX light chain. Full-length coagulation factor IX has a sedative effect on cells, which is counteracted by activated coagulation factor IX in vitro. Thus, coagulation factor IX may play roles before, during, and after the coagulation process.

Keywords: activation peptide; adhesion; coagulation factor; de-adhesion; EGF motif; migration

Introduction

Coagulation factor IX (F9) is essential for normal coagulation. Hemophilia B, which results in abnormal bleeding, is caused by mutations in the gene encoding FIX (Lawn, 1985). The FIX protein consists of an N-terminal light chain, a C-terminal heavy chain, and an intervening connecting region. During the coagulation process, FIX is cleaved at two Arg residues (Arg192 and Arg236 in mice) by activated factor XI or by the factor VIIa/TF complex (Zogg and Brandstetter, 2009). Cleavage releases the connecting region, also known as the activation peptide, resulting in FIX activation. Activated FIX consists of a light chain and a heavy chain linked by disulfide bonds. Activated FIX cleaves and activates factor X.

The functions of the various FIX domains have been intensively studied. The light chain consists of a Gla domain, an epithelial growth factor (EGF) domain, and a von Willebrand factor binding domain. The Gla domain at the N-terminus of the light chain plays an important role in FIX localization. It is essential for binding to phosphatidylserine on activated platelets during hemostasis. The Gla domain also mediates binding to endothelial cells via collagen IV (Gui et al., 2002). This appears to be one of the reasons that intravascular recovery of FIX is only approximately 50% effective in hemophilia B patients treated with FIX (Lichtman, 2006). The pattern of FIX distribution suggests that FIX functions not only in a circulating form but also in a fixed form in tissues or cells. The EGF domain is known to play supportive roles for other domains. A trypsin domain located within the heavy chain requires the EGF domain for enzymatic activity. Additionally, the EGF domain stabilizes the Gla domain. However, EGF domain’s own function has not been elucidated. The FIX activation peptide has not been studied extensively, although it is known that liberation of the FIX activation peptide is essential for enzyme formation. To our knowledge, no other function of the activation peptide independent from its enzymatic activity has been reported.
It is thought that FIX circulates in the blood in the inactive form until it is called upon to mediate hemostasis following an injury. In the present study, we examined the effect of FIX on adhesive cells outside of the coagulation process. The EGF domain and the activation peptide exhibit significant effects on cell adhesion and migration, and these activities are regulated in the same manner as processing for coagulation.

Materials and methods

Cells and culture

A431 cells (derived from human oral squamous cell carcinoma) were grown in serum-free 64 medium (60% Opti-MEN [Invitrogen, Carlsbad, CA], 40% LHC-8 medium [Invitrogen]). Pro5 cells (derived from mouse embryonic endothelial cells) and Cos7 cells were cultured in modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (Biowest, Nuaille, France). The results of immunoblotting verified that the serum did not contain full-length FIX or its light chain. Human umbilical vein endothelial cells (HUVECs) were grown in Medium 200 (Invitrogen) with Low Serum Growth Supplement (Invitrogen).

Reagents

Recombinant alkaline phosphatase (AP)-containing fusion proteins were prepared as previously described (Kitano et al., 2008). Briefly, we generated several F9 deletion mutants by RT-PCR and cloned them into AP-tag4 vector (GenHunter, Nashville, TN) for production of AP-tagged FIX as a secreted protein in CHO cells cultured in 64 medium. The DNA constructs included mouse full-length FIX (amino acids 47–471, accession number P16294) without the N-terminal propeptide, a fragment of FIX lacking the heavy chain (amino acids 47–236), the FIX light chain (amino acids 47–191), the first EGF domain of FIX (amino acids 97–130), the activation peptide (amino acids 192–236), and the EGF domain of coagulation factor VII (F7, amino acids 87–123, accession number P70375) or coagulation factor X (F10, amino acids 86–122, accession number O88947). An AP tag without FIX was used as a control. The AP activity in the conditioned medium was measured by adding 10 μL of 0.05% Zwittergent (Calbiochem, La Jolla, CA) in PBS to each well of a 96-well plate, followed by 20 μL of the conditioned medium. The enzyme reaction was initiated by adding 200 μL of substrate (1 mg/mL p-nitrophenyl phosphate [Sigma, St. Louis, MO] in 1 mM MgCl2 and 1 M diethanolamine, pH 9.8) to each well, and the absorbance at 405 nm was measured after 30–60 min. Native human FIX and active FIX were purchased from Thermo Scientific (Waltham, MA). Antibodies that recognize FIX, E-cadherin, integrin α3β1, integrinβ1, paxillin, and β-catenin were purchased from Abcam (Cambridge, MA). Antibodies recognizing VE-cadherin were purchased from Cell Signaling Technology (Beverly, MA).

Wound-healing assay

Cells were plated at a dilution consistent with confluence in a 5-mm diameter plastic ring on a plastic tissue culture dish coated with fibronectin. After 6 h, the ring was removed and migration was evaluated by time-lapse microscopy using a Cellwatcher (Corefront, Tokyo, Japan) in 64 medium at 37°C. The velocity of migration was assessed 2 h before and 2 h after administration of 0.1 pmol/mL of FIX or recombinant FIX fragments. A buffer for native FIX or an AP tag without FIX was used as a control. The front line of migrating cells was marked every 30 min, and the average migration velocity was estimated by determining the speed at which the marked point advanced on a line perpendicular to the front line. The migration velocity was normalized to that determined before administration of the recombinant proteins. Data are reported as the mean (±SD) of 20 marked points. All experiments were repeated more than three times.

Modified Boyden chamber assays

Cell migration was evaluated using a Chemotaxicell (Kurabo, Osaka, Japan) equipped with 8-μm micropores. In the top chamber, cells were incubated in αMEM (Invitrogen) containing 0.1 pmol/mL of recombinant FIX fragments. The bottom chamber was filled with αMEM containing 10% fetal bovine serum. After 1 h, membranes were stained with toluidine blue and the migrating cells were counted under a microscope. An AP tag without FIX was used as a control. Migration was evaluated on the basis of the ratio of the number of migrating cells treated with AP-tagged protein to the number of migrating non-treated cells. Data are reported as the mean (±SD) of 20–25 fields. All experiments were repeated three times.

Immunohistochemistry

To investigate adhesion to matrix, A431 cells were plated on fibronectin-coated coverslips for 1 h and treated with recombinant FIX for 5 min. To investigate intercellular adhesion, A431 cells or HUVECs were cultured for 48 h on fibronectin-coated coverslips and treated with recombinant FIX for 1 h. Cells were fixed in PBS with 4% paraformaldehyde and then permeabilized in PBS with 0.1% Triton X-100. Cells were then incubated with primary antibodies, and staining was detected with Alexa Fluor 488 anti-rat IgG (Invitrogen), Alexa Fluor 568 anti-mouse IgG (Invitrogen), or Alexa Fluor 488 anti-mouse IgG (Invitrogen). When necessary, cells were
then stained with Alexa Fluor 568-phalloidin to detect actin or
stained with Hoechst 33342 to detect nuclei. Cells were examined using a fluorescent microscope (Axioskop 2; Carl Zeiss, Oberkochen, Germany). Images acquired using the Axioskop 2 at 100× final magnification were analyzed using Axiovision software (Carl Zeiss). For quantitative evaluation of intercellular adhesion, the intensity of yellow fluorescence in immunohistochemistry was measured using Popimaging software (Digital Being Kid, Kanagawa, Japan).

Western blotting
Cells were cultured overnight in 3.5-cm diameter dishes, after which 1 pmol/mL AP or AP-tagged FIX fragment was added at 0, 5, 10, and 30 min and cells were then harvested with sample buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (ATTO, Tokyo, Japan). Membranes were incubated with primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies, and immunoreactive proteins were detected using an ECL Advance Western blotting detection kit (Amersham, Piscataway, NJ).

De-adhesion assay
Cells were plated at a sub-confluent concentration in a 96-
well plate and cultured overnight. Next, 1 pmol/mL of the recombinant proteins with AP tags were added and the plate was shaken for 30 min at 1,200 rpm on an OPM-103 microplate mixer (As One Corporation, Osaka, Japan). Detached cells were aspirated with a pipette and counted using a hemocytometer. Recombinant AP was used as a negative control. The number of detached cells in the negative control experiment was arbitrarily set to 1. Results are expressed as the mean ± SD.

Migration assay with local infusion of recombinant EGF-F9
Cells were cultured for 1 h on fibronectin-coated coverslips. Glass needles were prepared by pulling GD-1.5 glass capillaries (Narishige, Tokyo, Japan) with a PP-830 puller (Narishige) and placing them 50 μm from the edge of a cell. Medium containing 0.1 pmol/mL of control protein or recombinant EGF domain of FIX was infused at a rate of 0.1 mL/h using an infusion pump (Terumo, Tokyo, Japan). Photographs were taken every 5 sec using AquaCosmos software (Hamamatsu).

Statistical analysis
Wilcoxon’s rank sum test was used to assess the statistical significance of differences in means. The χ² test was used to evaluate the equivalence of frequencies. Statistical significance was set at P < 0.05.

Results
Native and activated FIX regulate cell migration
We first evaluated the migration of A431 cells using a wound-healing assay (Figure 1A). Migration of cells was recorded using time-lapse video photography and phase-contrast microscopy (Supplementary Movie 1). In this assay, cells normally migrate at the rate of 5–30 μm/h. We then compared the migration velocity before and after treatment with FIX. Treatment with native FIX led to a 20% decrease in cell migration velocity, whereas treatment with activated FIX led to a 73% increase in cell migration velocity. Administration of activated FIX led to retrograde movement within 5–10 min (Supplementary Movie 2). The cell sheet appeared to detach from the bottom of the dish and then quickly shrink. Migrating cells in the front line are shown in Figure 1B. In comparison with mock-treated cells, cells treated with native FIX remained dense along the front line. In contrast, cells treated with activated FIX clearly dispersed away from the front line.

To investigate how the active domains of FIX affect cell migration, we generated several different cell lines to determine the specificity of the effect of FIX on cell migration (Figure 2). As observed with A431 cells, embryonic yolk sac-derived cells, pro5 cells, and cos7 cells responded to full-length FIX (amino acids 47–471) and the light chain (amino acids 237–471) but was lost when the activation peptide (amino acids 192–236) was deleted. Deletion of the activation peptide reversed the effect of FIX from suppression to enhancement of migration. Further deletion analysis localized the region responsible for enhanced cell migration to the EGF motif (amino acids 97–130; hereafter, EGF-F9). The activation peptide by itself was sufficient to inhibit cell migration.

Because the results of the wound-healing assay could be affected by cell proliferation, stimulation of cell migration by activated FIX fragments was confirmed using a Boyden chamber assay (Figure 1D). In the Boyden chamber assay, the suppressive activity of full-length FIX was retained following deletion of the heavy chain (amino acids 237–471) and lost following deletion of the activation peptide (amino acids 192–236). Treatment of cells with EGF-F9 led to an increase in the number of migrating cells, and treatment with the activation peptide resulted in a decrease in the number of migrating cells.

We then examined several different cell lines to determine the specificity of the effect of FIX on cell migration (Figure 2). As observed with A431 cells, embryonic yolk sac-derived cells, pro5 cells, and cos7 cells responded to full-length FIX (amino acids 47–471) and the light chain (amino
acids 47–192). Thus, the effect of FIX appears to be nonspecific with regards to cell type, as the effect was demonstrated in cells of diverse origin (i.e., epithelial [A431], endothelial [pr05], and mesenchymal [cos7]). These data indicated that cell migration can be suppressed by native FIX with the activation peptide and stimulated by activated FIX with the EGF motif.

To elucidate the mechanism of the effect of EGF-F9 on cell migration, we examined the activation of signaling proteins involved in cell migration. Phosphorylation of ERK1/2, Akt-Thr308, and Akt-Ser473 was examined by Western blotting analysis. None of these proteins were phosphorylated within 30 min of EGF-F9 administration. Fragments of FIX regulate cell adhesion to matrix

Migrating cells treated with active FIX appeared to detach from the matrix (Supplementary Movie 2) within several minutes. We therefore hypothesized that active FIX disrupts the adhesion of cells to matrix. Cell adhesion to matrix was evaluated with A431 cells by counting the cells that detached from the culture plate surface after treatment with 1 pmol/mL of recombinant FIX fragments and subsequent shaking (Figure 3A). After administration of FIX fragments, cells did not move when the plates were gently shaken and instead appeared to adhere to the bottom of the plate. After treatment with FIX fragments that contain the EGF motif followed by vigorous shaking, however, some cells detached from the plate. Interestingly, the activation peptide inhibited detachment of cells treated with EGF-F9. Cells shaken after treatment with EGF-F9 became round in appearance. In contrast, cells treated with the activation peptide were spread out and epithelial in appearance (Figure 3B). Next, the effect of active FIX dose was examined in a de-adhesion assay (Figure 3C). The active form of FIX showed a dose-dependent effect beginning at a concentration of 0.1 pmol/mL.

Immunocytochemistry experiments were conducted to evaluate integrin-mediated adhesion (Figure 3D). Because integrin α3β1 staining was more intense than that observed for integrin α2β1, α5β1, and αvβ3 under our experimental conditions (data not shown), we examined the distribution of integrin α3β1 in this assay. In control cells, integrin α3β1 co-localized with paxillin along the cell edge and in the filopodia. Moreover, we observed a

Figure 1 Effect of various FIX fragments on cell migration. A: Effects of native (IX) and activated (IXa) FIX on cell migration. B: Upper panels, macroscopic observation of cell migration. Lower panels, high-magnification views along the edge of the cell disc. Bars in upper panels represent 5 mm and bars in lower panels represent 100 μm. C: Effect of FIX deletion mutant proteins on the migration of A431 cells in the wound-healing assay. D: Effect of FIX deletion mutant proteins on the migration of A431 cells in the Boyden chamber assay. Fragment 97–130 is the EGF-like domain. Fragment 192–236 is the activation peptide. Data are presented as the mean ± SD. *P < 0.05, **P < 0.01, n = 3.
punctate distribution of integrin α3β1 and paxillin in the filopodia. Treatment with EGF-F9 decreased staining along the cell edge and in the filopodia within 5 min. To determine whether EGF-F9 treatment disrupts filopodial adhesion, we microscopically counted the number of non-adherent moving filopodia. The number of moving filopodia was 1.56 ± 1.56/cell in control cells and 7.88 ± 2.96/cell in EGF-treated cells (P < 0.01). These results indicate that EGF-F9 treatment disrupts adhesion of cells to matrix, an effect that is more prominent along the edges of the cell disc and at the filopodia.

It has been reported that weak adhesion of cells to matrix facilitates cell migration (Arnold et al., 2008; Cavalcanti-Adam et al., 2008). To confirm that de-adhesion is involved in migration, we examined cells subjected to local infusion of EGF-F9. Local infusion has been used to evaluate positive chemoattractant factors in vitro. In the present study, we used this approach to determine if the centroid of a cell moves away from an injection needle. Figure 4A shows typical changes resulting from local infusion of EGF-F9. After infusion of recombinant EGF-F9 from a fine glass needle, the cell detached at the edge of the stimulated side. Quantitative evaluations based on shifts in cell centroid revealed that 72% of the cells treated with EGF-F9 moved away from the infusion site, compared with only 28% of control cells (Figure 4B). Some of the cells either did not move or moved toward the infusion site, as they detached around the entire perimeter, not merely at the stimulated site. These data indicate that cell de-adhesion could be a mechanism of the enhancement in cell migration mediated by EGF-F9.

**Figure 2** Effect of various FIX fragments on the migration of different cell types. The effect of various FIX fragments on the migration of A431 (open bars), Pro5 (closed bars), or Cos7 (shaded bars) cells was examined using a wound-healing assay (A) and Boyden chamber assay (B). Numbers indicate amino acid residues. Fragment 47–471 is full-length FIX. Fragment 47–192 is the light chain. Data are presented as the mean ± SD. *P < 0.05, **P < 0.01, n = 3.

**Fragments of FIX regulate intercellular adhesion**

Because A431 cells treated with the activation peptide became more like epithelial cells than control cells in the de-adhesion assay (Figure 3B), we hypothesized that FIX containing the activation peptide enhances intercellular adhesion. Moreover, the dispersed distribution of cells observed in the cell migration assay (Figure 1B) suggests that EGF-F9 might disrupt intercellular adhesion. We, therefore, analyzed the effect of FIX and its fragments on intercellular adhesion of A431 cells using immunocytochemistry with antibodies against E-cadherin and β-catenin. Control cells adhered to each other and exhibited curved boundaries (Figure 5). In contrast, when treated with full-length FIX or activation peptide (amino acids 192–236), the cell boundaries were straight and sharp, and when treated with the light chain, the cell boundaries were faint and discontinuous.

Because intercellular adhesion is critical for permeability of the endothelium, intercellular adhesion of HUVECs was also examined. In the cell migration assay, HUVECs treated with EGF-F9 were more dispersed and some gaps were observed between cells (Figure 6A). In contrast, cells treated with the activation peptide migrated in a papillary arrangement without cell dispersion. The migration velocity was determined using a cell migration assay (Figure 6B). Treatment with EGF-F9 increased the migration velocity by 50%; in contrast, treatment with activation peptide decreased the velocity by 50%. Using immunohistochemistry with anti-VE-cadherin and anti-β-catenin antibodies, we examined the intercellular adhesion of HUVECs cultured to confluence. In control cells, staining indicative of intracellular adhesion appeared mesh like. Treatment with EGF-F9 resulted in the appearance of gaps in the cell monolayer (Figure 6C) and loss of intracellular adhesion (Figure 6D).
**Figure 3** Effect of various FIX fragments on adhesion of cells to matrix. A: De-adhesion assay with various FIX deletion mutants. A431 cells were treated with the indicated FIX fragment and shaken for 30 min on a plate mixer. Detached cells were counted using a hemocytometer. The number of detached cells with the control AP protein was arbitrarily set as 1 (n = 6). Data are presented as the mean ±SD. **P < 0.01. B: Cells after 30 min of shaking in the presence of the indicated FIX fragment in the de-adhesion assay. Bar represents 100 μm. C: Dose-dependent effect of FIXa in the de-adhesion assay (n = 6). Data are presented as the mean ±SD. **P < 0.01. D: Immunocytochemistry of cells treated with a control protein or recombinant EGF domain (fragment 97–130) using anti-integrin α3β1 and anti-paxillin antibodies. Rightmost panels show magnified views of the areas indicated by rectangles. Scale bar represents 10 μm.

**Figure 4** Effect of local infusion of EGF-FIX on cell translocation. A: Time-lapse images of cells locally exposed to the recombinant EGF domain of FIX. Numbers in panels indicate minutes. Scale bar represents 10 μm. B: The locomotion of cells locally exposed to control protein (left) or recombinant EGF domain (right). Arrowheads indicate the site of recombinant EGF domain infusion. The cross-point of the diagonal dotted lines indicates the location of cells before the stimulus, and dots indicate the location of cells after 10 min of stimulation. Cells that moved into the upper 90° quadrant were found to have migrated away from the infusion site (n = 32).
Treatment with the activation peptide increased the level of intercellular staining and the disappearance of gaps induced by EGF-F9 treatment.

The function of EGF domains of proteins homologous to FIX

All vitamin K-dependent proteins (FIX, FX, FVII, protein C, and protein S) contain EGF domains. The amino acid sequences of various vitamin K-dependent proteins were compared to determine whether the EGF domains share the consensus sequence CXDXXXXYXCXC (Figure 7A). Because it was revealed that coagulation factors FX and FVII share the consensus sequence with FIX, the effect of FX and FVII on cell adhesion was examined using a de-adhesion assay. The results of this experiment showed that these factors induce de-adhesion of cells in a manner similar to EGF-F9 (Figure 7B).

Discussion

In the present study, we found that in addition to its role in the coagulation process, FIX has several novel effects on cells. Native FIX has a sedative effect on cell behavior, suppressing cell migration and increasing adhesion to matrix and enhancing intercellular adhesion. In contrast, active FIX has an activating effect on cell behavior, increasing cell migration, weakening cell matrix adhesion, and disrupting intercellular adhesion. As is the case with its function in the coagulation process, these novel functions of FIX are also regulated through protein processing. The activity of native FIX was localized to an activating peptide that is removed by cleavage during the coagulation process. Because the activating peptide is functional both alone and within native FIX, it could exert its effects both before and after processing. The activity of activated FIX, in contrast, was localized to the EGF domain of the light chain. The EGF
Figure 6 Effect of various FIX fragments on intercellular adhesion of HUVECs. A: Effect of control AP protein, fragment 97–130, and fragment 192–236 on migration of HUVECs. Arrowheads indicate gaps between cells. B: Effect of EGF-F9 (97–130) and the activation peptide (192–236) on cell migration (n = 3). Data are presented as the mean ± SD. *P < 0.05, **P < 0.01. C: Immunocytochemistry using antibodies to VE-cadherin (green) and β-catenin (red). Cells were treated with control AP protein, fragment 97–130, or fragment 192–236. Scale bar represents 50 μm. D: Comparison of fluorescence intensity at the intercellular adhesion sites in immunocytochemistry staining for VE-cadherin and β-catenin (n = 6). Data are presented as the mean ± SD. *P < 0.05, **P < 0.01.
domain was found to be active in the absence of the activation peptide and, therefore, likely exerts its function as coagulation progresses. Because the effect of EGF-F9 on cells is obvious within 5 min, it may function during the very early stages of tissue injury.

During coagulation, the activation peptide is released, and then FIX functions as it is unlocked. The activation peptide must remain with the light chain–heavy chain dimer to inhibit the enzymatic function of FIX. The activation peptide and EGF-F9 may affect cells differently. Because EGF-F9 and the activation peptide function in serum-free medium, these domains could work independently of each other.

It is difficult to speculate regarding the molecular mechanism of the activation peptide’s activity based only on homology, as it does not share homology with other known proteins. Low homology with chick F9 suggests that the activation peptide functions identified in the present study are restricted to mammals (Figure 7B). The activities observed in our study (i.e., suppression of cell migration, increased adhesion to matrix, and enhanced intercellular adhesion) are similar to the effects associated with cAMP-mediated signaling (Fukuhara et al., 2005; Kooistra et al., 2007; Adamson et al., 2008; Pannekoek et al., 2009). Noradrenalin, adrenomedullin, and prostaglandin E2 increase the level of cAMP and suppress cell migration and adhesion, resulting in decreased endothelial permeability. It is possible that the activation peptide functions through this signaling cascade.

In contrast to the activation peptide, several proteins are homologous to EGF-F9. EGF-F9 shares a calcium-binding EGF motif with numerous proteins (Winship and Dragon 1991). One of the calcium-binding EGF motifs includes the sequence CX(D/N)XXXX(Y/F)XCXC, in which the D or N residue is hydroxylated. It has been reported that the EGF motif of Del1, CVDLGNSYLCRC, loses its activity when the D residue is replaced by N or when the Y residue is replaced by F (Kitano et al., 2008). Thus, the sequence CXDXXXXYXCXC could represent a novel subfamily. This motif is shared by several proteins, such as FIX, coagulation factor VII, coagulation factor X, Del1, and Notch (Chillakuri et al., 2012). Coagulation factors FVII and FX share the sequence CXDXXXXYXCXC in their EGF domain, whereas the anti-coagulation factors protein C and protein S do not contain this sequence, although they have a similar structure. These data suggest that the consensus amino acid sequence plays a common role in processes related to coagulation.

Kitano et al. (2008) reported that EGF-F9 and the EGF domain of Del1, which is homologs to EGF-F9, are capable of increasing the gene transfer efficiency of non-viral vectors by modulating endocytosis. It has been reported that endocytosis is involved in cell migration. EGF-F9 may, therefore, exert its effects on cells via modulating endocytosis (Jekely et al., 2005).

Loss-of-function experiments may not be appropriate for investigating how FIX affects cells in vivo. Many studies have examined hemophilia B, and F9-knockout mice have been generated; however, the effect of FIX on cells remains poorly understood (Lawn, 1985; Lin et al., 1997; Sabatino et al., 2004). Hoffman et al. (2006) investigated wound healing after punch biopsy in F9-deficient mice. In F9-targeted mice, macrophage migration and dermal wound healing is significantly retarded. However, it is difficult to determine whether the phenotype is due to loss of EGF-F9 function, loss of the activation peptide, or repeated bleeding during the wound healing process. The redundancy of function...
associated with FVII and FX also makes interpretation difficult. Moreover, some coagulation-related proteins reportedly stimulate cell migration (Madsen et al., 2007; Mueller et al., 2008; LaRusch et al., 2010). Although no proteins homologous to the activation peptide have been reported, it is possible that proteins that activate cAMP could rescue loss of the activation peptide. Aside from redundancy, another difficulty encountered in analyzing F9-knockout mice is the opposing effects EGF-F9 and the activation peptide have on cells. Deletion mutants of the F9 gene could offset each other.

The significance of FIX and its fragments should be demonstrated in gain-of-function experiments. The EGF motif (i.e., CXDXXXYCXCXC) is probably active in vivo, because EGF-F9 and the EGF motif of Del1 were shown to improve in vitro and in vivo gene transfer when injected intravenously with the DNA complex via a non-viral vector (Mamiya et al., 2012). In the present study, EGF-F9 increased the rate of migration in several different cell types in vitro. Gain-of-function experiments in model mice for wound healing would thus be suitable to demonstrate the significance of EGF-F9 in vivo. Gain-of-function experiments should also be of value in studies of the activation peptide. Endothelial hyper-permeability resulting from abnormal coagulation is the cause of several serious diseases, such as thrombosis, disseminated intravascular coagulopathy (DIC), and acute respiratory distress syndrome (ARDS). Experiments using animal models of these diseases may be helpful in demonstrating the significance of the activation peptide and evaluating applications for it.

Conclusion

FIX has significant effects on cells. The active form of FIX decreases cell adhesion and increases migration. The activity is localized to the light chain of FIX and is activated by removal of the activation peptide. In contrast, the inactive form of FIX or FIX fragments containing the activation peptide enhance cell adhesion and suppress cell migration. The two conflicting functions of the FIX domains may thus regulate cell behaviors associated with bleeding, hemostasis, and wound healing.

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