Expression of Hirudin Fusion Proteins in Mammalian Cells
A Strategy for Prevention of Intravascular Thrombosis

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Background—Intravascular thrombosis occurs in disorders of diverse pathogeneses, including allograft and xenograft rejection. In this in vitro study, we describe an approach for tethering the specific thrombin inhibitor hirudin to plasma membranes as part of a genetic strategy for regulating intravascular coagulation.

Methods and Results—An HLA class I leader sequence was fused with hirudin linked to domains 3 and 4 of human CD4 and intracytoplasmic sequence from either CD4 or human P-selectin. The constructs were transfected into mouse fibroblasts, Chinese hamster ovary (CHO)-K1 cells, immortalized porcine endothelial cells (IPECs), and a pituitary secretory cell line (D16/16). Thrombin binding to the hirudin fusion proteins expressed on fibroblasts and CHO-K1 cells could be blocked by an anti-hirudin monoclonal antibody and by pretreatment of thrombin with either the synthetic tripeptide thrombin inhibitor PPACK or native hirudin. Hirudin expression significantly modified the procoagulant phenotype of IPECs in human plasma, leading to prolongation of clotting times. Hirudin-CD4–P-selectin fusion proteins accumulated in adrenocorticotropic hormone–containing granules in D16/16 cells, with no cell surface expression except on activation with phorbol ester, when hirudin relocated to the outer membrane.

Conclusions—Hirudin fusion proteins were expressed on mammalian cells, where they reduced local thrombin levels and inhibited fibrin formation. Regulated expression was achieved on activated cells by use of the cytoplasmic sequence from P-selectin. In vivo, these fusion proteins may prove useful transgenic or gene therapy agents for preventing intravascular thrombosis. (Circulation, 1998;98:2744-2752.)

Key Words: anticoagulants ■ coagulation ■ transplantation ■ thrombosis

Thrombotic vascular occlusion can occur in several multisystem disorders, may complicate local procedures such as angioplasty and vascular surgery, and features prominently in the pathology of hyperacute rejection of allografts or xenografts, where it leads to catastrophic and irreversible infarction of the transplanted organ. In this setting, the procoagulant state within the graft develops from the simultaneous effects of complement activation (dependent on the deposition of anti-graft endothelial cell [EC] antibody), subendothelial tissue factor exposure, loss of proteoglycan-linked anticoagulant molecules such as antithrombin III and tissue factor pathway inhibitor (TFPI) from EC surfaces, and downregulation of thrombomodulin, which is internalized after EC activation. In xenografts, another factor is the failure of some xenogeneic anticoagulant molecules to function efficiently across a species divide.

Intraluminal expression of EC-tethered regulators of coagulation is a particularly attractive strategy in the context of xenotransplantation because widespread intravascular deposition of fibrin is not only the end point of hyperacute rejection but is also a prominent feature of delayed xenograft rejection. Other situations in which membrane-anchored anticoagulant proteins might have a therapeutic use include prevention of complications after angioplasty or vascular surgery, when transient expression within a single vessel might be a safer and more effective way to inhibit thrombosis than systemic anticoagulation.

We have previously described an engineered membrane-tethered anticoagulant protein based on the soluble plasma protein TFPI. In this report, we describe genetic constructs encoding other fusion proteins based on the leech anticoagulant hirudin. When expressed constitutively on the surface of mammalian cells, anchored by a portion of human CD4, hirudin retained potent thrombin-binding activity and prevented fibrin generation in vitro as determined by a plasma recalcification assay. To avoid the potential complications that would accompany constitutive anticoagulation, the hirudin-CD4 constructs were modified by the addition of
P-selectin sequence to the cytoplasmic tail. This modified construct was efficiently targeted to intracellular secretory granules and was only released onto the cell surface after cellular activation. We envision these constructs will have therapeutic potential as agents for gene therapy or as transgens for expression in donor animals bred especially for xenotransplantation.

Methods

Antibodies and Reagents

The following antibodies were used in this study: mouse anti-hirudin and mouse anti–hirudin-thrombin complex (courtesy of Dr J.M. Schlaeppi, Ciba-Geigy Ltd, Basel, Switzerland); OKT-4 (ATCC); rabbit anti–human adrenocorticotropic hormone (ACTH) (Department of Histochemistry, Hammersmith Hospital, London, UK); mouse anti–human TFPI (American Diagnostica); rabbit anti–human prothrombin immunoglobulin (Dakopatts); mouse anti–human P-selectin (Becton Dickinson); FITC-conjugated swine anti-rabbit immunoglobulins and sheep anti-mouse immunoglobulins (Dakopatts); and mouse IgG1 and mouse IgG2a controls (Dakopatts). Native full-length hirudin was supplied by Biopharm. d-Phe-Pro-Arg chloromethyl ketone dihydrochloride (PPACK) was from Calbiochem, the chromogenic substrate H-d-Phe-Pip-Arg-pNA was from Calbiochem, and a synthetic amidolytic substrate S-2238 for 10 minutes at 37°C.

cDNA Constructs and Expression Vectors

The leader sequence of HLA-A2.1, encoding amino acids 1-24, was fused to hirudin by polymerase chain reaction with overlapping extension. The same technique was used to introduce sequences encoding glycine-serine–rich linkers (G1 to G3) onto the C-terminus of human CD4 (CD4166-435), a cassette cloning strategy was used. The P-selectin cytoplasmic region (P-selectin) was introduced into some constructs by polymerase chain reaction with overlapping extension, replacing CD4166-435. All primers used in the present study were designed from published cDNA sequences, and primer sequences are available on request from the authors. Intermediate constructs were subcloned into pBluescript SK(+) (Stratagene) for DNA sequencing. All constructs were predicted to encode peptides with wild-type sequence, except for the construct encoding CD4166-435, in which V328 was predicted to be mutated to A328. Complete HLA-hirudin-G1 to G3-CD4166-435 and HLA-hirudin-G1/G2-CD4166-435-P-selectin constructs were cloned into the expression vector pHβApr-1gpt. Full-length human P-selectin cDNA was subcloned into the sister vector, pHβApr-1neo. In all cases, the orientation of ligation was determined by restriction endonuclease mapping.

Cells, Transfections, and Flow Cytometric Analysis of Expression Levels

The mouse fibroblast line DAP.3, mouse pituitary cell line D16/16, and immortalized porcine endothelial cell (IPEC) clone A8 were all grown in DMEM (Gibco). Chinese hamster ovary (CHO)-K1 cells were maintained in RPMI 1640 (Gibco). Cultures were supplemented with 5% to 10% FCS, 10 μg/mL gentamycin, and 1-glutamine. Cells were transfected with either calcium phosphate according to standard protocols,10 electroporation, or a modified targeted-liposome technique. All stable transfectants were grown in medium containing mycophenolic acid (final concentration, 12 μg/mL), xanthine, and hypoxanthine or G418 as appropriate. For staining, a standard protocol was used with 2.5% FCS in PBS as buffer. Cells (10^6) were analyzed in an EPICS XL-MCL flow cytometer (Coulter).

Immunohistochemistry

Methanol-fixed D16/16 cells were permeabilized with 0.1% saponin and, after blocking with appropriate reagents, incubated with rabbit anti-human ACTH immunoglobulins and a mouse anti-hirudin monoclonal antibody (mAb) overnight. Cells were washed twice and incubated with Texas Red–conjugated rabbit anti-IgG (Vector Laboratories) or FITC-conjugated swine anti-rabbit immunoglobulins. After blocking experiments, cells were first incubated with anti-hirudin antibodies or appropriate control immunoglobulins for 30 minutes on ice and washed before being incubated with thrombin for 1 hour at 37°C as described above.

Inactivation of Thrombin Assessed by Amidolytic Assay

Either PPACK or a dodecapeptide corresponding to the C-terminal sequence of hirudin was incubated at 100-fold molar excess with 210 nmol of thrombin in 50 μL of Tris-buffered saline, 0.1% BSA, pH 7.4 for 1 hour at 37°C. In parallel, thrombin was treated with a 10-fold molar excess of native hirudin for 1 hour at 37°C. Thrombin activity was assessed by absorbance at 405 nm in a Thermomax microplate reader (Molecular Devices Ltd) after incubation with the substrate S-2238 for 10 minutes at 37°C.

Modulation of EC Procoagulant Phenotype in Human Plasma by Hirudin Expression

We mixed 10^3 hirudin-CD4-transfected IPECs or control untransfected IPECs with normal human plasma (Sigma) and 12 mmol/L CaCl2 in glass tubes (Corning Inc). The time for a fibrin clot to form at 37°C was determined in triplicate by a standard tilting technique. In some experiments, IPECs were first preincubated for 10 hours with human recombinant interleukin-1α (IL-1α) (10 ng/mL; Boehringer Mannheim) to induce tissue factor expression before washing and inclusion in clotting assays.

Results

Hirudin Fused With HLA Class I Signal Peptide and Linked to Domains 3 and 4 of Human CD4 Is Expressed as a Transmembrane Protein

The different hirudin constructs used in the present study are shown in Figure 1. Three different glycine-rich linkers (designated G1 to G3) were used (Figure 1A) to minimize the risk of steric interference of the interaction between hirudin and thrombin by the immunoglobulin-like third domain of CD4. On stable DAP.3 transfecants, hirudin-CD4 was easily detected at the cell surface (Figure 2), and its expression appeared uninfluenced by the length of the glycine linker. If trypsin was used to harvest cells from culture, the hirudin moiety was cleaved off whereas the CD4 anchor was left intact (Figure 2G and 2H), indicating that a trypsin cleavage recognition sequence may exist between hirudin and CD4.

Hirudin Anchored to the Cell Surface Binds Thrombin

After incubation with thrombin at 37°C, only hirudin-CD4-expressing DAP.3 cells and not untransfected controls bound
thrombin in a dose-dependent manner (Figure 3A and 3B); the type of glycine linker had no influence on thrombin binding. However, binding was inhibited if transfectants were preincubated with an anti-hirudin mAb (Figure 3C). Thrombin binding was also detected with an mAb directed against the hirudin-thrombin complex (Figure 3D). Because this antibody recognizes neither hirudin in the absence of thrombin nor thrombin complexed with endogenous thrombin receptors, these observations, taken together, demonstrate specific thrombin binding by the surface-expressed hirudin-CD4 fusion protein.

Interaction Between Thrombin and Hirudin-CD4 Is Abolished When the Catalytic Site of Thrombin Is Blocked

The amino-terminal domain of native hirudin binds to the active (catalytic) site of thrombin, whereas the carboxyterminal binds to the separate anion-binding exosite of thrombin. Thrombin binding to the hirudin-CD4 fusion protein was assessed after preincubation of the enzyme with either native hirudin, the tripeptide active site inhibitor PPACK, or a synthetic COOH-terminal hirudin dodecapeptide. Before analysis of binding, residual thrombin-dependent catalytic activity was analyzed with a small oligopeptide substrate (S-2238). As expected, thrombin preincubated with dodecapeptide cleaved S-2238 normally, whereas both native hirudin and PPACK completely abolished thrombin amidolytic activity (data not shown). As can be seen in Figure 3E, thrombin pretreated with hirudin or PPACK was not bound by cell-surface hirudin-CD4, whereas treatment with the hirudin dodecapeptide only slightly reduced thrombin binding. In conclusion, these results indicate that hirudin-CD4 tethered to the cell surface specifically and strongly bound thrombin via its catalytic site.

Hirudin-CD4 Expressed by IPECs Binds Thrombin and Inhibits Coagulation in Human Plasma

Figure 4A and 4B demonstrates that when expressed on IPECs, hirudin-CD4 bound thrombin similarly as when expressed on transfected DAP.3 cells. In a simple clotting assay, untransfected IPECs shortened the clotting time of citrated human plasma after recalcification to ~170 seconds, compared with a control clotting time of 370 seconds in the absence of cells (Figure 4C). After preincubation of untransfected IPECs with IL-1α, clotting times were further reduced to <100 seconds owing to the expression of tissue factor (as confirmed by flow cytometric analysis; data not shown). In contrast, the clotting times recorded with IPECs expressing hirudin-CD4 were consistently prolonged compared with those with untransfected control cells, even after preincubation of IL-1α and induction of tissue factor expression. This effect was due to the presence of cell surface–expressed hirudin on the transfected IPECs, as demonstrated by the attenuation of the prolongation of clotting by anti-hirudin mAb (Figure 4D).
Hirudin-CD4 With a Targeting Sequence From the COOH-Terminal Domain of P-Selectin Is Functionally Similar to Hirudin-CD4

Two constructs, with a targeting sequence derived from human P-selectin but with different glycine linkers, were synthesized (Figure 1B) and used to establish stable CHO-K1 transfectants. These cells were chosen because they lack secretory granules. In control experiments, cells transfected with wild-type P-selectin expressed protein at the cell surface (data not shown), which confirms what others have documented. Consistent with this, both hirudin-CD4–P-selectin constructs were expressed at the cell surface of the CHO-K1 transfectants (data not shown). Once again, the length of the glycine linker had no influence on surface expression.

Thrombin binding to these constructs was investigated in the same manner as for the hirudin-CD4–transfected DAP.3 clones and found to be identical in all respects (data not shown). We concluded from these results that inclusion of the P-selectin cytoplasmic tail had no adverse influence on the thrombin-binding activity of hirudin.

Hirudin-CD4–P-Selectin Is Stored in Secretory Granules in Pituitary Cells and Can Be Released on Activation

To examine whether hirudin-CD4–P-selectin accumulated in intracellular storage granules, we transiently transfected a secretory mouse pituitary cell line (D16/16) with either hirudin-CD4–P-selectin or hirudin-CD4. This particular cell...
Figure 3. Flow cytometric assessment of specific binding of thrombin to fibroblasts expressing hirudin-CD4. A and B, DAP.3 cells transfected with HLA-hirudin-G1-CD4 (▲), HLA-hirudin-G2-CD4 (●), HLA-hirudin-G3-CD4 (●), or the negative control HLA-DR (○) were incubated with thrombin for 1 hour at 37°C. Percentage positive cells (A) and mean fluorescence intensities (B) are shown. Cells were labeled with an anti-prothrombin immunoglobulin fraction. Representative of 2 analyses. C, DAP.3 cells transfected with HLA-hirudin-G3-CD4 were incubated with either 10 μg/mL anti-hirudin mAb (▲) or an irrelevant control antibody (anti-human TFPI) (○). After 30 minutes on ice, cells were incubated with thrombin for 1 hour before analysis with the anti-prothrombin immunoglobulin. Representative of 3 analyses. In D, HLA-hirudin-G3-CD4 expressing DAP.3 cells were incubated with thrombin and stained with either...
line was used for 2 reasons. First, the cells have been previously described to express ACTH in specific storage granules that are discharged to the cell surface on activation with phorbol esters. Second, pig aortic ECs do not contain Weibel-Palade (W-P) storage granules, and other vascular ECs rapidly lose their W-P bodies on culture in vitro.

In D16/16 cells transfected with hirudin-CD4–P-selectin, hirudin was detected in granules evenly distributed in the cytoplasm (Figure 5A; green fluorescence). The same pattern of granule distribution was detected when cells were examined for ACTH expression, which implies colocalization with hirudin (Figure 5B; red fluorescence). This finding was verified and was even more striking in specimens examined for both hirudin and ACTH (yellow granules, indicating colocalization, Figure 5C). Moreover, D16/16 cells that were transfected with hirudin-CD4 cDNA (lacking the P-selectin sequence) did not accumulate hirudin in intracellular granules but expressed high levels of hirudin at the cell surface (Figure 5D). When these transfected cells were examined for both ACTH and hirudin staining, no colocalization was observed (orange granules, Figure 5F), which contrasts markedly with the cells transfected with hirudin-CD4–P-selectin (Figure 5C).

In support of this interpretation, D16/16 cells stably transfected with hirudin-CD4–P-selectin did not express detectable hirudin at the surface after being stained with anti-hirudin mAb (Figure 6A). However, after stimulation with anti-prothrombin immunoglobulins (△) or a specific anti-hirudin-thrombin mAb (●). Representative of 3 analyses. E, Active-site blocking of thrombin abolishes thrombin binding to hirudin-CD4 at the cell surface. DAP.3 cells expressing hirudin-G2-CD4 were incubated with thrombin or thrombin pretreated with various inhibitors and subsequently stained for thrombin binding with anti-prothrombin immunoglobulins or anti-thrombin-hirudin mAb according to the standard procedure (see Methods and Results). Representative of 2 experiments.
phorbol myristate acetate (PMA) for 30 minutes, hirudin was relocated to the cell surface (Figure 6B). Furthermore, PMA-activated D16/16 cells specifically bound thrombin, as shown with the anti-hirudin-thrombin–specific mAb (Figure 6C), unlike the nonactivated cells (not shown). Thus, using the granule-containing pituitary cell line D16/16, we clearly demonstrated that hirudin-CD4 –P-selectin could be targeted to specific storage granules and that functional fusion proteins were released and exposed at the cell surface on cellular activation.

**Discussion**

In the present study, using novel fusion proteins, we have demonstrated both constitutive expression of cell surface–tethered hirudin and stimulation-dependent relocation of hirudin from secretory granules to the cell surface. Linked to an appropriate mammalian signal sequence and membrane anchor, hirudin was expressed at the cell surface, where it bound exogenous thrombin via its active site and inhibited the formation of fibrin in an in vitro clotting assay that used human plasma. When the C-terminal sequence of the hirudin-CD4 fusion protein was replaced by a targeting sequence derived from P-selectin, it accumulated in storage granules of secretory cells and could be relocated to the cell surface on activation. Furthermore, this relocated hirudin was active, because specific thrombin binding could be visualized by flow cytometry.

The targeting and regulated expression of the P-selectin–tagged fusion protein were predictable from recent data concerning the intracellular processing of P-selectin. Indeed, previous work has shown that fusion of the cytoplasmic sequence of P-selectin can redirect protein expression into storage granules. We have shown for the first time that expression of hirudin can also be manipulated by targeting to storage granules as part of a novel genetic strategy to tether functionally active anticoagulant molecules to EC surfaces.

The efficacy of recombinant hirudin in various preclinical and clinical models of thrombosis is well documented. At least 1 soluble fusion protein containing hirudin has been shown to retain thrombin binding activity in vitro. Hirudin


has also been successfully targeted to specific sites by use of covalently linked antibodies or Fab fragments to fibrin and E-selectin (CD62E), which is expressed on activated ECs.

More recently, adenovirus-mediated gene transfer leading to expression of recombinant soluble hirudin in smooth muscle cells of injured rat carotid arteries was shown to be effective in the inhibition of neointima formation, which suggests that local delivery of hirudin may constitute an attractive intervention strategy for arterial proliferative disease.

These approaches may, however, be problematic in that they rely on treating thrombosis that is already well established, whereas our genetic approach is designed to inhibit trace thrombin generation before clot formation. We envision that the hirudin-CD4 constructs could be used proactively in situations in which thrombotic complications are anticipated, including vascular surgery and transplantation. After therapy with the hirudin-CD4–P-selectin constructs, constitutive hirudin expression would be absent on normal, quiescent endothelium but would be rapidly expressed on type 1 EC activation induced by complement activation or small amounts of thrombin, owing to the discharge of W-P bodies. This strategy should have clear advantages over other targeted anticoagulant strategies that rely on constitutive expression.

In transplantation, EC activation and P-selectin expression occur in allografts subject to prolonged hypoxia and hypothermic preservation, and these grafts are susceptible to reperfusion injury. Similar changes occur during hyperacute rejection and delayed xenograft rejection. In these settings, expression of hirudin-CD4–P-selectin fusion is expected to have beneficial effects on graft survival by inhibiting intraluminal fibrin deposition. In combination with complement inhibitory proteins at the EC surface, the expression of hirudin activity in xenografts may be a potent strategy in prevention of the microvascular thrombosis associated with all types of vascular rejection.

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