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Gene Transfer and Functional Expression in Mammalian Cells of the Medicinal Leech Anti-coagulant Hirudin Using Adeno-Associated Virus Vectors

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Hirudin, a protein secreted by the medicinal leech, is a potent and specific inhibitor of thrombin. As an initial step towards producing a vector for vascular gene therapy we have constructed a recombinant adeno-associated virus (rAAV) vector containing two copies of hirudin variant-1 driven by a CMV promoter. rAAV was produced using a two-plasmid system and purified using iodixanol density centrifugation and heparin affinity chromatography. Anti-thrombin analysis of conditioned media from plasmid transfected and rAAV transduced 293 cells showed that both secreted biologically active hirudin.

Key words: Hirudin, anti-coagulant, thrombin, (AAV), vascular gene therapy

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Introduction

Hirudin is a potent thrombin inhibitor originally derived from the medicinal leech (Fink, 1989). Recombinant technology is now used to produce it in quantities sufficient for research and development (Johnson, ***et al.***, 1989; Bischoff, ***et al.***, 1989). Unlike heparin, hirudin acts directly on thrombin, rather than through other clotting factors. They have a high binding affinity and specificity for thrombin. The mechanism of hirudin-thrombin binding appears to be unique (Braun ***et al.***, 1988; Grutter ***et al.***, 1990). Findings from animal and human studies indicate that, when administered intravenously, hirudin follows an open two-compartment model with first-order phamacokinetics. Approximately 60% of a parenteral dose is eliminated unchanged via glomerular filtration (Richter ***et al.***, 1988).

The use of hirudin has been studied for maintaining adequate blood flow in the region of reimplanted digits and for the preservation of stored platelets. It may reduce metastasis in certain cancers and may play a role in managing complications of atherosclerosis. Bleeding does not appear to pose a problem. Hirudin offers great promise for research purposes and in treating clinical diseases that involve hemostatic disorders (Hand ***et al.***, 1999).

Hirudin's potential medical utility stems from the drug's interactions with the serine protease thrombin which catalyzes the conversion of fibrinogen to fibrin leading to blood clotting. Other serine protease inhibitors block or alter the actions of thrombin, but hirudin appears to have a unique mechanism of interaction related to the molecular structure of hirudine and thrombin. At the heart of the hirudin-thrombin interaction are three regions of the thrombin molecule: a catalytic site, an apolar site, and an anion-binding exosite. Other sites may be involved, but hirudin apparently does not require an interaction with the basic specificity pocket of thrombin (Agnelli ***et al.***, 1990). In the interaction of multiple sites on both molecules, no single interaction is dominant. Thus, hirudin appears to have considerable flexibility in its binding capacity, which contributes to the strong affinity for thrombin (Just ***et al.***, 1991).

One method of measuring the hirudin activity of a sample is to determine the capacity of the sample to neutralize a known quantity of thrombin. The activity of hirudin is commonly measured in antithrombin units (ATU). One ATU is defined as the quantity of hirudin needed to neutralize 1 unit NIH of thrombin at 37^{0}C with the fibrinogen used as a substrate (Johnson ***et al.***, 1989). Hirudin activity can also be measured by assessing changes in prothrombin time, activated partial thrombin time and/or other clotting tests, these tests can vary in their sensitivity. Hirudin activity can also be measured by competitive binding assays employing hirudin labeled with ^{125}I (Richter ***et al.***, 1988; Wagenvoord ***et al.***, 1993). An ELIZA method has been developed for measuring concentrations of hirudin in buffer, urine and plasma (Spinner ***et al.***, 1988). The assay is reported to be rapid, sensitive and reproducible, making it suitable for pharmacokinetic studies of hirudin in parenteral or topical administration to humans.

Because of this high affinity and specificity for thrombin, hirudin is uniquely suited to a variety of research and therapeutic purposes. Studies in animals show that hirudin can prevent thrombosis and neointimal proliferation after arterial injury (Just *et al.*, 1991; Sarembock *et al.*, 1991; Talbot *et al.*, 1991; Brill-Edwards *et al.*, 1992) and in humans has been used successfully as an adjunct to thrombolytic therapy and coronary angioplasty (Cannon and Braunwald, 1995). Importantly, no clinically significant side effects have been reported in these studies, suggesting that hirudin is both effective and safe in treating haemostatic disorders.

The aim of this study was to produce a vector for vascular gene therapy. Adeno-associated virus (AAV) was chosen as a delivery vehicle for several reasons, including its ability to persist in immunocompetent hosts, its lack of pathogenicity and its capacity to infect numerous human and animal cell types.

Materials and Methods

Vector construction

The plasmid pBSHV1.2, containing the cDNA encoding for hirudin variant 1 fused to the human growth hormone signal peptide (HV-1.2) (Rade *et al.*, 1996) was used to construct a double copy hirudin expression cassette, HIH, by cloning a polio internal ribosome entry site (IRES) element between two copies of the HV-1.2 sequence. The AAV shuttle plasmid pTR-UF5, containing the enhanced GFP gene under the expression of a CMV immediate-early promoter with a polyadenylation signal and flanked by two AAV serotype 2 inverted terminal repeats (Conway *et al.*, 1999) was subsequently used to produce plasmid pUF5HIH by replacing the eGFP gene with the HIH expression cassette (Fig. 1).

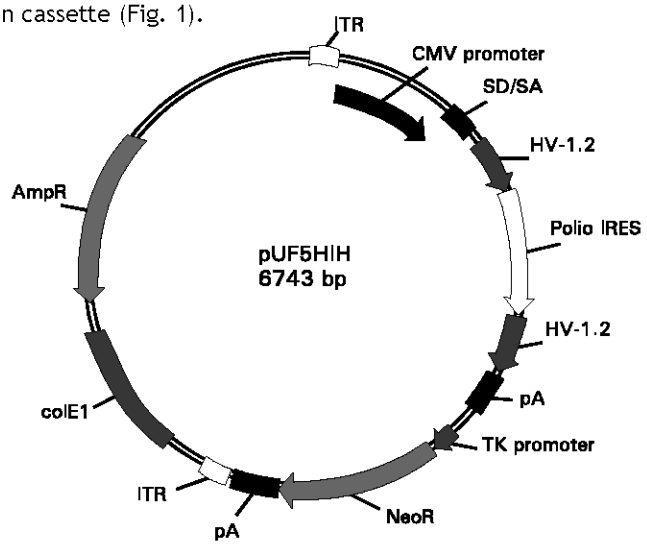

FIG. 1: Plasmid map of pUF5HIH. The vector was constructed by cloning a ~1.2 kb hirudin variant 1 (HV-1.2) cDNA cassette (two copies of HV-1.2 separated by a polio IRES) into a ***Not***I digested AAV serotype 2 vector under the control of a CMV promoter

Cells and production of rAAV

293 and 293T cells were propagated in DMEM, supplemented with 10% (v/v) FBS and incubated at 37°C, 8% CO_2. Recombinant AAV (rAAV) was produced in 293T cells using a two-plasmid system (Grimm ***et al.***, 1998) and virus purified using iodixanol density centrifugation followed by heparin affinity chromatography columns (Sigma) (Zolotukhin ***et al.***, 1999). Determination of rAAV titre was done by dot-blot hybridisation.

Cell transfection/transduction, SDS-PAGE and western blot analysis

293 cells were plated into a 6-well dish 24 hours prior to treatment at ~7.5×10^5 cells/well. Transfection with pUF5HIH was by $CaPO_4$ co-precipitation, and transduction of rAAVUF5HIH particles was at ~2000 particles/cell. G418 selection (400 μg/ml) was applied after 48 hours and continued until all untransfected/untransduced cells had died. Two colonies were selected from each experiment, expanded and plated into 1ml of media in a 24-well dish at ~1×10^5 cells/well. Media samples, collected at 24 hour intervals, were boiled for three min, electrophoresed through 4-12% polyacrylamide Bis-tris gels (NuPAGE®; Invitrogen) and proteins blotted onto Hybond™ ECL™ membranes using the NOVEX® Xcell II blotting apparatus (Invitrogen). Membranes were probed with a polyclonal rabbit anti-hirudin IgG antibody (1:1000; American Diagnostica) followed by an HRP-conjugated goat anti-rabbit IgG antibody (1:2500; Jackson Laboratories). Detection of hirudin was done by the ECL™ system (Amersham Pharmacia Biotech).

Hirudin activity

The activity of hirudin was determined using the Accuclot™ Thrombin Time Reagent and Accuclot™ Control I (human serum) (Sigma Diagnostics) in conjunction with an Amelung KC1 micro analyser. Culture media was taken from both treated and untreated cells at 24-hour intervals and three timed readings (50 μl/reading) recorded for each.

Statistical analysis

Comparison between means was performed by the Student's ***t*** test with each data point representing the mean±S.E. of three independent timed readings where $p<0.01$ was considered as level of significant.

Results and Discussion

Hirudins are small proteins derived from the salivary glands of the medicinal leech. The three principal variants (HV-1, HV-2 and HV-3) consist of 65 or 66 amino acid residues with a MW of ~7000 kDa and are identified as monomer, dimer and trimer based on their molecular mass (Johnson ***et al.***, 1989). Their potential medical utility stems from the molecules' strong interactions with the serine protease thrombin, which catalyses the conversion of fibrinogen to fibrin (Markwardt, 1970). By blocking both the catalytic site and the anion-binding exosite of thrombin, hirudin inhibits the interaction of thrombin both with fibrinogen and with thrombin receptors that are present on the surface of platelets and vascular cells.

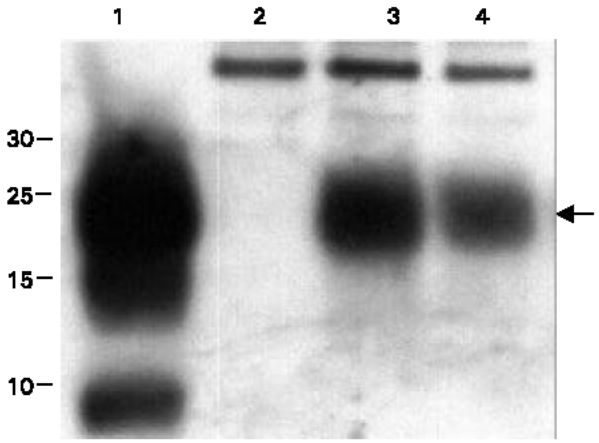

Fig. 2: Western blot of 10-day-old conditioned media from pUF5HIH transfected and rAAVUF5HIH transduced 293 cells probed with a polyclonal rabbit anti-hirudin IgG antibody (1) recombinant hirudin from yeast (2) conditioned media from 293 cells alone (3) conditioned media from 293 cells transduced with rAAVUF5HIH (4) conditioned media from 293 cells transfected with pUF5HIH. The trimeric form of the expressed hirudin is arrowed. Molecular weight markers are in kDa

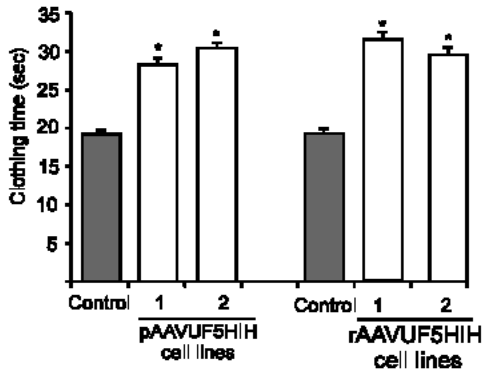

Fig. 3: Anti-thrombic activity of secreted hirudin from conditioned media. Eight-day-old conditioned media from untreated 293 cells plus two independent pUF5HIH transfected and two independent rAAVUF5HIH transduced 293 cell lines were mixed individually with human serum and Accuclot™ Thrombin Time Reagent and the time taken to form a clot recorded. Comparison between means was performed by the Student's *t* test with each data point representing the mean ± s.d. of three independent timed readings. *$p<0.01$ versus untreated 293 cell conditioned media

Direct inhibitors of thrombin have considerable promise as therapeutic agents in the treatment of cardiovascular disease. Retrovirus-mediated secretion ***in vitro*** of biologically active hirudin from the HV-1.2 cassette has been previously described (Rade ***et al.***, 1999). In the present experiments two copies of this cassette, separated by a polio IRES, was cloned into the AAV shuttle plasmid pTR-UF5 to produce plasmid pUF5HIH from which rAAV particles (rAAVUF5HIH) were subsequently generated. Western blots of conditioned media from pUF5HIH transfected and rAAVUF5HIH transduced 293 cells showed that both contained hirudin (Fig. 2), although very little was observed until after eight days incubation. However, unlike native hirudin or recombinant hirudin propagated in yeast, the species present was almost exclusively the trimeric form. In contrast to this, a human endothelial cell line (HUVEC) transfected with recombinant hirudin HV-1.1 resulted in secretion of primarily monomeric and dimeric forms (Rade ***et al.***, 1999). This anomaly is most likely due to the differential processing by the 293 cell line but may also be related to the different signal peptides used to direct hirudin secretion between HV-1.1 (tissue plasminogen activator) and HV-1.2 (human growth hormone).

To test the anti-thrombic activity of the secreted hirudin we added conditioned media from pUF5HIH transfected, rAAVUF5HIH transduced and untreated 293 cells to human serum followed by Accuclot™ Thrombin Time Reagent and measured the time taken to convert fibrinogen to fibrin. Media samples from the above cell lines were tested every 24 hours and the clotting times compared to a time matched negative control. A significant increase ($p<0.01$) in clotting time over the base level was observed after the pUF5HIH transfected and rAAVUF5HIH transduced cell lines had been incubated for eight days (Fig. 3). No significant differences were observed within the two plasmid or two rAAV derived cell lines, nor was there any significant difference between the cell lines. This suggests that the rAAVUF5HIH particles produced from the pUF5HIH vector contain a correctly packaged genome, which is fully functional. The efficacy of this vector can now be examined ***in vivo*** using animal models of vascular disease.

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