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MINI-REVIEW

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Current status of the anticoagulant hirudin: its biotechnological production and clinical practice

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Abstract Hirudin is a potent thrombin inhibitor originally derived from the medicinal leech, *Hirudo medicinalis*. Owing to its high affinity and specificity for thrombin, hirudin has been intensively investigated for research and therapeutic purposes. The investigation of hirudin has contributed greatly to the understanding of the mode of action of thrombin and the clotting system. Hirudin and several hirudin analogues have also been demonstrated to have several advantages as a highly specific anticoagulant over the most widely used drug, heparin. Due to the great demand for hirudin in physicochemical and clinical studies, various recombinant systems have been developed, using bacteria, yeasts, and higher eukaryotes, to obtain the biologically active hirudin in significant quantities. After 10 years of clinical applications, two recombinant hirudins and a hirudin analogue have gained marketing approval from the United States Food and Drug Administration, for several applications. Clinical trials are currently ongoing for other treatments for thrombotic disease. As a consequence, it is conceivable that hirudin may expand its therapeutic utility over heparin in the near future.

Introduction

Hirudin is a low molecular weight anticoagulant peptide (~7 kDa) excreted naturally from the salivary glands of the medicinal leech, *Hirudo medicinalis* (Markwardt 1955). It is specific for thrombin, the ultimate regulatory enzyme of the blood coagulation cascade and is extremely potent as an inhibitor: the inhibition constant (K_i) has been reported as approximately 10^{-11}–10^{-14} M (Dodt et al. 1984; Stone and Hofsteenge 1986). Hirudin not only prevents fibrinogen from clotting but also prevents other thrombin-catalyzed reactions, such as the activation of factors V, VIII, and XIII, and platelet activation (Hoffmann and Markwardt 1984). The direct inhibitory action of hirudin on thrombin has valuable therapeutic potential as an anticoagulant. The most important application of hirudin is in treating arterial thrombosis, which causes cardiovascular diseases, such as heart attack and stroke. Although heparin is widely used as an anticoagulant, it can not inactivate fibrin-bound thrombin, a major stimulus for thrombus growth. It also requires endogenous cofactors, such as antithrombin III, for mediating its anticoagulation effect. Furthermore, heparin induces serious bleeding and an adverse immune reaction, heparin-induced thrombocytopenia (HIT; Warkentin et al. 1995). In contrast, hirudin inhibits thrombin directly, both in the free and in the thrombus-bound state, and does not require any endogenous cofactors. Moreover, it does not induce antigenic reactions or a high degree of bleeding when administered to animals or humans (Markwardt 1994).

Owing to such advantages over heparin, hirudin has prompted tremendous efforts towards its development as a pharmaceutical (Walenga et al. 1989; Markwardt 1994). There is pharmaceutical interest in the direct use of hirudin itself as a drug and in the development of new, low molecular weight, synthetic hirudin analogues (Maraganore et al. 1990; Lombardi et al. 1999). For the development of a protein as a therapeutic reagent, a large quantity of the purified protein is usually necessary for extensive biochemical investigations and clinical studies. Natural hirudin, however, is produced only in trace amounts from leech extracts (~20 µg/leech head; Markwardt 1970). Exploiting recombinant DNA technology, several recombinant organisms, including *Escherichia coli*, yeasts, and transgenic plants, have been developed to provide hirudin in quantities sufficient for research and development.

During the past decades, clinical applications for recombinant hirudins and hirudin analogues have been intensively investigated for various symptoms caused by thrombosis. To date, two recombinant hirudins and a

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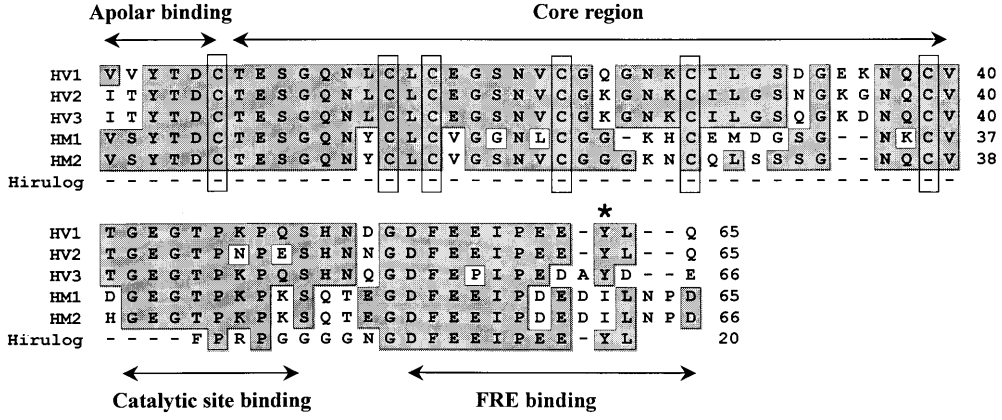

Fig. 1 Sequence alignment for hirudin isoforms (hirudin variants *HV-1*, *HV-2*, *HV-3* from *Hirudo medicinalis*; *HM1*, *HM2* from *Hirudinaria manillensis*) and a synthetic hirudin analogue, *Hirulog*. Six cysteine residues in all hirudin variants are *boxed*. The *asterisk* indicates the position of the sulfated tyrosine in hirudin. *FRE* Fibrinogen recognition exosite of thrombin

hirudin analogue have gained marketing approval from the United States Food and Drug Administration (FDA) as anticoagulants for patients with different thrombotic diseases. In addition to these approved indications, clinical trials for many other thrombotic diseases are also underway. In this paper, we review natural hirudins and synthetic hirudin analogues with respect to their structure, function, and methods for biotechnological production. We also summarize the results of the approved applications of hirudin in clinical practice and discuss the problems of hirudin for expanded therapeutic use instead of heparin.

Hirudin and its analogues

Hirudin was first isolated as an anti-thrombotic substance and biochemically characterized in the 1950s (Markwardt 1955). The amino acid sequence of hirudin variant-1 (HV-1) was later identified in 1976, with ValVal as the N-terminal sequence and a sulfated tyrosine in position 63 (Bagdy et al. 1976), but its complete primary structure was only determined in 1984 (Dodt et al. 1984). Several hirudin-like proteins have been also identified from various species of leeches, *H. medicinalis*, *H. nipponia*, and *Hirudinaria manillensis* (Scharf et al. 1989; Scacheri et al. 1993; Yang and Yin 1997). Native hirudin is not a single, homogeneous protein, but rather includes several isoforms. Three variants, designated HV-1, HV-2, and HV-3, have been isolated from *Hirudo medicinalis* (Dodt et al. 1984; Harvey et al. 1986; Tripier 1988). They show a high degree of similarity to each other and exhibit point differences (Fig.1). All three variants of the 65–66-amino acid proteins from *H. medicinalis* contain six cysteines (forming three disulfide bridges) and a sulfated tyrosine in position 63 or 64. The reduction of any one of the disulfide bridges or modification of the free carboxyl groups sharply decreases hirudin's affinity for thrombin (Bagdy et al. 1976). The amino acid composition of hirudin is characterized by a remarkably high content of acidic amino acids at the C-terminal and by the absence of arginine, methionine, and tryptophan. Two novel hirudin variants have also been isolated from *Hirudinaria manillensis*, named HM1 and HM2. They show sequence differences from other known hirudins, mainly in the central part and in the C-terminal region of the polypeptides (Fig. 1). A striking difference is the lack of a sulfated tyrosine residue in the C-terminal portion of the molecule, which is replaced by aspartic acid (Scacheri et al. 1993).

The crystal and solution structures of various hirudins have been studied by x-ray crystallography and nuclear magnetic resonance techniques (Haruyama and Wuthrich 1989; Grutter et al. 1990; Nicastro et al. 1997). The basic elements of the structure are a compact, hydrophobic core comprising the N-terminal half of the molecule and an extended, very hydrophilic C-terminal region. The core region has three disulfide bridges that restrict the configuration of the core. The x-ray crystal structure of the thrombin–hirudin complex revealed the enzyme–inhibitor interaction mode at molecular level (Rydel et al. 1990). Hirudin generates its action by binding directly to thrombin at multiple sites: the N-terminal globular domain binds near the active site of thrombin, while the extended C-terminal segment, which is abundant in acidic residues and includes a sulfated tyrosine, makes both ionic and hydrophobic interactions with the fibrinogen recognition exosite (FRE) of thrombin (Rydel et al. 1990). Unlike other serine proteinase inhibitors, which interact with their target enzyme predominantly in the active site region, binding of hirudin apparently does not require an interaction with the basic specificity pocket of thrombin (Grutter et al. 1990). No single interaction is dominant in the interaction of multiple sites on both molecules. Thus, hirudin appears to have a considerable flexibility in its binding capacity, which contributes to its strong affinity for thrombin.

The function of the sulfated tyrosine residue is still unclear, but it appears to enhance the thrombin exosite-binding of hirudin. Unsulfated hirudin shows around tenfold reduced affinity for thrombin (Hofsteenge et al. 1990). Recombinant hirudins expressed in several microorganisms differ from native forms in lacking the sulfate group on the tyrosine; and thus they are described as desulfatohirudins. In vitro tyrosine sulfation of desulfatohirudin by tyrosylprotein sulfotransferase from leech

Table 1 Expression of recombinant hirudin in several heterologous hosts. *I* Intracellular, *S* secreted, *S** secreted into periplasmic space

Hosts	Yields	Comments	References
Bacteria			
Escherichia coli	~1 mg/l (I); 200~300 mg/l (S*)	Reduced activity of the N-terminal methionine modified hirudin; expression of authentic N-terminal hirudin into periplasmic space; no sulfation in Tyr	Bergmann et al. (1986); Dodt et al. (1986); Bender et al. (1990); De Taxis du Poet et al. (1991)
Streptomyces lividans	0.25–0.5 mg/l (S)		
Yeasts			
Saccharomyces cerevisiae	40–500 mg/l (S)	Efficient secretion into culture supernatant; no sulfation at Tyr; *S. cerevisiae*-derived hirudin gained market approval from FDA; *H. polymorpha*-derived hirudin is being evaluated in phase III clinical trials	Mendoza-Vega et al. (1994); Sohn et al. (1995); Weydemann et al. (1995); Rosenfeld et al. (1996)
Hansenula polymorpha	1,500 mg/l (S)		
Pichia pastoris	1,500 mg/l (S)		
Fungi			
Acremonium chrysogenum	7 mg/l (S)	No detectable hirudin under intracellular expression	Radzio and Kück (1997)
Insect cells	0.4 mg/l (S)	HV-1 showed higher expression than HV-2	Benatti et al. (1991)
Mammals			
BHK cells	0.05 mg/l (S)	Sulfation at Tyr	Skern et al. (1990)
Plants			
Oilseed rape, tobacco, Ethiopian mustard	0.55 anti-thrombin units/mg oil body protein	Oleosin–hirudin fusion expression; endopeptidase cleavage recovers active hirudin	Parmenter et al. (1995); Chaudhary et al. (1998); Giddings et al. (2000)

and cattle rendered the protein indistinguishable biochemically and biologically from natural hirudin (Niehrs et al. 1990). The positive effect of tyrosine sulfation on the binding activity to thrombin has also been investigated in several peptides mimicking the FRE-binding element (C-terminal) of hirudin (Payne et al. 1991; Muramatsu et al. 1994).

Analysis of various fragments and mutations of the hirudin molecule has shown that only the last ten amino acid residues of the C-terminal tail are necessary for specific binding and inactivation of thrombin (Krstenansky et al. 1987; Mao et al. 1988; Chang 1990). Within this sequence, the alteration of amino acids at positions 56, 57, 59, 60, or 64 can alter the binding efficiency of the molecule (Krstenansky et al. 1987); and position 56 (Phe) is critical for anti-thrombotic activity (Mao et al. 1988). Twelve of the 17 C-terminal residues participate in electrostatic or hydrophobic interactions with the FRE of thrombin. About half of C-terminal hirudin residues at 55–65 are hydrophobic and all participate in non-polar thrombin–hirudin interactions (Haruyama and Wuthrich 1989). The interactions between thrombin and hirudin have served as a model for the design of numerous peptide-competitive inhibitors for thrombin (Lombardi et al. 1999). The synthetic inhibitors essentially consist of a N-terminal catalytic site-directed fragment, a spacer of variable length and composition, and the C-terminal hirudin tail as FRE-binding element. Hirulog (Angiomax or bivallirudin), which has been approved for marketing by the FDA, is a synthetic peptide (~20-mer) designed to interact both with the catalytic site and the FRE of thrombin by joining two functional parts of hirudin with a four-glycine spacer (Fig. 1; Maraganore 1993). Similar to hirudin, it interacts with thrombin at multiple loci, forming a high-affinity complex (Maraganore 1993). Sulfation of tyrosine at position 19, which corresponds to Tyr-63 in hirudin, showed three to four times greater inhibition of thrombin amidolytic activity than the non-sulfated form (Bourdon et al. 1991). Although the K_i of Hirulog to thrombin (about 2 nM) was considerably higher than that of natural hirudin, it appeared to have several advantages over native hirudin, such as a short half-life, a narrow therapeutic window and a reverse dose effect (Billett 2001).

Biotechnological production of hirudin

During the recent past, the intensive biochemical investigation and clinical application of hirudin as an anticoagulant have shown it to be a very important biomedical application of a great interest. After several laboratories cloned the hirudin gene, using a cDNA library or synthetic gene (Dodt et al. 1986; Harvey et al. 1986), considerable effort has been made to obtain recombinant hirudin in significant quantities, using several heterologous gene expression systems (Table 1). Expression of recombinant hirudin was first reported in *E. coli*, the most widely used host for producing foreign proteins (Bergmann et al. 1986; Dodt et al. 1986; Harvey et al. 1986). Recombinant hirudin produced from *E. coli* showed a high specific activity, approximately 13,000 anti-thrombin units (ATU)/mg, which is comparable to the specific activity of native hirudin. This indicated that the intracellular environment of *E. coli* permitted identical folding to that of native hirudin. However, the major drawback

in producing recombinant hirudin intracellularly in *E. coli* was that a mixture of two hirudin forms was produced, with an additional N-terminal methionine residue (Met^+) and without (Met^-), corresponding to the translation initiation codon. The Met^- form is of high specific activity, whereas the Met^+ hirudin contains extra methionine and displays low anti-thrombin activity (about >5,000 ATU/mg; Courtney et al. 1989). In addition, only low expression levels (~l mg/l) were obtained, due to proteolysis of the recombinant hirudin within the cell. Periplasmic expression of hirudin was attempted in *E. coli*, using an alkaline phosphatase signal sequence (Dodt et al. 1986) and an outer membrane protein OmpA signal sequence (De Taxis du Poet et al. 1991), which were fused with the hirudin gene. Both systems were able to secrete hirudin into the periplasmic space with a high efficiency, up to a concentration of 200–300 mg/l. The purified hirudin was correctly processed: the leader peptide was completely removed and the amino acid sequence corresponded to natural hirudin, but the tyrosine residue at position 63 was not sulfated (De Taxis du Poet et al. 1991). Secretory production of hirudin was also described in other prokaryotic systems, such as *Bacillus subtilis* (Marki and Wallis 1990) and *Streptomyces lividans* (Bender et al. 1990). In the case of *S. lividans*, a synthetic hirudin gene was fused to the DNA encoding the signal peptide of the alpha-amylase inhibitor tendamistat, derived from *S. tendae*. Though immunoblots revealed an efficient translocation of hirudin through the plasma membrane, the yield of hirudin was quite low (around 0.25–0.5 mg/l). The recombinant hirudin produced from *S. lividans* also showed a low specific activity (about 20 times less than the reference hirudin), which might be due to a different N-terminal sequence or to incorrect processing of the precursor (Bender et al. 1990).

To circumvent the problems of using bacteria as host, recombinant hirudin has been expressed and produced predominantly as a secreted protein using yeast systems, including *Hansenula polymorpha*, *Pichia pastoris*, and *Saccharomyces cerevisiae*. The first efficient secretion of biologically active recombinant hirudin (HV-2) from *S. cerevisiae* was described by Loison et al. (1988). The mature hirudin-coding sequence was placed in frame with the prepro leader sequence of the *S. cerevisiae* mating factor α1 gene as a secretion signal. Biologically active hirudin was identified in the culture supernatant with the authentic N-terminal of native hirudin. Subsequently, yeast secretory expression of HV-1 in *S. cerevisiae* was also reported using the *GAP* promoter and the *PHO5* signal sequence (Janes et al. 1990). The recombinant hirudin secreted in *S. cerevisiae* has been reported with a specific activity of 13,000 ATU/mg protein (Loison et al. 1988). As found in the recombinant hirudin from *E. coli*, it was also found to lack the sulfate residue at position 63 (Riehl-Bellon et al. 1989). HPLC and chemical analysis of yeast recombinant hirudin revealed the presence of two derivatives lacking the last Gln (position 64) or the penultimate Leu (position 63) besides the major full-length protein of 65 amino acids. When expressing recombinant hirudin in mutant strains defective in all but one of the three major known carboxypeptidases, it turned out that both the vacuolar carboxypeptidase (yscY) and the alpha-factor precursor-processing carboxypeptidase (yscα), participated in the C-terminal degradation of recombinant hirudin. Another vacuolar arboxypeptidase (yscS) had no effect on hirudin truncation. Thus, using mutant strains disrupted in two protease genes, *PRC1* and *KEX1*, coding for yscY and yscα, respectively, could greatly improve the expression level of intact recombinant hirudin in *S. cerevisiae* (Hinnen et al. 1994).

Several efforts have been made to improve the production process of recombinant hirudin in *S. cerevisiae*. Modification of expression elements in vectors, such as signal sequence (Achstetter et al. 1992), selection marker (Hottiger et al. 1995), and promoters (Choi et al. 1994), and optimization of fermentation (Mendoza-Vega et al. 1994; Sohn et al. 1995) have improved the hirudin yield up to 500 mg/l. The stable maintenance of plasmids carrying a hirudin-expression cassette greatly influenced the expression yield of recombinant hirudin (Janes et al. 1990; Hottiger et al. 1995). Recently, a multiple chromosome integration system to circumvent segregational instability of the expression vector has been developed using the δ-sequence of the yeast retrotransposon, Ty1, for the stable expression of hirudin in *S. cerevisiae* (Kim et al. 2001). Production of hirudin in the integration system increased over two-fold, compared with the 2μ-based episomal system. As an alternative way to improve the production yield in the yeast system, two promising expression systems using non-conventional methylotrophic yeasts, such as *H. polymorpha* and *P. pastoris*, have been investigated for the expression of hirudin (Weydemann et al. 1995; Rosenfeld et al. 1996). The yields of secreted recombinant hirudin in these methylotrophic yeasts were in the range of grams per liter and were at least threefold higher than the production level of recombinant hirudin achieved in *S. cerevisiae*. In addition, the C-terminal proteolysis was much less pronounced in *H. polymorpha* and *P. pastoris*, compared to the situation in *S. cerevisiae* (Weydemann et al. 1995; Rosenfeld et al. 1996). Thus, non-*Saccharomyces* yeasts have proven to be more efficient systems, compared with *S. cerevisiae*, for the production of multigram quantities of biologically active recombinant hirudin on an industrial scale.

Industrial scale fed-batch cultivation and purification of recombinant hirudin has also been investigated using recombinant yeast (Lehman et al. 1993; Mendoza-Vega et al. 1994; Chung et al. 1999). Because yeast-derived recombinant hirudin can be recovered from the culture supernatant, purification steps are generally simple. Recombinant hirudin with purity over 97% can be obtained, using a couple of column chromatography techniques, including ion-exchange chromatography, affinity chromatography, and reverse phase HPLC (Bischoff et al. 1993; Rosenfeld et al. 1996). A specially designed purification scheme has been also tried, to facilitate the purification of recombinant hirudin using

immobilized metal-affinity chromatography (IMAC; Chung et al. 1994). Based on the affinity of hirudin to IMAC via a single surface-accessible histidine at position 51, hirudin variants were constructed to exhibit an increased metal-binding affinity in an attempt to apply a metal-affinity partitioning process as a primary separation step for the purification of hirudin. The hirudin variants were genetically engineered to contain additional surface-accessible histidines and were produced by recombinant *S. cerevisiae*. Upon the addition of a small amount of Cu(II)-iminodiacetic acid-polyethylene glycol (PEG) ligand to a PEG/dextran two-phase system, the hirudin variants with two or three surface-accessible histidines were more selectively partitioned into the PEG-rich phase than the wild type. Although the scheme could greatly facilitate the purification of recombinant hirudin, the specific activities of the engineered hirudins were reduced (Chung et al. 1994). To obtain recombinant hirudin of therapeutic purity in large quantities, the fed-batch fermentation of recombinant *S. cerevisiae* expressing hirudin under the control of the *GAL10* promoter was carried out and a subsequent downstream processing with the preparative-scale column chromatography systems was developed. Using a two-step chromatographic process (anion-exchange chromatography followed by reverse phase HPLC), the recombinant hirudin can be purified to >98% with an overall recovery yield of 84% (Chung et al. 1999). Reducing the ionic strength of the initial fermentation broth from a conductivity of 19.9 mS/min to 4.3 mS/min by desalting with Ion Clear Bigbead (Sterogene Bioseparations, USA) and diluting the broth with distilled water greatly shortened the overall processing time, which resulted in a remarkable decrease in hirudin degradation during the purification process and thus improved the yield of recombinant hirudin. This process shows the highest recovery yield ever reported for the purification of recombinant hirudin by yeast (Lehman et al. 1993; Sohn et al. 1995; Rosenfeld et al. 1996). A process flow diagram for the purification of recombinant hirudin from *S. cerevisiae*, currently carried out in the authors' laboratory on a pilot scale, is shown in Fig. 2.

Other eukaryotic expression systems besides yeasts have been tried for the production of recombinant hirudin. The secretion of biologically active leech hirudin has been reported in baculovirus-infected insect cells (Benatti et al. 1991), the filamentous fungus *Acremonium chrysogenum* (Radzio and Kück 1997), and BHK cells (Skern et al. 1990). In particular, the recombinant hirudin expressed in BHK cells was shown to be sulfated, by labeling experiments using [^{35}S] sulfate. Recently, transgenic expression in plants has emerged as another powerful system for high-level production of recombinant hirudin. So far, oilseed rape, tobacco, and Ethiopian mustard have been engineered to produce hirudin (Parmenter et al. 1995; Chaudhary et al. 1998; Giddings et al. 2000). Targeting recombinant hirudin to an oil body, using a plant oleosin as carrier protein, simplified the process of purifying hirudin from other proteins. Since the oleosin–hirudin fusion protein was targeted to the oil body membrane in seeds, it could be separated from the majority of other seed proteins by flotation centrifugation. Recombinant hirudin was released from the surface of the oil bodies by endoprotease treatment and was then partially purified through anion exchange chromatography and reverse-phase chromatography. The oleosin fusion protein system provides both a unique route for the large-scale production of recombinant proteins in plants and an efficient process for the purification of the desired polypeptides. Oilseed rape transgenic for hirudin is now grown commercially in Canada by SemBioSys (Boothe et al. 1997).

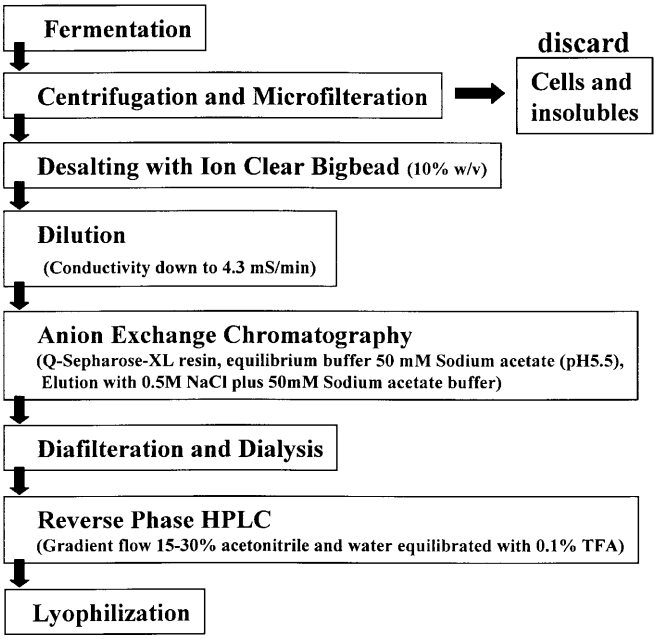

Fig. 2 Process flow diagram for the purification of recombinant hirudin from the culture broth of *Saccharomyces cerevisiae*

Hirudin and its analogues in clinical practice

Since hirudin is the most potent and specific thrombin inhibitor currently known, clinical applications for recombinant hirudins and hirudin analogues have been intensively investigated. As direct thrombin inhibitors, hirudin and its analogues have offered advantages over indirect thrombin inhibitors in the management of patients (Anard 1999). To date, two recombinant hirudins and an analogue have gained FDA approval for marketing. One of them is lepirudin (Refludan), which is a biotechnologically manufactured desulfatohirudin from *S. cerevisiae* and has a N-terminal of Leu-Thr. The direct inhibition of thrombin with lepirudin has been evaluated as a treatment for HIT, the most frequent and dangerous side effect of heparin therapy. HIT is an adverse immune reaction, which occurs in up to 3% of patients receiving unfractionated heparin (UFH; Warkentin et al. 1995) and is caused by IgG antibodies that recognize complexes of heparin and platelet factor 4. Activation of platelet and

endothelial cell by HIT antibody generates thrombin in vivo. Thrombin generation increases the risk of thromboembolic complications, which frequently result in limb amputation or death (Warkentin 1999). Lepirudin has been proven as a safe and effective thrombin inhibitor in patients with HIT and is approved in both Europe and the United States for the treatment of HIT-associated thrombosis (Manfredi et al. 2001). In addition to the first approved indication of lepirudin in HIT, the recently completed trials [Organization to assess strategies for ischemic syndromes (OASIS) I, II, Global use of strategies to open occluded coronary arteries (GUSTO) IIb] for use of lepirudin in unstable angina indicate a potentially new indication (GUSTO 1996; OASIS 1997; Greinacher and Lubenow 2001).

Another recombinant hirudin, desirudin, which differs from lepirudin only in the first two N-terminal amino acids (Val-Val), has a licensed indication for the prevention of deep vein thrombosis (DVT). DVT is a significant and frequent complication of major orthopedic surgery of the pelvis, hip, or lower limb. Low molecular weight heparin (LMWH) or low-dose UFH was mainly used for prophylactic regimens for DVT after surgery. Recently, desirudin was found to be superior to either UFH or LMWH in the prevention of DVT after total hip alloplasty (Eriksson 2000; Matheson and Goa 2000). Desirudin is currently licensed only for the prevention of DVT in patients undergoing elective hip and knee replacement surgery. It has also been studied in acute myocardial infarction (GUSTO 1996), unstable angina (Rao et al. 1996), and hemodialysis (Van Wyk et al. 1995).

In December 2000, Angiomax (Hirulog or bivalirudin), which is a short synthetic peptide of 20 amino acids designed on the basis of the structure of hirudin, was approved by the FDA for marketing. Angiomax is indicated for use as an anticoagulant in patients with unstable angina undergoing percutaneous transluminal coronary angioplasty (PTCA). Coronary thrombosis (a clot in the coronary artery) is a major risk during and after PTCA, leading to death, myocardial infarction, or the need for a revascularization procedure. Clinical data showed a 22% reduction in the risk of death, myocardial infarction, or revascularization for Angiomax-treated patients, compared with heparin-treated patients. Furthermore, Angiomax also demonstrated a 62% reduction in the incidence of major hemorrhage for Angiomax-treated patients, compared with heparin-treated patients (White et al. 1997). Beyond the first approval indication for PTCA, further clinical studies are ongoing or in preparation worldwide to determine the clinical effectiveness and safety of Angiomax in patients with acute myocardial infarction or unstable angina and in patients undergoing percutaneous coronary intervention with HIT, or suffering HIT with a thrombosis syndrome.

Conclusions

Hirudin is a powerful, direct, and specific anti-thrombotic agent that can be used in many thrombotic diseases in which heparin is routinely used. For the gradual substitution of heparin with hirudin, it should be comparable to LMWHs with respect to effectiveness and cost. Although recent clinical trials indicate that LMWHs can reduce the side effects of UFH, washed platelet assays with high sensitivity showed that LMWH also showed considerable cross-reaction with HIT antibodies (Newman et al. 1998). Thus, there are still great demands for a new anticoagulant showing no or little side-effect. At present, the cost of recombinant hirudin is about five times higher than that of LMWH. One reason for the higher cost of therapy with hirudin appears to be a relatively short half-life (around 2–3 h after intravenous injection; Zoldhelyi et al. 1993). Several approaches to increase the half-life of hirudin have been developed, using the covalent binding of hirudin to dextran (Markwardt et al. 1990), or to PEG (Zawilska et al. 1993). PEG–hirudin proved to be a safe, direct thrombin inhibitor in patients and showed a five-fold reduction in total plasma clearance, compared with non-conjugated hirudin (Poschel et al. 2000). Because a large proportion of the production cost of recombinant protein is spent on protein purification, it is important to select an expression system which can provide a simple purification process. Currently, the yeast expression system appears to be the system of choice, mainly due to the high level expression and the efficient protein secretion that can greatly facilitate the purification process. Two commercial recombinant hirudins (lepirudin, desirudin), which gained FDA approval for human use, are being produced from recombinant yeast, *S. cerevisiae*. In addition, BASF/Knoll is evaluating *H. polymorpha*-derived hirudin in phase III clinical trials. A simple purification of hirudin using the oleocin fusion technique developed in transgenic plants can also provide a cost-effective way to produce hirudin in quantity. As mentioned earlier, several applications of hirudin or hirudin-like substances have already been approved for human therapeutic uses. Furthermore, clinical trials for many other thrombotic diseases are intensively ongoing. Thus, improvement of hirudin half-life, enhancement of recombinant hirudin activity by sulfation, and development of a cost-effective production system can gradually expand the therapeutic utility of hirudin over heparin.

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