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Heparin-
Present and Future

Heparin – Present and Future

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Preface

Heparin was discovered in 1916, the first clinical trial was published in 1939, but only in the 1970s the mechanism of its anticoagulant activity was understood. During this last decade, a number of conceptual and practical advances had led to a reassessment of many of the basic and clinical properties of heparin. The use of heparin at low doses to prevent arterial thrombosis, preparation and availability of low molecular weight heparins to predictably prevent venous thrombosis after surgery, and the knowledge that antithrombotic, anticoagulant, and hemorrhagic properties of heparin are not necessarily correlated are only a few examples of the progress of the knowledge of heparin.

The aim of this meeting was to transfer these advances to clinicians and nonspecialists, because their improved knowledge of the properties of heparin should allow better results from its clinical use to be obtained. Important biological activities of heparin, other than the anticoagulant and antithrombotic, were addressed. In particular, the effects of heparin on angiogenesis and smooth muscle cell proliferation were discussed in detail. These seem to be the new address of study and, likely, of clinical application.

The organizers of the meeting were pleased by the overwhelming support received from the experts in this field who accepted to participate.

We would like to thank Italfarmaco who generously sponsored this Symposium, and we hope that this volume will be useful both for clinical and new researchers in the heparin field.

G.G. Neri Serneri

Special Lecture

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Heparin and Thrombosis: A Seventy Year Long Story

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Take from the altar of the past,
the fire – not the ashes.
Jean Jaurès

Key Words. Heparin · Low-molecular-weight heparin · Deep vein thrombosis · Anticoagulation

Abstract. Heparin was a chance discovery made by a medical student who was searching for a clot-promoting activity in various tissue extracts and found an inhibitor of coagulation. It has taken 20 years from the discovery of heparin in 1916 to its therapeutic use (1937). This long delay was mainly due to problems with the purification and extraction on large scale of the active material. Standard unfractionated heparin is a mixture of mucopolysaccharide chains of various length that may range from 5,000 to 30,000 daltons. Heparin is only effective as an anticoagulant in the presence of a plasma protein, termed antithrombin III, with which it forms a complex. High- and low-affinity heparin are two types that readily bind or do not bind to antithrombin III. The pharmacokinetics of unfractionated heparin are compatible with a model based on the combination of a saturable and a linear mechanism. Low-molecular-weight heparins have been produced by a variety of techniques, and their molecular weights range from 3,000 to 9,000 daltons. These preparations have a ratio of anti-Xa activity to anti-II activity of approximately 4, while it is 1 for unfractionated heparin. After intravenous administration of low-molecular-weight heparins, the half-life of the antifactor Xa activity is considerably longer than for unfractionated heparin, while the antifactor II half-lives are similar. In contrast to unfractionated heparin, low-molecular-weight heparin is virtually completely absorbed after subcutaneous administration, and its biological half-life is almost twice as long. In spite of certain differences with regard to the ratio between factor Xa and IIa inhibition, the various low-molecular-weight preparations show a rather similar absorption pattern. Low-molecular-weight heparins interact less with platelets than unfractionated heparin; nevertheless, a lower bleeding incidence with low-molecular-weight heparin for equivalent antithrombotic efficacy has yet to be established in man.

Heparin, like so many other biological substances, was discovered incidentally. William H. Howell, Professor of Physiology at Johns Hopkins University, was in 1916 trying to isolate tissue thromboplastin from organ extracts, a well-known accelerator of coagulation. His coworker, Jay McLean, a young medical student, was instructed to make extracts from brain, heart, and liver. He noticed that the clot-promoting thromboplastic property of the liver extracts deteriorated upon storage. In fact, the oldest batches even prolonged the clotting time of test plasma.

McLean [1] has written the history of the discovery shortly before his fatal illness in 1957, and this account was published after his death:

'Howell gave me the problem of determining the value of the thromboplastic substance of the body. He thought this to be cephalin, obtained from brain, but, of course, knew the thromboplastic material from brain to be a mixture – a crude extract, though a powerful thromboplastic agent. He made this by macerating brain tissue, spreading it on glass panes, drying it over a gas flame in an oven, extracting it in ether, decanting, concentrating the ether extract, and finally by precipitation by alcohol. This precipitate was his thromboplastic substance. He used it in blood clotting experiments. It was kept in a glass vessel with ground glass cover (vaselined), as it was observed that access to air decreased its ability to accelerate clotting. In three months it was decayed. My problem was to determine what portion of this crude extract was the active accelerator of the clotting process and to that end, to prepare cephalin as pure as possible and determine if it had thromboplastic action. I was also to test the other components of the crude ether-alcohol extract. In my reading of the German chemical literature on phosphatides, I found articles describing extracts of heart and liver secured by a process similar to that for obtaining cephalin from brain. Therefore, the products might be brain and liver cephalin, but were named cuorin (from the heart) and heparphosphatides (from the liver). I suggested this research programme as a logical supplement to the problem Dr. Howell had assigned to me. He had not known about cuorin or heparphosphatides. I prepared cuorin and heparphosphatides and both were brown, not yellow like cephalin and lacked its fishy like smell. Both had a much less accelerating effect on blood coagulation than cephalin. The more the material was "purified" (ether extract into hot alcohol), the weaker the thromboplastic activity became. The same process of extraction was used for brain, heart and liver. Yet in the brain the end product was almost all cephalin, but in the heart and especially in the liver it was something else which was mixed with cephalin. Many batches were made of both cuorin and heparphosphatide which were tested from time to time to determine whether or not the extract lost its thromboplastic activity as did that of the brain. If I had not saved them, I would probably not have found heparin. This was a fortuitous decision. The various batches were tested down to the point of no thromboplastic activity, but two of those first prepared appeared not only to have lost their thromboplastic activity but actually to retard slightly the coagulation. I had in mind, of course, no thought of an anticoagulant, but the experimental fact was before me; and I retested again and again until I was satisfied that an extract of liver (more than heart) possessed a strong anticoagulant action after its contained cephalin had lost its thromboplastic action.'

McLean [1] described his discovery in 1916 and referred to the compounds carrying the anticoagulant activity as 'the phosphatids from heart and liver' [2]. Howell and Holt [3] proceeded with the extension of McLean's work and introduced for the first time the term heparin, purified to some degree the material [4], and published a detailed report on its chemical and physiologic reactions [5]. McLean attempted several times to return to experimental work in the heparin field, but was engaged in clinical practice and was only honoured after his death as the discoverer of heparin.

At that time Charles H. Best, assistant director at the Connaught Laboratories in Toronto, had been intimately involved with the preparation of insulin and of beef liver extracts for administration to patients.

Based on this experience and with the help of a young organic chemist, Arthur Charles, he conducted chemical work on heparin [6–9]. This group showed that heparin could be found in many organs throughout the body, and they isolated heparin in 1933 in a highly purified state. It then appeared that heparin contains large quantities of hexosamine, which was later shown to be glucosamine, amounting to one mole of hexosamine per mole of uronic acid.

In the meantime Eric Jorpes and his coworkers Hjalmar Holmgren and O. Wilander at the Chemistry Department of the Karolinska Institutet in Stockholm found by metachromatic staining that the site of storage of heparin was the granules of the so-called mast cells discovered by Paul Ehrlich in 1877. This group, using the Charles and Scott procedure of extraction, also found that the liver capsule called after Glisson is extremely rich in mast cells and contains ten times more heparin than the liver parenchyma itself [10]. Since then, possibly too much functions have been assigned to these mast cells than the order of nature reasonably can have bestowed upon them.

The Canadian heparins studied in Toronto in thrombotic models in dogs after mechanical or chemical injuries of veins were rather crude preparations [11]. These materials were even used as an anticoagulant in transfusing human patients but produced severe headache, chills, and nausea [12]. Using a more purified Swedish heparin preparation, Hedenius and Wilander [13] have performed the first intravenous heparinization on themselves, outside the hospital. Their finding that 100 mg or more of heparin is needed for anticoagulating a human being for a few hours caused at first an almost desperate feeling [14]. Indeed, all the chemistry work had been performed with a supply of 6 g, which had only been obtained with great labour. Fortunately, a pharmaceutical company (Vitrum) got interested in the project and produced in a few years relatively purified heparin on a larger scale.

Twenty years elapsed between the discovery of heparin (1916) and its therapeutic use (1937). The first clinician to use heparin in patients was the Swedish surgeon Clarence Crafoord [15] who had studied pulmonary embolism in postoperative patients and treated some of them by embolectomy. He was criticized because he 'made his patients haemophiliac' for a time. He treated 325 postsurgery patients in his department, and his colleague Per Wetterdal did similarly in 309 patients during the postpartum period at the Department of Obstetrics of the same University of Lund. A frequency of 3–4% of serious or fatal pulmonary embolism was expected in these patients based on previous clinical experience, but practically no incident of that kind occurred in the patients receiving heparin prophylaxis; moreover, bleeding and other complications were considered to be acceptable. Soon hereafter, clinical trials with heparin were also started in Toronto by Murray et al. [16] at the Toronto General Hospital. These authors treated 260 patients and reported results equally as good as the Swedish team. Once the prophylactic value of heparin was established, its effectiveness in the treatment of patients with established venous or arterial thrombosis was soon demonstrated in small series of patients as well in Sweden as in Canada. In the early 40s large-scale clinical studies with heparin were instituted in America and in Switzerland.

The clinical use of heparin had already started when it was discovered that heparin

was effective as an anticoagulant only in the presence of a plasma component, which at that time was termed heparin cofactor [17–19], but has since been isolated [20, 21] and renamed antithrombin III. A second heparin-dependent inhibitor of thrombin, the heparin cofactor II, has more recently been identified and purified from human plasma [22].

From 1940 to 1980 heparin has been administered intravenously, either in repeated bolus injections or as a continuous infusion. The finding by Kakkar et al. [23] that a prophylactic dose of heparin given *subcutaneously* did not lead to antithrombin III depletion and was effective and safe in postoperative patients resulted in a more practical, highly cost-effective, and attractive approach to the prevention of deep venous thrombosis [reviewed by Lindblad, 24].

In the meantime, considerable progress had been made in purification, chemistry and mode of action of heparin. The anticoagulant activity of heparin is primarily related to its ability to accelerate the formation of a molecular complex between antithrombin III and the serine proteases of the coagulation system, thereby blocking their enzymatic activity. The term antithrombin III is a misnomer for several reasons, as this protein inhibits not only thrombin, but also the activated forms of numerous coagulation factors (XII, XI, IX, and X) as well as of plasmin and kallikrein. However, the inhibition of thrombin and factor Xa is particularly important and clinically relevant.

In pharmaceutical-grade heparin (average molecular weight 12,000–15,000 daltons), most anticoagulant activity is accounted for by a small functional fraction of the molecules, those with high affinity to antithrombin III. The remaining molecules have only a very limited anticoagulant effect, but may still increase bleeding in experimental animals [25], inhibit the activation of prothrombin by factor Xa [26, 27], or potentiate the action of high-affinity, low-molecular-weight fractions [28]. Furthermore, heparin molecules with low affinity for antithrombin III appear to inhibit hyperplasia of vascular smooth muscle [29], can activate lipoprotein lipase [30], suppress aldosterone secretion, and can induce platelet aggregation [31].

At higher than therapeutic concentrations, heparin and heparin-like mucopolysaccharides have an additional effect by catalyzing the inhibition of thrombin by another plasma protein, heparin cofactor II [22].

Fragments or fractions can be obtained by hydrolytic cleavage of heparin molecules and isolated by a variety of techniques, including gel and ultrafiltration, solvent extraction, and enzymatic or thermal depolymerization. Fragments below 10–20 monosaccharide units per heparin molecule (5,000 daltons), while containing the essential pentasaccharide-binding sequence to antithrombin III, are not long enough to permit binding to thrombin; they, therefore, inhibit only factor Xa [32, 33]. Even a synthetic pentasaccharide of only 5 monosaccharide units (molecular weight approximately 1,700 daltons) contains the domain that binds to antithrombin III (but not to heparin cofactor II) [34] and possesses a high specific activity in vitro against factor Xa, but little activity against thrombin [35–38]. Heparin preparations weighing more than 5,000 daltons maintain their inhibitory property against factor Xa, but, with increasing chain length, gain a progressively stronger inhibitory capacity against thrombin. The unexpected discovery that heparins of low molecular weight prolong the clotting time moderately (indicating no thrombin inhibition), but are

still capable of potentiating the inhibition of factor Xa, raised the hope of dissociating the antithrombotic property (anti-Xa) from the anticoagulant property (inhibition of thrombin) which then would avoid the haemorrhage-inducing effect of unfractionated heparin. The rationale for this assumption is that it would be of importance in inhibiting the cascade system, with its multiplying effect, at as early a stage as possible without altering normal haemostasis. With low-molecular-weight heparins, the latter conditions could be fulfilled due to their limited inhibition of thrombin which would allow the local accumulation of the latter enzyme for normal haemostasis. It was subsequently shown in animal experiments that an anti-Xa activity is a prerequisite, although not sufficient in itself, for a thrombosis-preventing effect. Heparin molecules, large enough to retain some thrombin-blocking action are indeed also necessary. The lack of correlation between blood levels as measured by anti-Xa assay and impairment of stasis thrombosis in animals described some years ago [39–44] has recently been confirmed [45]. Indeed, it appears that inhibition of thrombin is a more effective way of preventing thrombosis, and the catalysis of thrombin inhibition provides a more reliable in vitro index for estimating possible antithrombotic effects of glycosaminoglycans [46]. It is possible that factor Xa can be 'protected' from the inhibitor action of the heparin-antithrombin III complex in prothrombinase by binding to phospholipid [47], platelets [48–50], or tissue factor [48, 51]. Some other factors, possibly a molecular-weight-dependent vascular wall interaction or a heparin-binding protein such as placental protein 5, may also contribute to the antithrombotic effect of glucosaminoglycans [40–42, 52–56]. In short, all these findings refute the earlier hypothesis that the antithrombotic properties of low-molecular-weight heparins are mainly due to their ability to catalyze the inhibition of factor Xa. It should also be noted that at a very early stage of its development, Thomas et al. [39] and Thomas and Merton [40] did question whether low-molecular-weight heparin would be associated with a lower incidence of haemorrhagic side-effects than unfractionated heparin, a point which until now has not been unequivocally proven in clinical trials directly comparing low-molecular-weight heparins with subcutaneous unfractionated heparin.

Low-molecular-weight heparins interact less with platelets than high-molecular-weight heparins [57–60]. It is logical that larger heparins would have greater affinity than smaller ones of equivalent sulphation, as the larger species would present more negatively charged areas for binding to positively charged regions on the platelet surface; a secondary factor may be the balance of sugar moieties [61]. Reduced bleeding in animal experiments [62, 63] may, therefore, be more related to a decreased effect on platelets than to the reduced antithrombin property of low-molecular-weight heparin [64, 65]; however, alternative explanations have also been suggested [66].

All low-molecular-weight heparins have a ratio of anti-Xa activity to anti-II activity of approximately 4, while it is 1 for unfractionated heparin. After intravenous administration of low-molecular-weight heparin, the half-life of the anti-Xa activity is considerably longer than for unfractionated heparin, while the anti-II half-lives are similar. In contrast to unfractionated heparin, low-molecular-weight heparins are completely absorbed after subcutaneous administration,

and their biological half-life is almost twice as long [for a recent review, see ref. 67].

For prophylaxis of postoperative deep vein thrombosis, a single daily subcutaneous injection of one of the various low-molecular-weight heparins results in a satisfactory protection with remarkably low bleeding risk. For the treatment of deep venous thrombosis, two daily injections of a low-moelcular-weight heparin are necessary. The presently recommended doses for each low-molecular-weight heparin differ and are less well established than for standard unfractionated heparin. Each brand of low-molecular-weight heparin should be considered as a distinct entity, due to distinct biochemical characteristics that determine their pharmacological properties. Thus, for each of the low-molecular-weight heparins the optimal dose in terms of effectiveness and safety is to be established.

The hope that oral heparin complexes [68] or liposome-encapsulated heparin [69] would become available is slowly becoming more realistic.

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