Heparins in Thrombosis and Cancer

Chapter 10

General Discussion and Summary

The presence of a tumor in a given subject predisposes the host to thrombotic complications, that have a negative impact on the patient's quality of life and life expectancy. Vice versa, the activation of the hemostatic system increases the risk of cancer progression, while a thrombosis may indeed be the first sign of an undiagnosed malignancy.

New therapeutic approaches, aimed to effectively disrupt this two-way connection, are strongly needed.

Heparins are well known drugs, widely used in the clinic for thromboprophylaxis and treatment of VTE, but these agents are also equipped with an array of potential anti-tumor properties. Among possible anti-tumor targets, the vascular endothelium is a fascinating target since it plays a role both in hemostasis and in tumor progression.

In the last decades, so-called low-molecular-weight heparins (LMWH), obtained by fractionation of the classical "old" unfractionated heparin (UFH), have progressively replaced UFH in daily clinical use, due to some clinical and biological advantages [1]. While LMWH have been successfully introduced for the clinical management of a wide range of patients with thromboembolic disorders (both for prevention and treatment), conflicting evidence points to potential targets beyond anticoagulation, in particular related to improved survival of patients with cancer as summarized in the Chapter 2. However, the biological mechanisms

underlying this dual action of heparins have not been completely clarified yet, as reported in Chapter 3.

In the present thesis, we then aimed to verify whether heparins, both LMWH and UFH, can exert anticoagulant and antitumor effects by means of multiple actions on the vascular endothelium, so to impair the tumor-hemostasis mutual relationship.

As illustrated in Chapter 4, we started by demonstrating that both LMWH (dalteparin) and UFH have an antithrombotic effect on endothelial cells (EC) by suppressing bacterial endotoxin inflammatory stimulus-mediated procoagulant tissue factor expression and by enhancing anticoagulant thrombomodulin expression and tissue factor pathway inhibitor release. Previous studies have reported an inhibitory effect of heparins (both UFH and LMWH) on EC tissue factor expression. However, these studies were conducted mainly in macrovascular EC, while we included also microvascular EC, the endothelial type most involved in pathological processes. Although the effects reported in Chapter 4 may have impact on hemostatic activation, no tumor cell was involved in that system.

We then employed the same experimental model by exposing the vascular endothelium to products derived from breast cancer and leukemic cells. As shown in Chapter 5, these tumor products elicit a rise in the procoagulant tissue factor expressed by the endothelium, but, similarly to what

was observed with the standard bacterial endotoxin in Chapter 4 and interleukin-1β in the same Chapter 5, heparins are able to counteract also this tumor cell-induced prothrombotic response.

Next step was to evaluate the interference of heparins on angiogenesis promoted by tumor cell derived products. In Chapter 6 we explored for the first time the effect of heparins on the interaction of tumor cells with human microvascular EC on two steps of angiogenesis: the EC proliferation, and the capillary-tube formation by the matrigel assay, which evaluates the final differentiation of EC into capillary-like tubule structures. HMEC-1 cells were incubated with tumor cell conditioned media (TCM) derived from human breast cancer and leukemic cells

or recombinant cytokines (i.e. VEGF, FGF-2, TNF-α) in the absence and presence of heparins. Capillary-like tube formation in Matrigel and EC cell proliferation were evaluated. The results show that all TCM induced a significant increase in total length of tubes formed by HMEC-1 in Matrigel, and that these increases were significantly counteracted (62 to 100% mean inhibition) by the LMWHs enoxaparin and dalteparin, but were significantly less affected by UFH. Similar results were obtained with the standard purified proangiogenic factors. Next, using the same experimental model (see Chapter 7), we observed a similar anti-angiogenic activity also for the “second

generation” LMWH bemiparin and for the “ultra-LMWH” RO-14. Ultra-LMWH are prepared by further heparin fractionation, and very few data are available about their biological activities.

We then extended our study by exploring the in vitro antitumor effect of LMWH on a pancreatic cancer cell line. This type of tumor carries the highest risk of thrombotic events amongst any other gastrointestinal cancers, with an incidence range of 17%–57%. Furthermore, the diagnosis of venous thrombosis in pancreatic cancer is associated with a poor overall survival. As shown in Chapter 8, the LMWH dalteparin, the "second generation" bemiparin and the ultra-LMWH RO-14 significantly prevented the capillary network formation induced by a pancreatic cancer cell line. Interestingly, the anti-angiogenic effect was higher for the LMWH compared with the ultra-LMWH RO-14. In addition, the same heparins showed a direct inhibitory effect on the migration of pancreatic cancer cells *in vitro*.

Finally, in Chapter 9, we report data about the interference of heparins in the adhesion of cancer cells to the vascular endothelium. The adhesion of tumor cells to the endothelium and its matrix, particularly in the microvascular bed, is a crucial step in the metastatic process [2] and organ infiltration. Here, we could demonstrate that heparins impair the adhesion of leukemic cells to endothelial monolayer, and that this is associated with a significant decrease of main

endothelial surface adhesion molecule expression. We are currently extending our studies by exploring the *in vitro* anti-adhesive effect of heparins on breast cancer cells. Altogether, these *in vitro* data further contribute to support the evidence of a possible antitumor *in vivo* effect of heparins, particularly LMWHs.

Research perspectives

The association between cancer and thrombosis was first described by Armand Trousseau in 1865 [3]. Since that time, it has become clear that cancer increases the risk of thrombosis [4]. We also know now that the activation of the clotting system may favour the progression of cancer [5-6]. In the last two decades, the research in this field has clarified many of the mechanisms that underlie the dialogue between the tumor and the hemostatic system. However, this has not led to significant improvements of the cancer patients' quality of life and life expectancy yet. This is likely because we first needed to reach a good comprehension of the biological pathways, before trying to hypothesize possible effective clinical strategies.

Antithrombotic agents, particularly heparins, are able to interfere with tumour cell proliferation and growth, angiogenesis, the development of distant metastases, resistance to chemotherapy and immune responses [7]. Therefore, it seemed feasible that antithrombotics with

antineoplastic properties may improve cancer survival. A review of older trials comparing LMWH with UFH or oral anticoagulants for the treatment or prophylaxis of VTE revealed an advantage with respect to survival rates in patients receiving LMWH, although these studies were not designed to evaluate the effect of the anticoagulants on cancer mortality [8]. More recent studies have been designed to assess the effect of LMWH on cancer survival rates, in addition to their efficacy in preventing VTE. However, data coming from these recent clinical trials are still insufficient/contradictory in terms of demonstrating a clear advantage given by heparins with regard to cancer patient survival. Particularly, it is unclear whether the possible beneficial effect of heparins is influenced by patient-related variables, such as tumor type, tumor stage and aggressiveness (metastatic or localized), or by administration timing, or by the intrinsic characteristics of the employed heparin preparation.

With our work we tried, on one hand to shed more light on the mechanisms underlying the possible beneficial effect of heparins in cancer, by studying the effect of these drugs on the vascular endothelium, on the other hand to understand whether these effects are restricted at particular tumor types and/or to some heparins.

With regard to the question of whether heparins are able to interfere with the prothrombotic and protumor actions of vascular endothelium, the answer is affirmative. Our studies clearly show that heparins impair the EC prothrombotic switch and the neoangiogenesis process triggered by tumor cells. This does not appear restricted by tumor type or aggressiveness. Heparins show to be effective even against tumor cells derived from very aggressive breast and pancreatic cancers.

The second question we tried to answer is whether some heparins are more effective than others. In line with other studies [9], we have observed that EC show different responses to UFH and LMWH. One explanation for this difference may reflect differences in the chemical composition of heparins. Heparins are heterogeneous preparations, derived from mammalian tissues, containing polysaccharide chains of different lengths and molecular weights (MW). UFH has a mean molecular weight of 12-15 kDalton (kDa), with molecules ranging from 3 to 37 kDa in weight. LMWHs are derived from UFH by controlled enzymatic or chemical depolymerization, and have mean molecular weights ranging from 3 to 8 kDa. LMWHs are, therefore, characterized by a much higher proportion of short chains compared with UFH. This leads to an increased anti-Xa/anti-IIa activity ratio, a greater bioavailability, a lower effect on platelets, and a longer half life, compared with UFH

[10]. It is possible that the different effects of heparins on EC observed in the present studies might result from the different proportion of short and long polysaccharide chains within the two heparins as proposed by other authors [11]. It has been indeed demonstrated that heparin fragments with MWs of 4.8- to 5.4-KDa inhibit the in vitro binding of $VEGF^{165}$ to $VEGF^{165}$ receptors on cultured bovine aortic arch EC, while fragments with MWs of more than 6.9 kDa enhance the binding [12]. As a consequence of the lower mean MW of the heparin fragments, it is possible that the LMWHs used here might prevent the binding of growth factors to their receptor on EC and thus inhibit their functions. As a consequence of these considerations, we can also hypothesize that the lower mean Mw of enoxaparin (4.5 kDa) compared to dalteparin (6.0 kDa) should be responsible for the major effect of enoxaparin over dalteparin in inhibiting capillary tube formation. Our results are in line with those of other studies that have explored the effects of heparins on angiogenesis in different experimental *in vivo* and *in vitro* models. In an animal model of angiogenesis, it has been shown that FGF-2- and $VEGF^{165}$-induced angiogenesis is more suppressed by a LMWH, or heparins enriched in 2.5 kDa and 5.0 kDa species, than by UFH and high-molecular-weight heparins [13-14]. Similarly, in an *in vitro* model of angiogenesis in HUVEC, LMWH in the range of 3-6 kDa significantly inhibited FGF-2- and VEGF- induced angiogenesis, whereas no

inhibition was observed with UFH, tetrasaccharide, pentasaccharide and octasaccharide [15].

So, we show here that LMWHs in low range of mean MW appear to be the most effective. However, during the course of our studies, heparins prepared by further fractionation of LMWH were made available by some pharma companies: these are called ultra-low molecular weight heparins (ULMWH). Since these ULMWH present an even higher anti-Xa/anti-IIa activity ratio (indeed anti-IIa acrivity is almost completelety absent in these preparations), that renders them very promising by the clinical point of view, we tested one representative of this new subclass of heparins in our models, i.e. the RO-14 (mean Mw 2.2 kDa), together with bemiparin, a “second-generation” LMWH characterized by a mean Mw of 3.6 kDa, that can be considered intermediate between the “first-generation” LMWH and the ULMWH. Our studies show that RO-14 and bemiparin possess an anti-angiogenic activity similar to those shown by LMWH. RO-14 also possesses a direct inhibitory effect on the migration of the pancreatic cancer cells. Of note, in clinics these ULMWH are under consideration for thromboprophylaxis in a variety of clinical conditions, including cancer. Although they seem equally effective as LMWH for the prevention of thrombosis, with less bleeding complications, little is known about their effect on cancer patient survival.

Our results provide background of, but also need for confirmation, by further studies.

The employment of tumor animal models would be of great help to further confirm the anticancer effects of heparins. Many approaches could be used. One of these could be the grafting of tumor in mice in the orthotopic site or by direct i.v. injection of tumor cells in the circulation as described by some authors [16-20]. Mice would then be treated with heparins. Many conditions could be explored: the drugs could be administered before tumor grafting, at the time of grafting, during malignant progression**,** or after the surgical removal of the primary tumor. Also, different tumor cell types could be employed (metastatic/non metastatic, different organ origin, etc.). Mice would then be monitored all along and sacrificed at the beginning and at the end of treatment, as well as after a period of drug washout. The rate of the primary tumor growth could be then evaluated. Metastatic dissemination could also be grossly monitored by optical imaging while at sacrifice the metastatized organs could be examined [17, 20]. Hemostatic activation of the mice could be analyzed in parallel to the oncologic study. In these models, all heparins studied for this thesis could be employed, particularly the new second-generation LMWH and the ULMWH. For the latter no data at all is available in cancer animal models.

A next step of our studies could also be the evaluation, in our models, of the so-called “non-anticoagulant” heparins [21]. Indeed, some heparin preparations have been modified so that while they have lost the anticoagulant properties they still retain other features, including anti-cancer effects [21]. This could render these modified heparins of interest in the cancer setting, when one aims to target tumor progression without altering the hemostatic status of the host and thus avoiding heparin side-effects (namely hemorrhages).

The last step should be to understand how to translate the new and the future information into better clinical strategies. Indeed, those studies that have evaluated the benefits coming from the treatment with anticoagulants on survival of cancer patients show conflicting results [22]. However, the majority of these studies have evaluated the effects of anticoagulants in cancer patients that also had a thrombosis. We still lack studies in which the evaluation of the effect of anticoagulants on cancer progression is the primary target [23], independently from their effect in preventing the hemostatic system activation and thrombosis-related deaths. So, we strongly need this type of clinical studies. Data coming from in vitro research and animal models could help to design clinical trials of such type with narrow, clear targets.

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