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Effect of sulfated glycosaminoglycans on tumor invasion and metastasis

Eliene O. Kozlowski, Mauro S. G. Pavao

Laboratorio de Bioquimica e Biologia Celular de Glicoconjugados, Programa de Glicobiologia, Instituto de Bioquimica Medica, Universidade Federal do Rio de Janeiro (UFRJ) and Hospital Universitario Clementino Fraga Filho (UFRJ), Rio de Janeiro, RJ 21941-913, Brazil

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1. ABSTRACT

Metastasis is the most devastating aspect of the tumor, being the main cause of morbidity and mortality in cancer patients. The events that lead to tumor invasion and metastasis depend fundamentally on the initial aquisition of a mesenchymal phenotype by the primary carcinoma, which provides the necessary machinary for invasion, intravasation, vascular transport, extravasation and tumor colonization. These events are orquestrated by different growth factors, proteoglycans and adhesion molecules, acting at the surface of cells. The anticoagulant heparin binds several of these molecules and can regulate the interactions that occur during tumor invasion and metastasis. For example, heparin modulates the binding of FGF-2 to its tyrosine kinase receptor during tumor proliferation, and the binding of growth factors involved in epithelial to mesenchymal transition during tumor invasion. It also binds P-selectin on activated platelets, preventing tumor cell-platelet interaction during hematogeneous metastasis. In this review, we discuss the role of sulfated glycosaminoglycans during tumor invasion and metastasis, and the possible therapeutic use of heparin analogs on cancer treatment.

2. INTRODUCTION

In nature, polysaccharides are produced as highly functional polymers with magnificent structural diversity. Sulfated polysaccharides abound in the Animal Kingdom and the glycosaminoglycans are one of the most abundant and best studied sulfated polysaccharides in vertebrates. Specific structural motifs in the chain enable these glycans to interact with several biomolecules involving in distinct biological roles. Recent advances in developmental biology and cancer biology have revealed numerous pivotal roles of these polymers in diverse biological processes, such as regulatory functions in development (1-3), axonal growth (4-6), angiogenesis (7-9), microbial pathogenesis (10, 11) and anticoagulation (12-14). Heparin is the best known glycosaminoglycan and its anticoagulant activity was the earliest pharmacological effect attributed to these molecules (15). However, several reports have shown highly relevant effects of heparin in other processes such as inflammation (16-18) and metastasis (19-25), angiogenesis (26, 27) and cell-cell adhesion (28). Although most of the work published focus on the effects of heparin or heparan sulfate, chondroitin sulfate and dermatan sulfate have also been shown to have similar effects (29-31). Here, we

discuss the effects of sulfated glycosaminoglycans in cancer, focusing on invasion and metastasis of tumor cells.

According to the WHO (World Health Organization), cancer is the leading cause of death in all parts of the world (32) and metastasis is the most devastating aspect of the disease. "Metastasis" is a Greek word meaning "changing the place". The expression "tumor metastasis" was first used in 1829 by the French physician Joseph Claude Recamier in his treatise "*Recherches du Cancer"*, referring to the movement of cells from a primary tumor to distant organs. Metastasis is a complex multistep process in which tumor cells acquire the ability of colonizing distant sites. Although the primary tumor can release millions of cells per day into the circulation (33), only ~0.01% of the cells that enter the circulation have the potential to form secondary tumors, and are called cancer initiating cells (34, 35). The cascade of events involved in metastasis includes: (1) the proliferation of transformed (malignant) cells to form a small tumor mass, which can be supported by simple diffusion of nutrients; (2) the induction of its own vascularization (angiogenesis); (3) detachment of tumor cells from the tumor and invasion through the basement membrane or to the surrounding stroma; (4) entering of cells into the blood circulation; (5) evasion from the immune system; (6) arrest of tumor cells in the capillary beds of distant organs; (7) invasion of the surrounding parenchyma and (8) proliferation of cells to form a tumor mass in a secondary organ.

Recent advances on cancer glycobiology have raised increasing interest in the role of several glycoconjugates on different steps of the metastatic cascade. Sulfated glycosaminoglycans, such as heparin, heparan sulfate, dermatan sulfate and chondroitin sulfate have been widely studied on different molecular events of tumor dissemination, such as proliferation (36), recruitment of leukocytes (37), invasion (38), and survival in blood circulation (21). The relevance of glycosaminoglycans in each one of those mechanisms is discussed below.

3. GLYCOSAMINOGLYCANS AND CELL PROLIFERATION

One of the hallmarks of cancer cells is their uncontrolled proliferation. Multiples genes and pathways are differently regulated, resulting in an altered cell cycle. In general, competent growth factors such as PDGF (Platelet-Derived Growth Factor) and FGF (Fibroblast Growth Factor) (39, 40), as well as EGF (Epidermal Growth Factor) and IGF-1 (Insulin-like Growth Factor) (41, 42) are required for a quiescent cell to enter in the G1 phase. The growth factor-growth factor receptor signaling usually culminates in the modulation of cyclins, specifically those from the cyclin D family that induce the phosphorylation of retinoblastoma protein (RbP), leading the cell to enter into the S-phase (43-45). Some of the signaling cascades initiated by growth factors are modulated by proteoglycans and/or glycosaminoglycans. Several studies have shown an increased expression of the proteoglycan decorin gene during the quiescence of cells in organogenesis and tissue differentiation (46, 47). For example, De Luca *et al.* (1996) observed an arrest of decorin-expressing tumor cells at the G1 phase that concomitantly expressed the cyclin-dependent kinase inhibitor $p21_{waf1}$ (48). It has also been shown that decorin directly interacts with Epidermal Growth Factor Receptor (EGFR) evoking a profound downregulation of the receptor, and inhibition of its downstream signaling activity (49-51). Albeit the signaling effect of decorin is attributed to the core protein, dermatan sulfate chains of decorin have been shown to interact with Transforming Growth Factor-b (TGF-b) (52) and FGF-2, acting as a co-receptor, and inducing cell mitogenic signaling (53). In melanoma, chondroitin sulfate proteoglycans control cell proliferation by enhancing focal adhesion kinase and activating the extracellular signal-regulated kinase (54).

Membrane heparan sulfate proteoglycans (HSPGs), such as Syndecans are classic mediators of cell growth and differentiation, acting by interacting and modulating different growth factors activities. Extracellular HSPGs can also bind to soluble ligands, increasing their local concentration and modulating ligand-receptors encounters (55). HSPGs markedly enhance signaling of FGF-2 and Hepatocyte Growth Factor (HGF) (see (56) for review). In particular, FGF-2 completely depends on heparan sulfate to transduce an intracellular signal through the formation of ternary complex HSPG-FGF-2-FGFR (57-60). Due to the HS-dependence of those growth factors, heparin is likely to be a good tool for inhibiting the growth factor-induced cell signaling. In fact, several works have shown that heparin shows anti-proliferative effects, acting as a competitor for HSPGs on the cell surface. Although the anti-cancer effects of heparin have been documented for decades (8, 21, 22, 61-63), the mechanisms involved in cell proliferation and tumor growth still need to be fully understood.

Apoptosis is a key process in cancer development and progression (64, 65). The ability of cancer cells to escape apoptosis and continue to proliferate is a hallmark of tumor cell and one of the major targets in cancer therapy (65). Sulfated glycosaminoglycans actively participate in the regulation of the apoptotic process (66, 67) by interacting with different growth factors. For instance, expression of glypican-3 is frequently silenced in mesotheliomas, ovarian and breast cancer (68-70). Re-expression of glypican-3 in tumor mammary cells increased the susceptibility to apoptosis, by modulation of Wnt, PKB/Akt and p38MAPK (71, 72). Modifications on glycosaminoglycans chains are likely to be involved in different susceptibility of cancer cells to apoptosis. Sulfatase-2 (SULF2), an extracellular 6-O-endosulfatase, which modifies the sulfation pattern of extracellular heparan sulfate chais, induces an increase in phosphorylation of the anti-apoptotic Akt kinase substrate GSK3□, leading to a reduction of the apoptotic index in human hepatocellular carcinoma cells. (73).

Syndecan-1 has been pointed as a powerful tumor suppressor. Syndecan-1 sheded from cell surface acts by reducing cell growth and inducing apoptosis of

myeloma cells *in vitro* (74). Morover, the expression of Syndecan-1 on myeloma cells is associated with a reduction of mortality in a mouse model of tumor (75). In tumors of epithelial cell lineage, syndecan-1 is usually downregulated, contributing to progression of malignancy. In contrast, overexpression of syndecan-1 is also related to unfavorable phenotypes in ovarian, breast, pancreatic and endometrial cancer (76-78). However, how syndecan-1 effectively affects tumor progression is still unknown. Recent work has shown that syndecan-1 ectodomain was effective in inducing apoptosis and inhibiting the growth of low expressing syndecan-1 prostate cells, through a PDK1/Akt/Bad signaling pathway (79). Analogs of syndecan-1 ectodomain, designed by conjugation of glycosaminoglycan chains with carbodiimide, are shown to inhibit myeloma and breast cancer cells viability by inducing apoptosis, without any apparent toxicity to the adjacent normal tissue (80). Unfractionated heparin was also shown to induce cell apoptosis, affecting cell growth in four oral squamous cell carcinoma cell lines, through suppression of Akt pathway (81). The low molecular weight heparin, dalteparin, was shown to induce lung adenocarcinoma A549 cells to early apoptosis (82). Despite interesting results, the lack of mechanisms to explain the effects of exogenous glycosaminoglycans and some contradictory data, since heparin did not affect apoptosis in several colon carcinoma cells (83, 84) and glioma (85), makes the use of glycosaminoglycan as antiapoptotic drugs a subject of discussion.

5. GLYCOSAMINOGLYCANS AND TUMOR INVASION

The ability of cancer cells to metastasize first depends on their ability to overcome local adhesive forces, migrate and invade the surrounding tissue. Cell migration is a result of increased cell motility, a highly coordinated multifactorial process, requiring continuous formation and disassembly of matrix adhesions (86-89). About 80% of the cancers are carcinomas that acquire a migratory phenotype through the epithelial-to-mesenchymal transition (EMT) program (90) (Figure 1A). EMT is a complex process whereby polarized epithelial cells transform into apolar fibroblastoid-like cells. A reduction of cell-cell adherence via a switching of cadherins (down regulation of E-cadherin and up regulation of N-cadherin) and acquisition of migratory capability are important changes observed upon EMT. In 2001, Zajchowski *et al.* (91) found that vimentin and other mesenchymal gene products, such as integrin a3, osteonectin, thrombospondin-1 and collagen I and VI were part of the gene signature predicting invasiveness of human breast cancer cells. Several well known invasive cell lines, such as BT-549, Hs578T, MDA-MB-157, MDA-MB-231, MDA-MB-435, MDA-MB-436, posses a basal/mesenchymal genotype (92). The activation of transcription factors leading to initiation of EMT and consequently metastasis may be triggered by many extra- and intracellular signals. The "scatter factor" or HGF was the first factor observed to induce EMT (93, 94) and since then, many growth factors such as EGF, VEGF, TGF-b, Wnt, SDF and PGE2 have been shown to induce EMT *in vitro* (95). The binding of some growth factors to their receptors on cell surface usually requires the presence of cell surface proteoglycans carrying heparan sulfate and/or chondroitin sulfate (HSPG and/or CSPG) (96, 97). It has been shown that either HS/heparin or dermatan sulfate bind to HGF with high affinity and play role as cofactors *in vitro*. Although the glycosaminoglycan region that is recognized by the growth factor remains to be elucidated, it has recently been shown that a tri-saccharide containing a single iduronic acid, flanked by monosulfated hexosamines, is enough to bind to HGF with high affinity (98). Dermatan sulfates isolated from ascidians, primitive chordate marine invertebrates have been shown to bind HGF and regulates its activity, enhancing cell signaling (99, 100). HGF stimulates skeletal muscle proliferation, differentiation and migration in synergy with FGF-2, in a DS-depending manner (101). Thus, it is possible that DSs and other glycosaminoglycans could influence the EMT process (Figure 1B). However, how dermatan sulfate and other glycosaminoglycans are involved in cancer-related signaling pathways still requires extensive investigation.

Invasion of tumor cells into surrounding tissues requires not only an increased mobility, but also a "degrading activity" on the extracellular matrix to allow migration of the cells. This activity is provided by secretion of degrading enzymes, such as matrix metaloproteases (MMPs) and heparanases (102, 103). Heparanase is a HS-degrading endoglycosidase preferentially expressed in human tumors. Its overexpression is strongly associated with an invasive phenotype and highly correlated with the metastatic potential of cancer cells (104). Heparanase cleaves HS at low sulfated regions, contributing to structural changes of the extracellular matrix and basement membrane underling epithelial and endothelial cells, which is fundamental for the invasion of cancer cells. HS fragments generated by heparanase cleavage are usually still of appreciable size and can act as signaling molecules for cancer and stromal cells, enhancing proliferation and migration (105-107). Due to the important role of heparanase in tumor invasion and metastasis, a variety of inhibitory molecules have been developed, including neutralizing antibodies, modified species of heparin (108, 109) as well as many polyanionic molecules, such as suramin and laminaran (110, 111).

6. GLYCOSAMINOGLYCANS AND HEMATOGENOUS METASTASIS

The surface of carcinoma cells shows altered glycosylation patterns (112-114), expressing highly branched or sialylated oligosaccharides, especially fucosylated glycans containing sialyl-Lewis^X and sialyl-Lewis^a. The presence of these oligosaccharides in tumor cells is directly associated with a poor prognosis because of tumor progression and metastatic spread (112) (113) (115). The sialyl-Lewis^X-oligosaccharides from the carcinoma cells are ligands of the 3 members of the selectin family of cell adhesion molecules. E-, P- and L-selectins are vascular receptors for certain normal glycoproteins that contain Sialyl-Lewis^{x,a} found on leucocytes and endothelium (116). Therefore, by mediating the interactions of tumor cells with

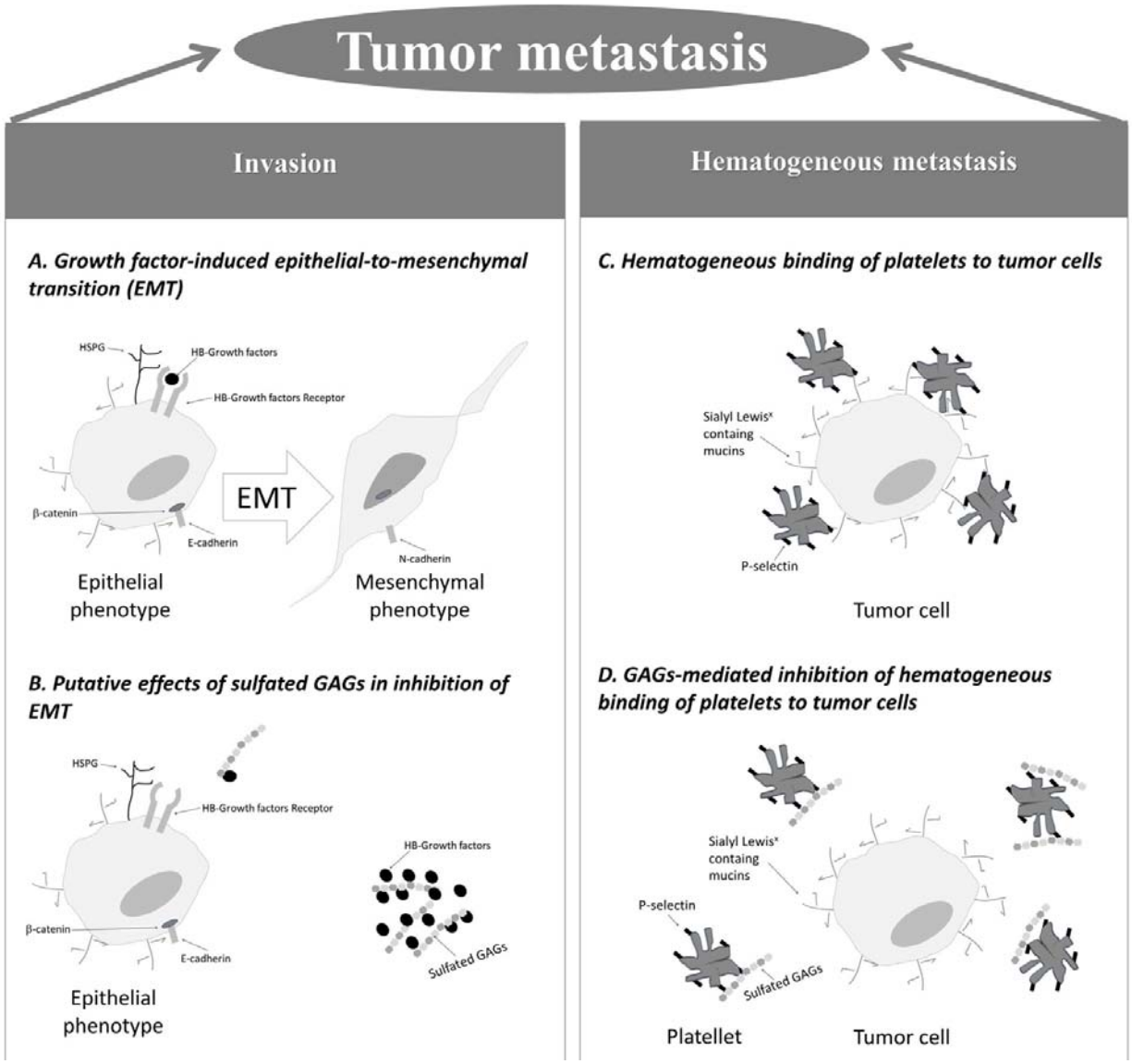

Figure 1. Putative effects of sulfated glycosaminoglycans on tumor metastasis.

platelets and endothelium, the selectins participate in metastatic spread of tumor (Figure 1C) (112) (113) (115).

Studies from several groups have indicated that tumor metastasis in experimental animals can be inhibited by heparin (117) (118) (119). Some clinical studies have also shown a benefic effect of heparin in some types of human cancer (120) (121) (122). However, the antimetastatic effect of heparin is not related to its anticoagulant action (117) (123), but to the heparin's ability to inhibit the interaction of tumor cells with platelets (Figure 1D). Heparin inhibits the binding of P- and L-selectin to their natural ligands (sialyl Lewis^{x,a}-rich oligosaccharides) (124). The inhibition of L- and P-selectin requires the presence of 6-O-sulfated glucosamine residues in the heparin molecule (125). Therefore, the heparin-mediated mechanism of metastasis attenuation involves the inhibition of the interaction of sialyl-Lewis^{x,a}-rich oligosaccharides on the surface of tumor cells and P-selectin on platelets (21). In the presence of heparin, tumor cells loose the protection conferred by platelets becoming susceptible to the potentially cytotoxic action of immune effector cells, which leads to the inhibition of metastasis. A single intravascular injection of heparin promotes the immediate attenuation of the interaction of tumor cell-platelet, with a marked reduction of metastasis 6 weeks after the initial steps of the metastatic cascade. The antimetastatic activity of heparin could not be demonstrated in P- or L-selectin-deficient mice, indicating the involvement of selectins in the antimetastatic activity of heparin (22). Although heparin might influence several steps of metastatic cascade, it is clear that the ability of heparin in inhibiting the binding of tumor cells to P-selectin can explain the clinical evidence that administration of heparin to cancer patients might influence survival (126).

Table 1. Anti-tumor activity of invertebrate glycosaminoglycans

Phylum	Family	Species	Gag (Disaccharide units)	Biological Activity	References
Echinodermata	*Holothuridae*	*Holothuria grisea* (*Ludwigothurea grisea*)	oversulfated CS (GlcA-GalNAc4S,6S) (GlcA3S residues) (sulfated fucose branches)	anti P- and L-selectin inhibitory activity; antimetastatic activity;	(18)
Chordata	*Ascidiidae*	*Phallusia nigra* (*Ascida nigra*)	oversulfated DS (IdoA2S-GalNAc6S) (80%) (IdoA-GalNAc6S)	HGF-SF binding activity; anti P- and L-selectin inhibitory activity; antimetastatic activity	(13, 127), (110)
		Styela plicata	oversulfated DS (IdoA2S-GalNAc4S) (70%) (IdoA-GalNAc4S)	HGF-SF binding activity anti P- and L-selectin inhibitory activity; antimetastatic activity	(13, (13) (110)

However, due to its high hemorrhagic risk the therapeutic use of heparin is still restricted. In addition, it has been reported that the dose required for P-selectin inhibition is higher than that required to achieve anticoagulation, which increases the chances of bleeding and prevents the clinical used of heparin to treat tumor metastasis. The use of low molecular weight heparin, which has a lower bleeding effect, is not an adequate alternative due to its weak inhibitory effect on the selectins. Our laboratory isolated and characterized various heparin analogs from marine invertebrates (Table 1). Several of these compounds, such as dermatan sulfates with different degrees of sulfation from the ascidians *Styela plicata* and *Ascidia nigra* (13) (127) and a fucosylated chondroitin sulfate from the sea cucumber *Ludwiguthurea grisea* (18) possess a high anti-selectin activity. Initial studies indicated that, after intravenous administration to animals, these compounds were able to inhibit P- and L-selectin-mediated events, such as inflammation and tumor metastasis (18), without producing significant bleeding. Therefore, the invertebrate glycans represent a potential therapeutic alternative to mammalian heparin to treat inflammation and tumor metastasis. More recently, the fucosylated chondroitin sulfate was shown to be absorbed after oral administration in rats, which is a great advantage in comparison to heparin, that needs to be administrated intravenous or subcutaneously (128). The antimetastatic effect of orally administrated fucosylated chondroitin sulfate remains to be tested.

7. THERAPEUTIC USE OF GLYCOSAMINOGLYCANS FROM MARINE INVERTEBRATES IN CANCER AND METASTASIS

Metastatic disease is responsible for most cancer-associated deaths and EMT along with hematogeneous metastasis, are critical steps in cancer progression. Therefore, inhibition of these events could be an effective approach to reduce the metastatic disease. Heparin has been show to modulate EMT-associated growth factors and also to inhibit P-selectin, leading to attenuation of metastasis. Previously, we showed that unique dermatan sulfates from the ascidians *Styela plicata* and *Pallusia nigra* (Table 1), composed by 2,4-O-sulfated and 2,6-O-sulfated disaccharide units, respectively, bind with high affinity (KD of ~ 33nM) to the EMT-related growth factor, HGF, modulating its MET-dependent intracellular signaling (99, 100). More recently, we demonstrated that the 2,6-disulfated dermatan sulfate from *P.nigra* inhibits the TGF-β-mediated migration of human mammary cells in a wound-healing cell migration assay (personal observation, not published) and also the binding of tumor cells to P-selectin, with IC_{50} value 2-fold lower than that of unfractionated heparin (129). To investigate the effect of the ascidian DSs *in vivo*, mice were injected with LS180 cells 10 minutes after treatment with PBS, UFH (1 mg/mouse) or ascidian dermatan sulfate (100 µg/mouse) and the presence of platelets-tumor cells aggregates in the lung microvasculature was evaluated. The ascidian DSs inhibited the adhesion of platelets to tumor cells in vivo in a concentration 10-fold lower than heparin. Metastasis in mice was also investigated in a long-term metastasis model, which showed that both ascidian DSs drastically reduced metastasis of murine colon carcinoma cells. Such effects were shown to be P-selectin-dependent. In conclusion, ascidian DSs can inhibit cell migration and reduce hematogeneous metastasis by EMT- and P-selectin mediated events and could be used therapeutically to prevent tumor invasion and metastasis.

Similar to the ascidian dermatan sulfate, several heparin analogs obtained from different marine invertebrate sources were described (for review, see (130)). Some of these compounds have been extensively studied in terms of structure, biological activity and mechanism of action, and evaluated in pre-clinical experiments in rodent animals with promising results. The most critical question related to therapeutics from natural sources is the technical and economic possibility to obtain very large quantities of the compounds in a constant and ecologically correct manner. Most of the invertebrate glycans can be obtained at a reasonable yield (about 0.5% of the dry weight, comparing to 0.022 % from pig intestinal mucosa (125), by procedures similar to those already employed in the preparation of pharmaceutical heparin. Several species of mollusks and sea cucumbers, including those containing high quantities of heparin analogs, have been successfully cultivated in different parts of the world (131, 132). Therefore, the conditions required to use marine invertebrates as source of natural therapeutic compounds have already been established. What is necessary now is a cooperative effort from scientists of related areas to specifically adapt and optimize current methodologies.

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Send correspondence to: Mauro S. G. Pavao, Hospital Universitario Clementino Fraga Filho, Rua Professor Rodolpho Paulo Rocco, 255, 4^{o} andar, 4a08, Cidade Universitaria, Ilha do Fundao, Rio de Janeiro, RJ, Brazil, Tel: 55 21 2562-2093, Fax: 55 21 2562-2090, E-mail: mpavao@hucff.ufrj.br

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