生物化学与生物物理进展 *Progress in Biochemistry and Biophysics*, 2006, 33(1): 72～76
www.pibb.ac.cn

肝素 6 位羧基修饰对抑制 P-选择素介导的 A375 细胞粘附活性的影响 *

魏 民[1] 田美红[2] 陈 琳[3] 曾宪录[1]**

([1]东北师范大学生命科学学院，长春 130024；[2]东北师范大学体育学院，长春 130024；[3]长春市中心血站，长春 130033)

摘要 已有的研究表明，肝素可以作为 P- 选择素的配体，显著抑制肿瘤转移过程中 P- 选择素介导的肿瘤细胞与血小板间的粘附. 但是，肝素被 P- 选择素识别所必需的确切寡糖结构信息仍很缺乏. 通过选择性化学修饰方法制备了 2 种低抗凝血肝素衍生物，即羧基还原肝素 （CR- 肝素）和羧基还原后再硫酸化肝素 （SCR- 肝素），系统地研究了它们对 P- 选择素介导的 A375 细胞粘附的抑制. 研究结果表明，显著失去抗凝血活性的 CR- 肝素仍能有效地抑制 P- 选择素介导的 A375 细胞粘附，说明肝素的 C6 羧基并不是被 P- 选择素识别所必需的. 而 SCR- 肝素所发生的 C6 羧基向羟甲硫酸酯基的转化却显著降低了抗粘附活性，说明 P- 选择素对肝素的识别并不只依赖于肝素的电荷密度. 研究结果为深入阐明拮抗 P- 选择素介导的肿瘤细胞粘附的分子机制提供了有价值的实验基础.

关键词 肝素，羧基还原肝素，P- 选择素，血小板，肿瘤细胞粘附
学科分类号 Q28，Q539，R73

选择素是新近发现的一个细胞粘附分子家族，包括 E-，P-，L- 选择素 3 个成员，其生理功能是在炎症发生时介导白细胞与血管内皮间的起始粘附[1,2]. 近年来，大量实验证据表明，选择素在肿瘤转移的过程中也起重要作用，其中 P- 选择素主要是介导了循环的肿瘤细胞与血小板间的起始粘附[3,4].

在临床上广泛使用的抗凝血药物肝素已被证明可以作为 P- 选择素的配体显著抑制肿瘤细胞与血小板的相互作用，从而可以作为预防和治疗肿瘤转移的候选药物前体[5,6]. 然而，肝素潜在的出血的副作用限制了其在抗肿瘤细胞粘附方面的深入研究和广泛应用. 另外，肝素被 P- 选择素识别所必需的确切寡糖结构信息仍很缺乏. 在本研究中，我们通过选择性化学修饰方法制备了 2 种低抗凝血肝素衍生物，即羧基还原肝素 (CR- 肝素) 和羧基还原后再硫酸化肝素 (SCR- 肝素)，系统地研究了它们对 P- 选择素介导的 A375 细胞粘附的抑制，并讨论了肝素糖醛酸残基上 C6 羧基的修饰对其抗粘附活性的影响，为将肝素用于预防肿瘤转移的临床实践提供了有价值的实验基础.

1 材料与方法

1.1 肝素衍生物的制备及抗凝血活性分析

肝素购于 Sigma-Aldrich Inc，150 U/mg，分子质量约为 20 000. 羧基还原肝素 (carboxyl-reduced heparin，CR- 肝素) 的制备采用 N- 环己基 -3-(2- 吗啉代己基)碳二亚胺对甲苯磺酸盐 (CMC； Sigma 公司) 酯化还原法[7]. 羧基还原后再硫酸化肝素 (sulfated carboxyl-reduced heparin，SCR- 肝素) 的制备采用三氧化硫 - 三乙胺复合物 (Sigma 公司) 硫酸化方法[7].

抗凝血活性分析采用激活部分促凝血酶原激酶时间 (aPTT) 测定法. 仪器为 ACL200 Automated Coagulation Laboratory (Japan)， Lyophilized silica aPTT 试剂盒.

*教育部科学技术研究重点资助项目(105060); 国家自然科学基金资助项目 (30570927).

** 通讯联系人. Tel: 0431-5099317, E-mail: zengx779@nenu.edu.cn

收稿日期：2005-06-24，接受日期：2005-09-10

1.2　蛋白质、抗体及细胞系

重组人 P- 选择素 /Fc 嵌合体蛋白 (P-Fc)，P- 选择素粘附阻断性单抗(9E1)，P- 选择素非粘附阻断性单抗 (P1) 均购买于 R&D Systems, Inc..

A375 (人恶性黑素瘤细胞系) 和 CHO (中国仓鼠卵巢细胞系) 均购买于中国科学院上海分院细胞库. 表达 P- 选择素的 CHO 细胞 (CHO-P 细胞) 通过对 CHO 细胞瞬时转染获得，PolyFect 转染试剂 (Qiagen 公司).

1.3　血小板的固定

首先使用 APES(3-aminopropyltriethoxysilane；Sigma) 处理玻片，然后将富含血小板的血浆 (PRP，2×10^8 /ml)与玻片结合 1 h[8].

1.4　流式细胞仪分析

美国 Backman-Counter 公司 Eltite 型号流式细胞仪，氩离子激光，激发波长 488 nm. 细胞重悬液为 PBS (含 1% FBS)，细胞悬液密度为 5×10^6/ml[9].

1.5　体外模拟毛细血管的层流实验

细胞重悬液为 IMDM (含 0.1% BSA)，细胞悬液密度为 1×10^6/ml. 将覆盖有血小板 /CHO-P 细胞的玻片装配于平行板流动小室 (GlycoTech，Rockville，MD)，然后放置在连接有彩色视频摄像装置的倒置显微镜载物台上. 用注射泵(Cole-Parmer Instrument Co.) 将 A375 细胞悬液注入至平行板流动小室内，以适合的流速维持层壁剪切压. 通过观看录像带人工计数[9].

2　结　　果

2.1　CR-肝素抑制 P-选择素与 A375 细胞的粘附

aPTT 分析表明，CR- 肝素与 SCR- 肝素均显著降低了其抗凝血活性 (表 1 和图 1).

Table 1　Anticoagulant activities[1] of heparin and modified heparins

	2 mg/L	4 mg/L	6 mg/L	8 mg/L	0.1 g/L	1.0 g/L
Control[2]	30.3	30.3	30.3	30.3	30.3	30.3
Heparin	72.9	>120				
CR-Heparin	31.2	31.3	32.3	32.6	51.2	>120
SCR-Heparin	30.9	31.2	31.3	32.5	43.9	>120

[1]Anticoagulant activity is represented in coagulation time (s), and values that are more than 120 s cannot be determined. [2] Plasma with no heparin and modified heparins.

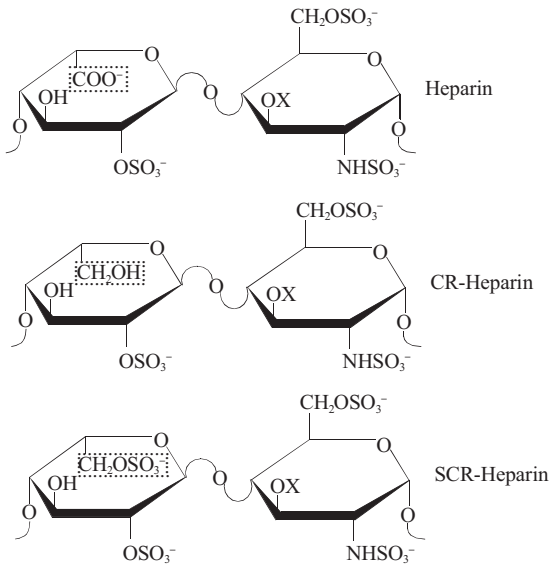

Fig. 1　Representative saccharide units of heparin and the modified heparins

Each saccharide illustrates a characteristic unit in the indicated preparation and does not represent the overall structure of the chains. The modified position and group were indicated by dotted square. X=H or SO_3^-.

已有的研究表明，肝素可以显著抑制 P- 选择素与 A375 细胞的结合[10]. 在本实验中，我们首先测定了在静止条件下 CR- 肝素与 SCR- 肝素对 P- 选择素与 A375 细胞粘附的抑制. 流式细胞仪分析结果表明，在 5.0 g/L 的高浓度条件下，肝素和 CR- 肝素显著抑制了 P- 选择素与 A375 细胞的粘附，粘附比例分别降低了 87%和 81%，而 SCR- 肝素的抗粘附活性很低，只有 52% (图 2a). 在随后的实验中，我们又检验了活性显著的 CR- 肝素在较低浓度水平下抑制 A375 细胞与 P- 选择素结合的能力. 实验结果表明，在 1.0 g/L 的浓度下，肝素和 CR- 肝素的预孵化使粘附比例分别降低了 62%和 36% (图 2b)，在 0.1 g/L 的浓度下，使粘附比例分别降低了 39%和 22% (图 2c). 以上结果说明，CR- 肝素仍然保留了明显抑制 P- 选择素与 A375 细胞结合的能力，抑制程度与其浓度正相关.

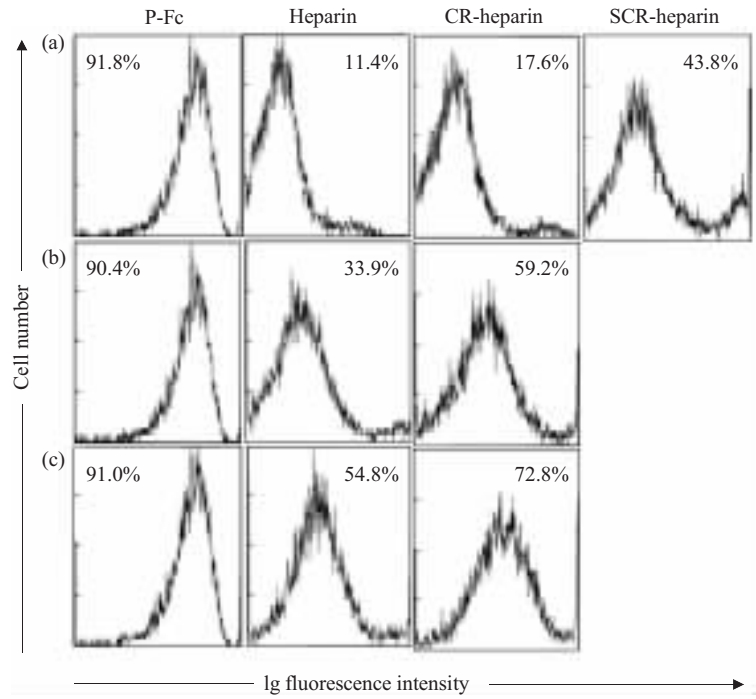

Fig. 2 Inhibition of P-selectin binding to A375 cells by heparin and modified heparins

(a) P-Fc was preincubated with heparin or modified heparins (5.0 g/L). (b) P-Fc was preincubated with heparin or modified heparins (1.0 g/L). (c) P-Fc was preincubated with heparin or modified heparins (0.1g/L). Then tumor cells were incubated with the blocked and unblocked P-Fc, following by an FITC-conjugated Ab against human IgG. The binding events were analyzed by flow cytometry. Results are presented as histograms of the lg(fluorescence intensity) from 10^4 cells. The proportion of positive cells is indicated in each histogram.

2.2 CR-肝素抑制 A375 细胞与 CHO-P 细胞在流动条件下的粘附

为进一步证实已有的结论，我们在本实验环节中使用了表达 P- 选择素 CHO 细胞 (CHO-P 细胞) 作为 P- 选择素的载体，并且采用了体外模拟毛细血管的层流实验条件，进一步分析了 P- 选择素与 A375 细胞在生理流动状态下的结合情况. 实验结果显示，CR- 肝素可以显著抑制 A375 细胞与 CHO-P 细胞的粘附. 与阳性对照相比，肝素及 CR- 肝素的预孵化使 A375 细胞与 CHO-P 细胞的结合分别减少了 90%和 74% (图 3).

2.3 CR-肝素抑制 A375 细胞与血小板在流动条件下的粘附

有确凿的证据表明，血小板与肿瘤细胞发生粘附是肿瘤细胞经血液转移的关键步骤，血小板表面的多种粘附分子参与了粘附过程，其中 P- 选择素主要是在起始粘附过程中起重要作用[4-6]. 为了进一步测定 CR- 肝素对 A375 细胞与血小板在流动条件下粘附的抑制，肿瘤细胞被注入底部覆盖有血小板的平行板流动小室内. 层流实验结果表明，CR- 肝

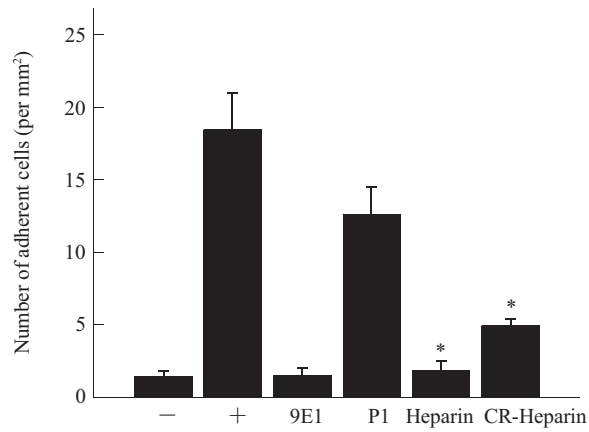

Fig. 3 Inhibition of A375 cells binding to CHO-P cells by heparin and CR-heparin under shear flow conditions

The adhesion of tumor cells to CHO cells (designated as −) and CHO-P cells (designated as +) at 0.3 dyn/cm² were measured respectively by videomicroscopy. For antibody inhibition experiments, CHO-P cells were preincubated with 9E1 or P1. For heparin inhibition experiments, CHO-P cells were preincubated with heparin or modified heparins (5.0 g/L). All values were represented to be the ($\bar{x}\pm s$) of adherent tumor cells in 10～20 fields of view using a 10× objective lens. Controls were run before and after each assay. All results are from three to six separate experiments. * $P < 0.05$ with respect to positive control.

素在 3 种测试浓度条件(5.0，1.0 和 0.1 g/L)下，均可以显著抑制 A375 细胞与血小板在流动条件下的粘附 (图 4).

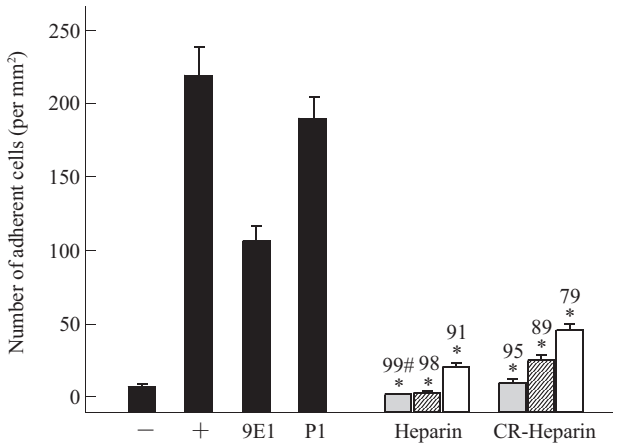

Fig. 4　Inhibition of A375 cells adhesion to surface-adherent platelets by heparin and CR-heparin at 0.3 dyn/cm² under shear flow conditions

Negative control was designated as "−", positive control was designated as "+". For antibody inhibition experiments, immobilized platelets were preincubated with 9E1 or P1. Adhesion of tumor cells to surface-adherent platelets was measured by videomicroscopy. All values were represented to be the ($\bar{x}\pm s$) of adherent tumor cells in 10～20 fields of view using a 10× objective lens. Controls were run before and after each assay. All results are from three to six separate experiments. *P < 0.01 with respect to positive control. # Values are percentages of the inhibition of tumor cells adhesion to platelets preincubated with indicated heparins compared with the adhesion to platelets with no heparins.■: Control; ▨: 5.0 g/L; ▨: 1.0 g/L; □: 0.1 g/L.

3　讨　　论

肝素是一种高度硫酸化的阴离子糖胺聚糖，具有多种显著的生物活性，尤其是它作为抗凝血药物在临床上的广泛使用已经超过了 70 年[11]. 肝素主要是由糖醛酸 -(1→4)-D- 葡萄糖胺的重复二糖亚单位所组成，但二糖亚单位上的 N- 硫酸化、O- 硫酸化和 N- 乙酰化等多种方式的取代使肝素产生了巨大而复杂的结构. 肝素的生物活性主要来自于它与蛋白质间的静电引力作用，即蛋白质侧链的阳离子基团与肝素的阴离子位点间的相互作用[11].

近年来的研究表明，肝素可以作为 P- 选择素的配体显著抑制 P- 选择素介导的细胞粘附. 但是，肝素分子结构中被 P- 选择素识别所必需的确切寡糖结构信息仍很缺乏. 我们在先前关于硫酸基团影响肝素抑制 P- 选择素介导 A375 细胞粘附的研究中发现，C2/C3 上的 O- 硫酸根对于 P- 选择素的识别并不重要，然而，C6/N 上硫酸根的消除却显著降低了肝素的抗粘附活性，说明肝素分子结构中 $GlcNSO_3(6OSO_3)$残基对于 P- 选择素的识别非常重要[12]. 在本研究中，我们又系统地分析了肝素的糖醛酸残基上 C6 羧基对其抗粘附活性的影响. 结果表明，C6 羧基的还原对于肝素的抗粘附活性没有明显影响，因此而产生的羧基还原肝素具有进一步深入研究的价值. 另外，对 C6 羧基还原后进行的硫酸化修饰，并没有使肝素的抗粘附活性得到恢复，这说明 C6 羧基转化为 C6 羟甲硫酸酯基，虽然使肝素的静电荷数没有发生改变，但可能改变了相关基团在空间的走向与排布，因此改变了 P- 选择素识别所必需的空间构象. C6 羧基的还原对于肝素抗粘附活性没有明显影响的原因，可能是由于 C6 羧基还原后得到的—CH_2OH 基团采用了与 C6 羧基相似的空间取向，因此保留了相当的抗粘附活性. 看起来，肝素的活性不但与其总电荷密度相关，而且还可能涉及到其电荷的分布、各基团在空间的取向、单糖组成顺序和硫酸根的位置及含量等诸多因素的影响. 本研究证明了显著失去抗凝血活性的 CR- 肝素仍能有效地抑制 P- 选择素介导的 A375 细胞粘附，为深入阐明拮抗 P- 选择素介导肿瘤细胞粘附的分子机制提供了有价值的实验基础.

参 考 文 献

1　Vestweber D, Blanks J E. Mechanisms that regulate the function of the selectins and their ligands. Physiol Rev, 1999, **79** (1): 181～213

2　魏 民, 曾宪录. 选择素与肿瘤转移. 生物化学与生物物理进展, 2003, **30** (2): 185～189
Wei M, Zeng X L. Prog Biochem Biophys, 2003, **30** (2): 185～189

3　Krause T, Turner G A. Are selectins involved in metastasis?. Clin Exp Metastasis, 1999, **17** (3): 183～192

4　McCarty O J, Mousa S A, Bray P F, *et al*. Immobilized platelets support human colon carcinoma cell tethering, rolling, and firm adhesion under dynamic flow conditions. Blood, 2000, **96** (5): 1789～1797

5　Borsig L, Wong R, Feramisco J, *et al*. Heparin and cancer revisited: mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. Proc Natl Acad Sci USA, 2001, **98** (6): 3352～3357

6　Varki N M, Varki A. Heparin inhibition of selectin-mediated interactions during the hematogenous phase of carcinoma metastasis: rationale for clinical studies in humans. Semin Thromb Hemost, 2002, **28** (1): 53～66

7　Wessel H P, Hosang M, Tschopp T B, *et al*. Heparin, carboxyl-reduced sulfated heparin, and trestatin A sulfate. Antiproliferative and anticoagulant activities. Carbohydr Res, 1990, **204**: 131～139

8 Buttrum S M, Hatton R, Nash G B. Selectin-mediated rolling of neutrophils on immobilized platelets. Blood, 1993, **82** (4): 1165～1174

9 Wei M, Tai G H, Gao Y G, *et al*. Modified heparin inhibits P-selectin-mediated cell adhesion of human colon carcinoma cells to immobilized platelets under dynamic flow conditions. J Biol Chem, 2004, **279** (28): 29202～29210

10 Ma Y Q, Geng J G. Heparan sulfate-like proteoglycans mediate adhesion of human malignant melanoma A375 cells to P-Selectin under flow. J Immunol, 2000, **165** (1): 558～565

11 Rabenstein D L. Heparin and heparan sulfate: structure and function. Nat Prod Rep, 2002, **19** (3): 312～331

12 Wei M, Gao Y G, Tian M H, *et al*. Selectively desulfated heparin inhibits P-selectin-mediated adhesion of human melanoma cells. Cancer Lett, 2005, **229**: 123～126

Effects of The Modification of Heparin 6-Carboxyl Group on Inhibitive Activity of P-Selectin-mediated A375 Cells Adhesion*

WEI Min[1], Tian Mei-Hong[2], CHEN Lin[3], Zeng Xian-Lu[1]**

([1]*School of Life Sciences, Northeast Normal University, Changchun* 130024, China;
[2]*School of Physical Education, Northeast Normal University, Changchun* 130024, China;
[3]*Changchun Blood Center, Changchun* 130033, China)

Abstract Several studies have demonstrated that heparin can significantly inhibit the P-selectin-mediated interaction of platelets and tumor cells during metastasis as a P-selectin ligand. However, little information is available about the specific oligosaccharide structures of heparin in recognition by P-selectin. Two chemically modified heparins, CR-heparin and SCR-heparin were prepared, to explore if such heparin derivatives can reduce the P-selectin-mediated A375 tumor cell adhesion. The results indicated that CR-heparin with low anticoagulant activity could significantly inhibit the P-selectin-mediated A375 tumor cell adhesion, demonstrating that 6-carboxyl group of the glucuronic acid in heparin may not be crucial for recognizing by P-selectin. In contrast, SCR-heparin reduced the inhibiting activity dramatically, suggesting that the recognition of P-selectin to heparin depend on not only densities of negative charge. These results provide valuable experimental evidence for clarifying the molecular mechanism of P-selectin-mediated tumor cell adhesion.

Key words heparin, carboxyl-reduced heparin, P-selectin, platelet, tumor cell adhesion

---

*This work was supported by grants from The Key Project of Chinese Ministry of Education (105060) and The National Natural Sciences Foundation of China (30570927).

**Corresponding author . Tel: 86-431-5099317, E-mail: zengx779@nenu.edu.cn

Received: June 24, 2005 Accepted: September 10, 2005