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赤魟软骨血管生成抑制因子的纯化及抗血管生成活性验证

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摘要:以赤魟软骨为原料, 通过盐酸胍抽提、离子交换层析、凝胶过滤、反相高效液相色谱等步骤, 首次获得赤魟软骨血管生成抑制因子-I(*Dasyatis akajei* cartilage angiogenesis inhibitory factor-I, DCAIF-I)。12%的SDS-PAGE电泳-考染显示为一条带,根据蛋白质的相对迁移率计算,分子量约为62 ku。通过鸡胚绒毛尿囊膜活性抑制模型进行活性分析,结果表明,活性物质处理组(DCAIF-I组)与阳性对照组(CS组)抑制效果明显,CAM上的血管发生大面积褪色,血管结构模糊, 分枝发生断裂, 血管密度减少。PBS对照组血管网清晰,呈叶脉状、放射状生长。血管定量分析结果表明,随着活性物质处理组DCAIF-I含量的增加,CAM上血管的数量减少更加明显,当DCAIF-I含量为1 μg时,对CAM上血管的抑制率达56%,结果表明,DCAIF-I对鸡胚绒毛尿囊膜新生血管生成具有明显的抑制效果,且抑制活性具有一定的浓度依赖性。

关键词:赤魟; 血管生成抑制因子; 鸡胚绒毛尿囊膜; 纯化; 肿瘤防治
中图分类号:Q 331; S 917 **文献标识码**:A

Purification and bioactivity of a novel angiogenesis inhibitor extracted from the cartilage of *Dasyatis akajei*

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Abstract: In the early 1980s, shark cartilage has been revealed to contain a protein, an angiogenesis inhibitor that significantly inhibits the development of blood vessels that nourish solid tumors, thereby restraining tumor growth. Since then, it has generated intense interest in both public and medical circles. The aim of this study was to determine the method of isolation and characterization of the angiogenesis inhibitor, and identify the bioactivity of angiogenesis inhibitor derived from the *Dasyatis akajei* cartilage. In this view, we study in detail the method of purification and characterization the bioactivity of a novel angiogenesis inhibitory factor derived from the *Dasyatis akajei* cartilage(DCAIF-I). By the 1.0 mol·L^{-1} guanidinium chloride extract of *Dasyatis akajei* cartilage, the extraction was then purified by Hitrap DEAE FF ion exchange chromatography, Superdex 75 10/300 GL gel filtration, and reverse-phase high performance liquid chromatography. The bioactivity of the products obtained is identified by the model of inhibiting the formation of the blood vessels of the chorioallantoic membrane of chicken embryo. Statistical analysis of blood vessels of DCAIF-I was used for quantitative analysis of the inhibitory effect of DCAIF-I. The pure inhibitor was homogeneous as a single band on a coomasie brilliant blue-stained 12% SDS-PAGE

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gel electrophoresis. DCAIF-I was a novel angiogenesis inhibitory factor which has a molecular weight about of 62 ku. The results of bioactivity identification of angiogenesis inhibitory factor indicates that the large area of blood vessels in active substance groups have heavy loss of color, vascular structure blurred with broken branches, accompanied by the decreased density of vessels. In control group, the leaflike vascular net is clear and grew radiatively. Quantitative analysis blood vessels of the chorioallantoic membrane of chick embryos (CAM) indicated that DCAIF-I can strongly inhibit the angiogenesis in the chorioallantoic membrane of chick embryos(CAM), 1 μg of DCAIF-I inhibited angiogenesis in 56% of the eggs. There is also a positive relation between the dosage and the effect. The results from the present study indicated that the DCAIF-I from *Dasyatis akajei* cartilage has angiogenesis inhibitory effect, and there is also a positive relation between the concentration and inhibitory effect. Therefore it is hoped that this angiogenesis inhibitor may provide a novel treatment for patients with malignancies, and perhaps even for those with nonmalignancies. It will be helpful for our knowledge of the molecular mechanisms of this angiogenesis inhibitor from *Dasyatis akajei*.

Key words: *Dasyatis akajei*; angiogenesis inhibitory factor; chick embryo chorioallantoic membrane; purification tumor therapy

原发性肿瘤的生长、入侵与转移都依赖于肿瘤的血管生成,抑制血管生长、把肿瘤血管作为治疗癌症的新靶点,有可能有效地控制肿瘤的生长与转移。因此,抗血管生成疗法引起许多研究者的兴趣。自 1975 年 Brem 和 Folkman 研究发现软骨能抑制肿瘤新生血管生长以来,许多研究者已从多种动物的软骨中发现有分子量不等的抑制肿瘤生长的活性因子存在,如 10 ~ 14 ku U-995 , 18 ku SCAIF-1, 80 ku SCAIF80, 10 ku SCF2 等[1-4],都具有一定的血管生成抑制活性,其中绝大部分来自于鲨鱼软骨。赤魟与鲨鱼同属软骨鱼类,且软骨资源总量及软骨含量远高于鲨鱼。研究表明,赤魟软骨成分中含有抑制血管生成、抗肿瘤的活性物质[5-6],但究竟是何种物质介导了赤魟软骨的这种活性,并且这种物质的本质是什么,国内外未见报道。本文首次从赤魟软骨中分离纯化获得了一种新的高纯度活性蛋白——赤魟软骨血管生成抑制因子-I (*Dasyatis akajei* cartilage angiogenesis inhibitory factor-I, DCAIF-I), 具有显著抑制新生血管生成的活性,为进一步在分子水平研究其抑制机理提供了理论基础,并为肿瘤治疗提供新的研究对象。

1 材料与方法

1.1 材料

赤魟软骨,购自中国水产舟山渔业总公司。盐酸胍、2-N-吗啡啉乙磺酸(MES)、十二烷基磺酸钠(SDS)、乙腈、硫酸软骨素(CS),均为 Fluka 公司产品;SDS-PAGE 低分子量标准蛋白为 Pharmacia 公司产品;牛血清白蛋白(BSA)、三羟基甲基氨基甲烷(Tris)均为 AMRESCO 分装产品。

1.2 抽提条件的优化

将 1 000 g 赤魟软骨放置在 5 000 mL 含 0.02 mol·L^{-1} MES 和质量体积分数为 0.02% 的 EDTA 的 1.0 mol·L^{-1}盐酸胍溶液中进行抽提,通过设计四因素三水平的正交试验对影响抽提效率的温度、抽提液 pH、摇床振荡速度、抽提时间进行了参数优化,以提取后溶液中所含蛋白质的浓度为考察指标,采用 Bradford 方法,以牛血清白蛋白为标准样品测定抽提液蛋白浓度。同时计算抽提产率:

产率(%) = 蛋白浓度(mg·mL^{-1}) × 60/10000。每次实验用的赤魟软骨为 10 g,所用的提取液定容为 60 mL 后再取样测定其中的蛋白质浓度。

1.3 DCAIF-I 的分离纯化

用盐酸胍溶液抽提得到的赤魟软骨粗提物于 4 ℃下以 9 000 r·min^{-1}离心 25 min,弃残渣;继续在 4 ℃下以 10 000 r·min^{-1}离心 25 min;上清液经超滤获取分子量在 3 ~300 的赤魟软骨粗提液,赤魟软骨粗提液经 pH 值为 7.2 的 0.02 mol·L^{-1} Tris-HCl 缓冲溶液(含 0.02 mol·L^{-1} NaCl 和 0.02% NaN_3)透析、Hitrap 26/10 Desalting(2.6

cm × 10 cm)柱脱盐、冷冻干燥;用 Hitrap DEAE FF 柱(1.6 cm × 10 cm) 进行离子交换,台阶梯度洗脱,流速 4.15 mL·min^{-1},收集组分,进行活性鉴定;Superdex 75 10/300 GL 柱(1.0 cm × 30 cm)层析:洗脱速度 0.6 mL·min^{-1},收集组分,进行活性鉴定;Shim-pack VP-ODS(0.46 cm × 15 cm) HPLC 柱层析:流动相为 40% 乙腈 +0.05% TFA,流速为 0.8 mL·min^{-1};收集组分,进行活性鉴定,透析,冷冻干燥,获得 DCAIF-I。活性峰采用鸡胚绒毛尿囊膜(CAM)血管生成抑制模型进行鉴定。

表 1　正交实验因素水平表

Fig. 1　The level and factor of orthogonal experiment

$L_9(3^4)$	温度 (℃) temperature	pH	摇床振荡速度 (r·min^{-1}) surge speed	抽提时间 (h) extraction time
1	20	4.8	100	24
2	25	6.0	130	48
3	30	7.2	160	72

1.4　DCAIF-I 纯度鉴定及分子量测定的 SDS-PAGE 电泳

参考 Sambrook 等[7]方法进行。以 5% 的浓缩胶以及 12% 的分离胶,采用垂直平板电泳。

1.5　DCAIF-I 抑制鸡胚绒毛尿囊膜(CAM)血管生成的测定

参考贺国安等[8]方法加以改进。滤纸片大小为 3 mm × 3 mm;加样前孵化时间为 4 d;加样量:DCAIF-I 分别为 1、4、8 μg;PBS 为阴性对照;4 μg 的硫酸软骨素为阳性对照,每个试验组 8 只种蛋。加样后继续孵化 24 h,以给药点为中心,以半径间隔 5 mm 将鸡胚绒毛尿囊膜划分为 3 个区域,拍照记录结果,计数各区域的血管数,进行数据统计。比较各剂量组与对照组之间的差异。

2　结果

2.1　DCAIF-I 抽提条件的优化

正交试验结果见表 2。结果表明,四个因素对抽提效率都具有显著性影响,其中抽提液的 pH 和抽提温度的影响更大,抽提时间次之,摇床振荡速度影响最小。因此,试验确定最佳提取条件为:抽提液 pH 7.2、温度 20 ℃、抽提时间 72 h、摇床振荡速度 160 r· min^{-1},此条件下抽提产率为 0.95%,抽提物中蛋白含量为 1.58 mg·mL^{-1}。

表 2　正交实验的设计与结果

Fig. 2　The condition and results of orthodoxy experiment

因素 factor	抽提时间 (h) extraction time	摇床振荡速度 (r·min^{-1}) surge speed	pH	温度 (℃) temperature	蛋白浓度 (mg·mL^{-1}) protein concentration
实验 1	3	1	1	1	1.29
实验 2	3	2	2	2	0.32
实验 3	3	3	3	3	1.56
实验 4	2	1	2	3	0.75
实验 5	2	2	3	1	1.05
实验 6	2	3	1	2	1.10
实验 7	1	1	3	2	0.60
实验 8	1	2	1	3	0.82
实验 9	1	3	2	1	0.83
K_1	0.754	0.880	1.066	1.061	
K_2	0.966	0.732	0.633	0.667	
K_3	1.061	0.977	1.078	1.046	
极差 R	0.307	0.245	0.445	0.392	

2.2　DCAIF-I 的分离与纯化

赤魟软骨的盐酸胍抽提产物透析、脱盐、冷冻干燥后,经 Hitrap DEAE FF 离子交换层析,获得 10 个明显洗脱峰(图 1),收集第 1 峰,浓缩后进行 Superdex 75 10/300 GL 分子筛层析,呈现 4 个明显的洗脱峰(图 2),将第 1 峰浓缩后进行反相 HPLC 层析(图 3),得到 3 个明显的洗脱峰,收集活性峰,浓缩冻干后得到 DCAIF-I。

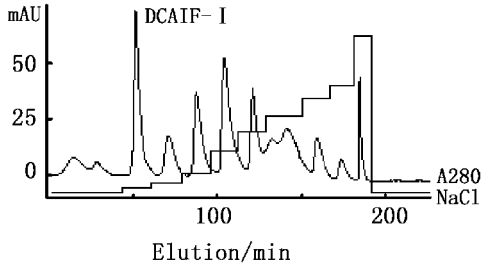

图 1　Hitrap DEAE FF 阴离子交换梯度洗脱曲线

Fig. 1　Elution profile of DCAIF-I in Hitrap DEAE FF Chromatography

The column was eluted at a flow rate of 4.15 mL · min^{-1}. Fractions of 2 mL were collected and assayed for CAM activity. A total of 10 distinct separations were eluted on the Hitrip DEAE FF column. DCAIF-I was contained in the first sepearation

2.3　DCAIF-I 的纯度鉴定及分子量测定

采用最优的分离纯化条件,1 000 g 赤魟软骨可制备 DCAIF-I 25 mg,所以 DCAIF-I 的提取率为 40 000∶1。SDS-PAGE 电泳结果(图 4)所示,考染显示一条带,说明 DCAIF-I 达到电泳纯,根

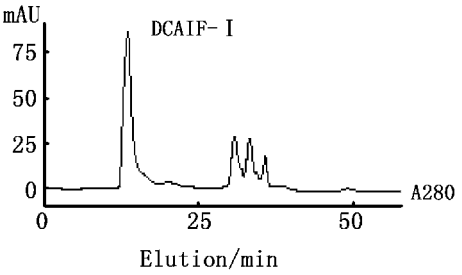

图 2 Superdex 75 10/300 GL 凝胶柱层析洗脱曲线

Fig. 2 Elution profile of DCAIF-I in Superdex 75 10/300 GL Chromatography

The column was eluted at a flow rate of 0.6 mL · min^{-1}. Fractions of 1 mL fractions were collected and assayed for CAM inhibitor activity. A total of 4 distinct separations were performed on the Supdex 75 10/300 GL gel filtration column. DCAIF-I was contained in the first separation

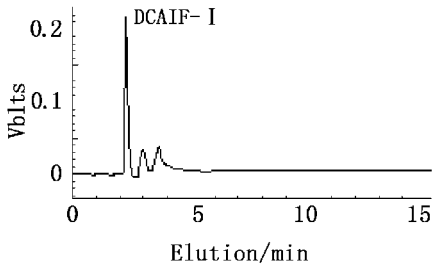

图 3 Shim-pack VP-ODS 柱反相高效液色谱图

Fig. 3 Elution profile of DCAIF-I in Shim-pack VP-ODS inversed phase high efficiency liquid Chromatography

The absorbed proteins were eluted with a 40% acetonitrile in 0.05% trifluoroacetic acid in water at a flow rate of 0.8 mL · min^{-1}. A total of 3 distinct separations were performed on the Shim-pack VP-ODS HPLC column. DCAIF-I was the first separation.

据标准蛋白质的迁移率,测得其分子量约为 62 ku。目前国内外尚未见获取同种蛋白质的报道,提示从赤魟软骨中分离获得的 DCAIF-I 可能是一种具有新颖结构的活性蛋白。

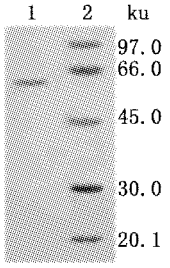

图 4 DCAIF-I 的 SDS-PAGE 电泳图谱

Fig. 4 SDS-PAGE profile of DCAIF-I

SDS-PAGE gel (12% w/v) of DCAIF-I was visualized by staining with coomasie brilliant blue R-250. The lane 1 well contained 15 μL of DCAIF-I and 5 of loading buffer. Sizes of molecular weight standard are indicated on the right lane 2.

2.4 DCAIF-I 对鸡胚绒毛尿囊膜血管生成的抑制 结果表明,PBS 对照组血管生长良好,血管数量多而密,与之相比 CS 对照组血管生长大量减少,管径较细且分支少(图 5),DCAIF-I 处理组与 CS 对照组呈现相似的结果,3 个记数区域内的血管数量均显著减少,且随着加样量的增加血管数量减少得更加明显(表 3)。实验还发现 DCAIF-I 血管抑制活性较强,1 μg 的 DCAIF-I 对 CAM 的抑制率达 56%,与 4 μg 硫酸软骨素的抑制效果相当。表明 DCAIF-I 能显著抑制 CAM 膜新生血管生成,并且抑制效果具有一定的浓度依赖性。

图 5 加样 24 h 后 CAM 上 DCAIF-I 的血管生成抑制效果

Fig. 5 Inhibitory effect of DCAIF-I on angiogenesis in the CAM at 24 h

由于实体瘤的进行性生长依赖于其诱导产生的血管网的建立,许多直接、间接的证据已经证明肿瘤生长是血管依赖的。新生肿瘤血管可持续不断地为肿瘤细胞提供营养及氧气,带走肿瘤代谢产物,而且肿瘤生长需要毛细血管内皮细胞的旁分泌作用,另外,新生的微血管是肿瘤浸润和转移的第一站,肿瘤微血管数量越多,肿瘤细胞进入血液循环的机会就越大。

表 3　加样 24 h 后 CAM 上 DCAIF-I 的血管生成抑制效果的统计学分析

Tab.3　Statistical analysis of inhibitory effect of DCAIF-I on angiogenesis in CAM at 24 h

组分 group	样本数 n	血管数目 quantity of blood vessels($\bar{x} \pm s$) 0 ~ 5 mm	5 ~ 10 mm	10 ~ 15 mm
control(PBS)	8	18.25 ± 1.65	24.32 ± 2.17	28.63 ± 2.76
CS(4 μg)	8	5.36 ± 0.65	10.23 ± 1.12	13.84 ± 1.72
DCAIF-I(1 μg)	8	6.28 ± 0.69	8.23 ± 1.08	14.84 ± 1.82
DCAIF-I(4 μg)	8	3.23 ± 0.56	5.32 ± 0.72	6.38 ± 1.16
DCAIF-I(8 μg)	8	1.51 ± 0.38	2.85 ± 0.58	3.92 ± 0.65

3　讨论

本文以赤魟软骨为原料,首次分离纯化得到一种新的血管生成抑制因子——赤魟软骨血管生成抑制因子-I(DCAIF-I),SDS-PAGE 考染鉴定为一条带,分子量约为 62 ku;生物学活性研究表明 DCAIF-I 对鸡胚绒毛尿囊膜(CAM)的血管生成具有明显的抑制作用,抑制效果与浓度呈正相关,1 μg 的 DCAIF-I 对 CAM 的抑制率与 4 μg 硫酸软骨素相当。提示纯化得到的 DCAIF-I 为高纯度的软骨血管生成抑制因子,能在整体水平上显著抑制新生血管生成,从而作为一种有效的抑制物为肿瘤的治疗提供新的选择,为进一步在分子水平研究其抑制机理提供了理论基础。

1971 年 Folkman 提出"肿瘤的生长是依赖血管的"[9]。血管生成因子的过量表达与血管生成抑制因子的调控能力的降低都是诱导新生血管生成的重要条件,这两个过程同时发生从而触发了肿瘤的生成[10]。基于肿瘤细胞的这一特性,抑制血管生成可能成为治疗肿瘤的有效策略。

据统计,软骨制剂在 15 000 例的临床应用中,仅发现 2 例出现轻微皮疹,在动物实验中也未发现明显的毒副作用。并且抗血管增生活性物质一般不会引起耐药性[11],Robert 等[12]认为血管生成抑制剂是无抗性的抗肿瘤药,因为其作用肿瘤的重要靶区在遗传性相当稳定的血管内皮细胞。抗血管增生多肽与化疗药物联合应用,可显著提高化疗药物的效应,大大降低其毒性[13]。因此,直接来源于赤魟软骨组织的 DCAIF-I 应该具有毒副作用小、无耐药性等特点,在治疗肿瘤及其它血管增生性疾病中有着广阔的应用前景。同时,国内外未见获得类似 DCAIF-I 的报道,值得进一步深入研究。

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