· Original article ·

Expression, purification and characterization of PCP-2EC/Fc fusion protein in mammal cells

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[**ABSTRACT**] **Objective**: To construct the extracellular region of PCP-2(PCP-2EC) and the immunoglobin IgG Fc fusi on protein expression vector, and then express and purify the soluble PCP-2EC/Fc fusion protein for the study of its function in neuronal adhesion. **Methods**: PCP-2 extracellular region was amplified and cloned into an expression vector pIGplus containing human IgG Fc; PCP-2EC/Fc fusion protein was expressed by COS-7 and 293 cells transfected by the constructed plasmid and purified by protein A. The purified fusion protein was used as substrate to study its function in neuronal adhesion. **Results**: PCP-2 extracellular region was cloned into IgG Fc expression vector successfully; PCP-2EC/Fc fusion protein was expressed and purified in mammal cells; and the purified fusion protein promoted neuronal adhesion. **Conclusion**: PCP-2EC/Fc fusion protein expression system is successfully constructed and the purified fusion protein can promote neuronal adhesion. These results lay a foundation for the research on the PCP-2 function in neuronal adhesion and the further functional study in the nervous system and other fields.

[**KEY WORDS**] PCP-2 extracellular demain; IgG; immunoglobulins, Fc; fusion protein; protein purification

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Protein tyrosine phosphorylation, controlled by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs), is a major mechanism of intracellular signaling and has been demonstrated to be involved in a large set of cellular events, including growth, differentiation, adhesion and migration[1,2]. Pancreatic carcinoma phosphatase 2(PCP-2) is a type Ⅰ transmembrane glycoprotein belonging to PTPs family[3]. The extracellular domain of PCP-2(PCP-2EC) displays Ig-like, fibronectin Ⅲ (FNⅢ), MAM(meprin, A5, PTPmu) motifs that resemble those present in many cell adhesion molecules (CAMs), which lead to speculation that it might be involved in cell-cell or cell-matrix interaction[4,5]. PTPμ and PTPκ, 2 subfamily members of PCP-2, have been demonstrated to mediate cellular adhesion through the CAMs-similar EC domains and play important roles in tumor cell migration and nervous system development[6-8]. In this study, the extracellular region of PCP-2 and the immunoglobin IgG Fc fusion protein was constructed, which lay a foundation for PCP-2 research in neuronal adhesions and further functional study in the nervous system and other fields.

1 MATERIALS AND METHODS

1.1 Materials New-born ICR mice were purchased from Shanghai Laboratory Animal Center; pIGplus vector was kindly provided by Dr. Frank S. Walsh; PBLIISK-PCP-2 was generously provided by Dr. Mitsuo Nishikawa; COS-7 and 293 cells and a polyclonal antibody against PCP-2 were kindly provided by Max-Plank Institute; DMEM and fetal bovine serum were obtained from Gibco BRL. Restriction endonucleases and T_4 DNA ligase were purchased from MBI; Lipofectinamine Reagent was obtained from Invitrogen. Protein A sepharose CL-4B was obtained from Amersham; and Nitrocellulose was obtained from Schleicher& Schuell. The other reagents were obtained from Sigma.

1.2 Construction of PCP-2EC expression vector PBLIISK-PCP-2 was cut by *Xho* Ⅰ and *Kpn* Ⅰ restriction enzymes and about 2-kilobase fragment encoding the 1-2 062 bp of PCP-2 was purified by 1% agarose gels electrophoresis. Then the plasmid was cut by *Kpn* Ⅰ restriction enzyme and the fragment containing PBLIISK-PCP-2Δ (1-2 062) was obtained and ligased. The plasmid PBLIISK-PCP-

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2Δ(1-2 062) was made as template and the fragment encoding the 2 062-2 341 bp of PCP-2 was amplified by PCR method. Sense- and antisense primer were (5′-TTC TCT AGG CGC CGG AAT TC-3′) and (5′-GCT CTA GAC ATC TCC TCC GAT CTC TG-3′), respectively. Reactions were amplified for 35 cycles, with an amplification profile of 94℃ 30 s, 50℃ 50 s, 72℃ 45 s. The PCR product was digested with *Kpn* Ⅰ and *Xba* Ⅰ (Fragment B). pIGplus was cut by *Xho* Ⅰ and *Xba* Ⅰ restriction enzymes and purified by 1% agarose gels electrophoresis (Fragment C). Fragment A, B and C were mixed and linked by T_4 DNA ligase. The recombinant plasmid, named pIGplus-PCP-2EC, was obtained after transformation and confirmed using *Xho* Ⅰ/*Xba* Ⅰ restriction cleavage and DNA sequencing consecutively.

1.3 Expression of PCP-2EC/Fc fusion protein COS-7 cells were transfected by the recombinant plasmid with Lipofectinamine and the provided protocol. COS-7 cells (2×10^5) were plated into 6-well culture plates on the day before transfection. When the plated cells were confluent by 50%-80%, transfection was performed with 0.8 μg pIGplus-PCP-2EC and 2 μl lipofectin per dish. After 24 h, the medium was replaced with serum-free DMEM. Conditioned medium was collected after 5-6 d, centrifuged to remove cell debris, adjusted to pH 8.0 with Tris base, then bound to 20 μl Protein A sepharose. Expression of the PCP-2EC/Fc fusion protein was detected by SDS-PAGE and Western blot with anti-PCP-2.

1.4 Purification of PCP-2EC/Fc fusion protein 293 cells were stably transfected with Lipofectinamine, and were selected with 500 μg/ml G-418. Established cell lines were then screened by Western blot with anti-PCP-2. High-expressing clones were then cultured in large scale with serum-free DMEM. Spent supernatants were harvested, and PCP-2EC/Fc fusion protein was purified by protein A affinity chromagraphy over a 1 ml Protein A sepharose CL-4B column. Fusion protein was eluted from the column as 0.5 ml fractions in 0.1 mol/L citrate buffer (pH 2.7) and neutralized using 50 μl of 1 mol/L Tris-HCl (pH 9.0). Eluted fractions were assayed for absorbance at 280 nm, and fractions containing fusion protein were pooled, dialyzed overnight in several liters of PBS (pH 7.4), and filter sterilized through 0.2 μm syringe filter units.

1.5 Neuronal adhesion assay[9] A total of 0.1 ml Nitrocellose were rapidly spread over the surface of 35 mm culture plates and allowed to dry under a flow hood. Test protein samples, PCP-2EC/Fc (50 μg/ml), Laminin-1 (20 μg/ml), Poly-*D*-Lysine (100 μg/ml) and IgG (200μg/ml) were applied in 2 μl droplets. After 1 min, the droplets were removed by aspiration and the substrate plates were then blocked twice with 1% BSA/DMEM. Cerebellar cells from postnatal day 6 mice were prepared by mechanical dissociation with fire-polished Pasteur pipettes and added to substrate plates in 5×10^5 in 2 ml 10% FBS/DMEM. After 2 h, cell debris and unattached cells were washed off and attached cerebellar cells were measured under micrography. The measurements were analyzed by ANOVA and SNK tests.

2 RESULTS

2.1 Construction of PCP-2EC expression vector

To avoid mismatch bases in long-length PCR and sequencing, we undertook the cloning of PCP-2 extracellular domain by cutting into 2 fragments: PCP-2 1-2 062 (Fragment A) was obtained from PBLIISK-PCP-2 as a 2 162 bp *Xho* Ⅰ/*Kpn* Ⅰ restriction fragment; PBLIISK-PCP-2Δ(1-2 062) is cut by PBLIISK-PCP-2 with *Kpn* Ⅰ and used as template to amplify the PCP-2 2 062-2 341, then digested with *Kpn* Ⅰ and *Xba* Ⅰ (Fragment B). The 2 fragments were then subcloned into the pIGplus expression vector to construct the recombinant plasmid: pIGplus-PCP-2EC. The plasmid was confirmed using *Xho* Ⅰ/*Xba* Ⅰ restriction cleavage (Fig 1) and DNA sequencing consecutively.

2.2 Expression of PCP-2EC/Fc fusion protein PCP-2EC/Fc fusion protein consists of extracelluar domain of PCP-2 and the Fc region of human IgG (hinge CH2-CH3) that makes the fusion protein easily secreted to the supernatants and easily purified using Protein A affinity chroma-

graphy. To express the fusion protein, COS-7 cells were transiently transfected by pIGplus-PCP-2EC with Lipofectinamine. They were collected 3-5 d after cultured with serum-free DMEM, then bound overnight with Protein A Sepharose. Analyzed by Western blot, anti-PCP-2 antibody detected a band of 140 000, which is the expected molecular weight of the PCP-2EC/Fc run under reducing conditions (Fig 2).

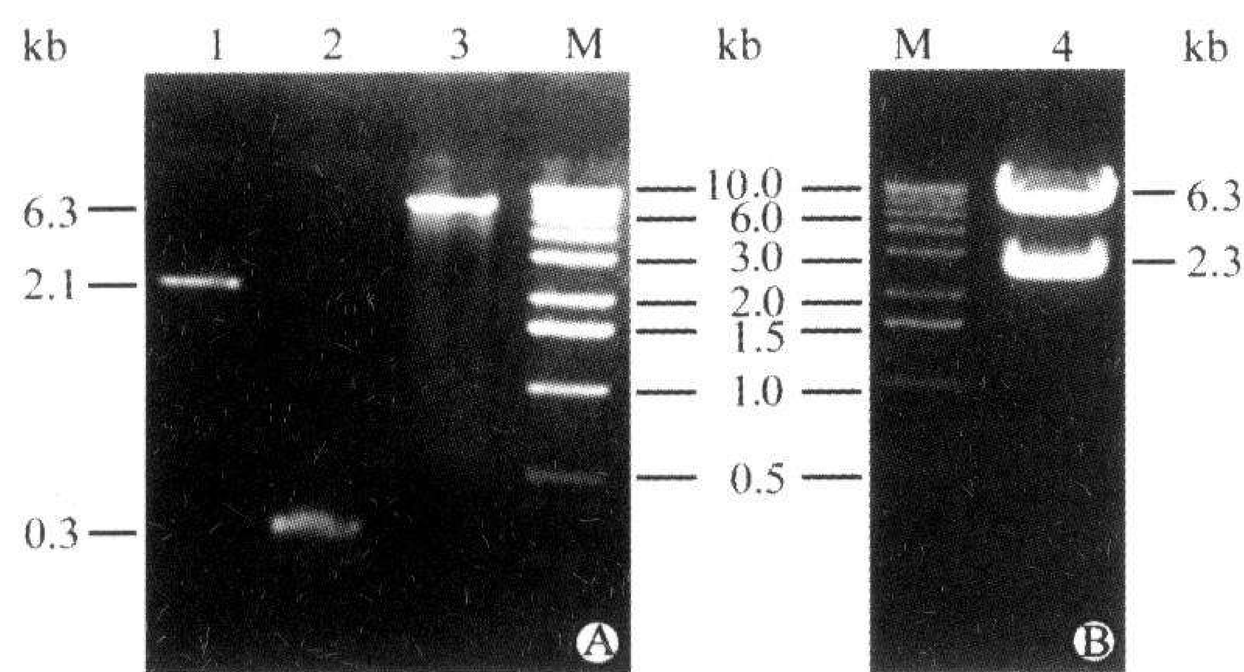

Fig 1　Gel electrophoresis of PCP-2EC cloning and recombinant plasmid pIGplus-PCP-2EC

A: Gelelectrophoresis of PCP-2EC cloning; B: Gel electrophoresis of pIGplus-PCP-2EC restriction enzyme digestion; 1: PCP-2(1-2 062), 2 kb; 2: PCP-2(2 062-2 341), 280 bp; 3: pIGplus, 6.3 kb; M: DNA marker(Generuler Plus, 1 kb); 4: pIGplus-PCP-2EC (*Xho* I + *Xba* I)

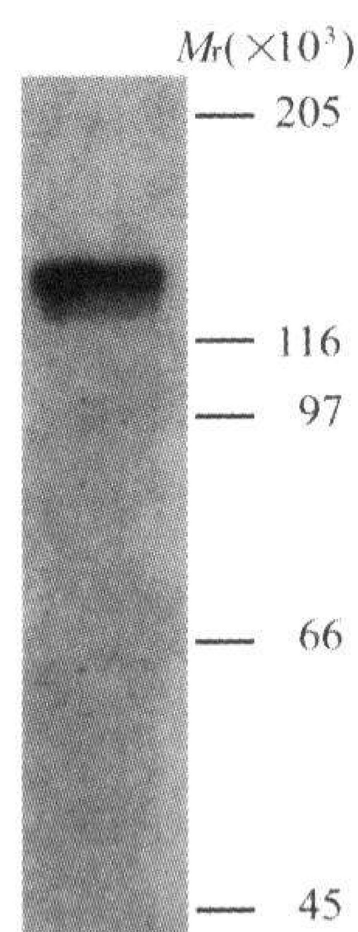

Fig 2　Western blot of protein expressed by COS-7 cells(α-PCP-2 NT)

2.3　Purification of PCP-2EC/Fc fusion protein　For the further functional study of PCP-2, we need to produce a large amount of purified PCP-2EC/Fc fusion protein. First, stably transfected 293 cell lines were established and large-scale cultured with serum-free DMEM to express the fusion protein. Supernatants were harvested, and PCP-2EC/Fc fusion protein was purified by protein A affinity chromagraphy. SDS-PAGE and Western blot showed that the purified PCP-2EC/Fc was measured at the concentration of 100 μg/ml or so (Fig 3).

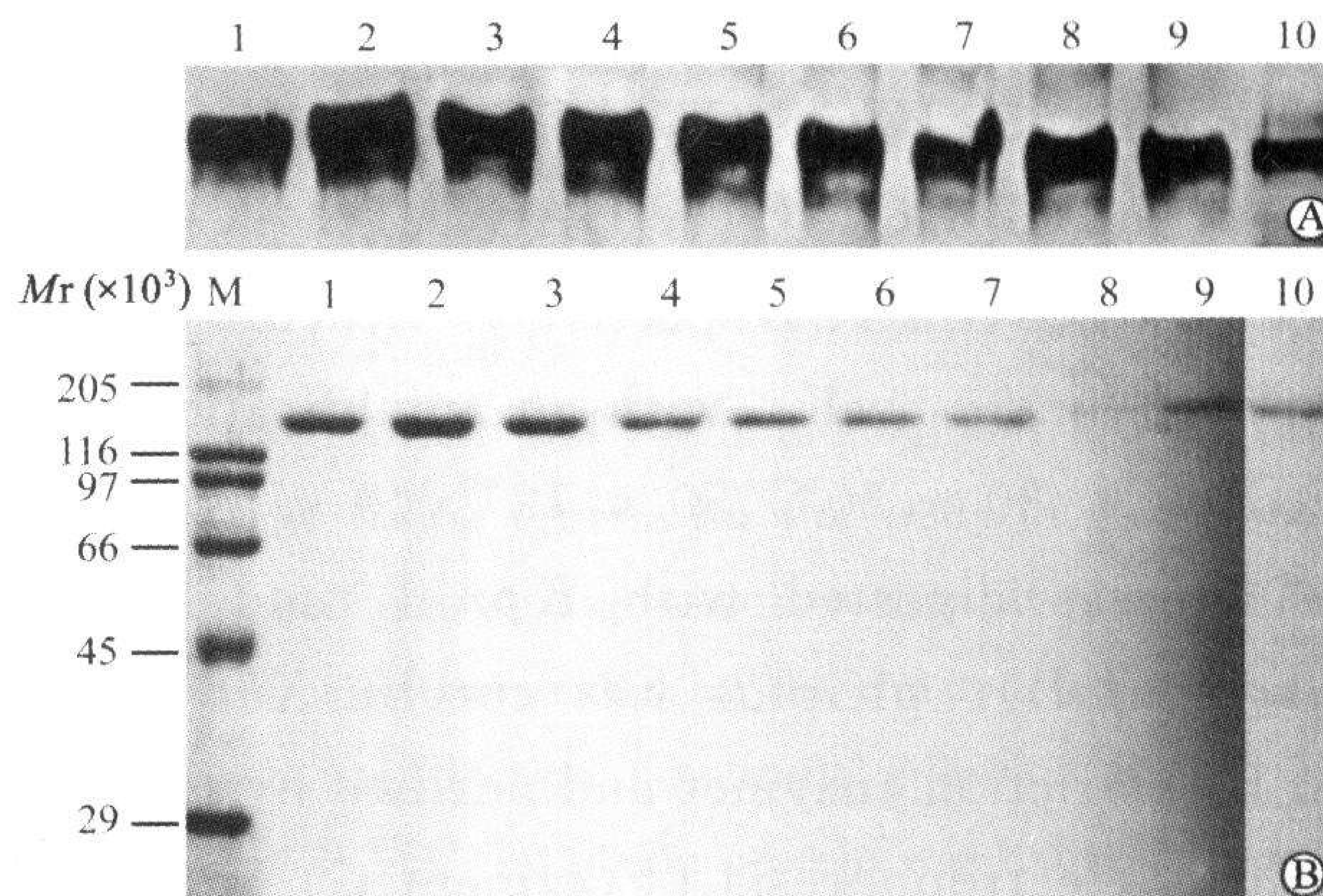

Fig 3　Purification of PCP-2EC/Fc fusion protein

A: Western blot of purified PCP-2EC/Fc protein(1-10); B: SDS-PAGE of purified PCP-2EC/Fc protein(1-10); M: Marker

2.4　PCP-2EC/Fc promotes neuronal adhesion　PTPμ and PTPκ, 2 subfamily members of PCP-2, are homophilic cell adhesion molecules and have been indicated to promote neuronal adhesion and neurite outgrowth as both purified molecules and soluble Fc fusion protein[8,10,11]. Fig 4 shows representative examples of neuronal adhesion in the presence of the PCP-2EC/Fc, suggesting that the PCP-2EC/Fc promoted neuronal adhesion, similar to other neuronal adhesion molecules Laminin-1 and Poly-*D*-Lysine. Control experiments were conducted to rule out nonspecific effects and/or effects from the Fc part of the protein. In this context, the IgG had no effect on neurite outgrowth, and the function of PCP-2EC/Fc was not associated with the Fc part of the protein.

3　DISCUSSION

Prof. WANG *et al*[3] has previously identified a human protein tyrosine phosphatase in human pancreatic adenocarcinoma cells. PCP-2 contains CAMs-like extracellular domain and phosphatase-activity intracellular domain. This combination gives PCP-2 the structural base to directly couple extracellular adhesion mediated events to intracellular signaling pathways in cell-cell or cell-matrix interaction[4,5]. PTPμ and PTPκ, 2 subfamily members of PCP-2, are homophilic cell adhesion molecules and have been demonstrated to promote neu-

ronal adhesion and neurite outgrowth in substrates as both purified molecules and soluble Fc fusion protein[8,10,11].

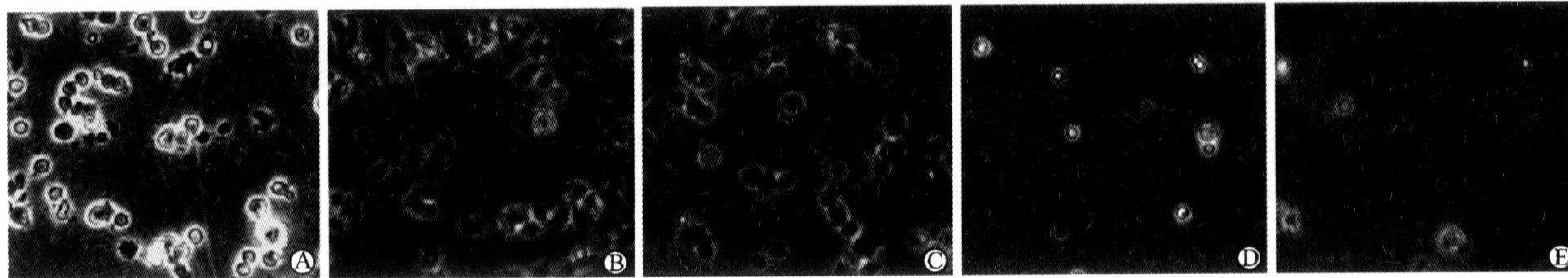

Fig 4 Detection of neuronal adhesion condition on different substrates(×200)

A:PCP-2EC/Fc(50 μg/ml);B:Laminin-1(20 μg/ml);
C:Poly-*D*-Lysine(100 μg/ml);D:IgG(200 μg/ml);E:Blank control

A major goal of this study was to construct the extracellular region of PCP-2 and an immunoglobin IgG Fc fusion protein. To avoid mismatch bases in long-length PCR and sequencing, we undertook the cloning of PCP-2 extracellular domain by cutting it into 2 fragments, then subcloned into the eukaryotic expression vector pIGplus containing the Fc region of human IgG (hinge CH2-CH3). And the PCP-2EC/Fc fusion protein were expressed in mammal cells and purified by protein A affinity chromagraphy.

To study PCP-2 function in the nervous system, we used the PCP-2EC/Fc fusion protein as substrate to culture the cerebellar cells from postnatal day 6 mice. PCP-2EC/Fc promoted neuronal adhesion, similar to other neuronal adhesion molecules Laminin-1 and Poly-*D*-Lysine, and the function of PCP-2EC/Fc did not come from the Fc part of the protein. The results indicate that PCP-2, as a neuronal adhesion molecule, may play an important role in maintaining the function of normal nerves and promoting the regeneration of injured nerves.

In conclusion, PCP-2EC/Fc fusion protein expression system was constructed successfully. The primary study indicated that the purified fusion protein promoted neuronal adhesion. These results lay a foundation for the research of the functions of PCP-2 in neuronal adhesion and the further functional study in the nervous system and other fields.

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PCP-2胞外区/Fc融合蛋白的真核表达、纯化及其生物学活性鉴定

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[摘要] **目的:**构建PCP-2胞外区(PCP-2EC)与人免疫球蛋白IgG Fc融合蛋白表达载体,在哺乳动物细胞中表达并纯化PCP-2EC/Fc蛋白,研究其在神经细胞黏附中发挥的作用。**方法:**以PBLIISK-PCP-2为模板扩增PCP-2EC片段,定向插入真核表达载体pIGplus;重组质粒分别转染COS-7细胞和293细胞,可溶性表达PCP-2EC/Fc融合蛋白,并通过金葡菌蛋白A特异性纯化;以此融合蛋白作为基质,观察原代培养神经元的黏附情况。**结果:**成功构建PCP-2EC/Fc融合蛋白表达载体,表达载体的构建与预期设计相符;进一步在哺乳动物细胞中表达并纯化PCP-2EC/Fc蛋白;PCP-2EC/Fc蛋白可以促进原代培养神经元的黏附作用。**结论:**本研究成功地构建了PCP-2EC/Fc融合蛋白真核表达载体,获得有活性的PCP-2胞外区可溶性分子,初步研究发现其在神经细胞黏附中发挥促进作用,为后续神经及其他领域中的功能研究奠定基础。

[关键词] PCP胞外区;IgG;免疫球蛋白类,Fc;融合蛋白;蛋白纯化
[中图分类号] Q 784 [文献标识码] A [文章编号] 0258-879X(2005)03-0300-05

·个案报告·

胆管丛状神经纤维瘤一例报告

Plexiform neurofibroma in biliary tract: a case report

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[关键词] 胆管肿瘤;神经纤维瘤,丛状
[中图分类号] R 735.8 [文献标识码] B [文章编号] 0258-879X(2005)03-0304-01

1 临床资料 患者,男性,55岁。因"胆管损伤再次手术后反复发热、黄疸4年,复发1周"于2003年12月入院。患者于4年前因胆囊息肉在外院行腹腔镜胆囊切除术,术后因黄疸再次手术探查诊断为"胆总管误扎损伤",行胆总管端端吻合术,吻合口放置支撑管半年后拔除。之后反复出现畏寒、发热、黄疸,不伴有腹痛、皮肤瘙痒、白陶土样便。经过抗感染、利胆等治疗可以缓解。1周前类似症状复发。即往有高血压病史10年,肾结石病史3年。体检:皮肤巩膜轻度黄染,右上腹轻压痛,肝脏未扪及,脾脏肋下未扪及。辅助检查:TB 43.7 μmol/L,DB 35 μmol/L,ALT 87 U/L,AST 63 U/L,γ-GT 434 U/L,AKP 100 U/L。乙肝三系及甲、丙肝抗体检查阴性。AFP及CEA检查阴性。CA19-9 435.2 U/L。CT示:"胆囊术后,肝内胆管扩张"。核磁共振胆胰管成像提示:"胆囊切除术后,肝总管与胆总管交界处一局限性狭窄"。术前诊断:高位胆管狭窄。治疗:行剖腹探查,术中扪及肝总管与胆总管交界处有约1 mm×0.8 mm大小肿块,见有黑色丝线样线头,似硬质瘢痕形成,继续分离显露肝门的肝总管及左右胆管,切开狭窄处予胆道探子探查未发现近端明显狭窄,远端未扪及结石,切除狭窄肝总管,胆总管远端缝闭,近端肝总管空肠Roux-en-y吻合及胆道支撑管引流术。手术恢复顺利。手术切除病灶胆管大体:胆总管下端长1.7 cm,周径1.5 cm,可见2个灰白色结节,直径分别为0.6 cm及0.5 cm。镜检:胆管被覆上皮无异型,壁内见多枚结节状肿物,瘤细胞长梭形,胞质淡红染,核呈波浪状,呈旋涡状排列。免疫组化标记结果:VI(+)、CD57(+)、S-100(+)、MBP(-)、NGFR(+),病理诊断结果:(肝总管下端)丛状神经纤维瘤。

2 讨 论 丛状神经纤维瘤是周围神经及其末梢的瘤样病变,表现为周围神经的变性、肿胀、增生,是神经纤维瘤的一种特殊类型。神经纤维瘤的分布和位置深浅变化很大,头颈部及浅表软组织者常见,但大而深者,恶变的可能性大。文献报道的胆管神经纤维瘤有胆管扩张或梗阻性黄疸的临床表现,均为手术病理检查明确,但胆管丛状神经纤维瘤未见报道。本例表现为胆管吻合术后的反复畏寒、发热、黄疸,术前影像学检查提示胆管扩张,经手术探查发现胆管狭窄段有残留的黑色丝线及灰白色结节,最终由病理及免疫组化确诊。丛状神经纤维瘤的病因尚不明确,本病例的病因可能与前次手术中胆管吻合方式及吻合采用的材料有关。我们认为在胆管吻合手术中应遵循黏膜对黏膜的吻合方式,同时尽可能采用可吸收线进行吻合,如需放置内支撑管应选用合适的口径。这有助于预防吻合口不同组织间的反应性增生及线头的长期刺激,减少丛状神经纤维瘤的发病机会。胆管丛状神经纤维瘤比较罕见,术中病理学检查是必要的,手术完整切除是惟一有效的治疗手段,恶变者手术后易复发。

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