doi: 10.13241/j.cnki.pmb.2015.05.010

脐带间充质干细胞诱导分化为类许旺细胞的实验研究 *

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摘要 目的：探讨通过化学诱导的方法，诱导脐带间充质干细胞分化为类许旺细胞，为组织工程神经寻找一种新的种子细胞来源。**方法**：通过酶消化法，分离获得原代脐带间充质干细胞。体外培养至第 3 代(P3)，以 1× 10^3/cm^2 接种于培养瓶中，待细胞长至亚融合状态，吸弃培养液，加入含 β- 巯基乙醇的培养液预诱导 24 h。然后，加入含有全反式维甲酸的培养液进一步诱导 72 h。最后，加入含有胶质细胞生长因子的培养液，作用两周。对诱导后的脐带间充质干细胞用免疫荧光及 RT-PCR 法，进行形态观察和表型鉴定。**结果**：经过上述诱导过程，脐带间充质干细胞的形态，由开始时的扁平、片状、多极，类似成纤维细胞状，逐渐变为长梭形，双极或三极，类似许旺细胞。同时，表达许旺细胞特异性标志 S-100、P75；而且 S100，P75 的 mRNA 水平也明显上调，并且可以观测到 GFAP 的 mRNA 条带。**结论**：化学诱导法，可以将脐带间充质干细胞诱导分化为类许旺细胞，有望为组织工程神经提供一种新的许旺细胞来源。

关键词：脐带间充质干细胞；类许旺细胞；诱导；胶质细胞生长因子

中图分类号：Q813；R318 **文献标识码**：A **文章编号**：1673-6273(2015)05-834-05

Differentiation of Human Umbilical Cord Mesenchymal Along a Schwann Cells Lineage*

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ABSTRACT Objective: To explore the potential differentiation of Schwann cells from human umbilical cord mesenchymal stem cells under the induction of chemical agents, which may provide a new source of seed cells for neural tissue engineering. **Methods:** The primary human umbilical cord mesenchymal stem cells were harvested by digestion and isolation. After the 3rd passage, the cells were plated at 1× 10^3/cm^2 in the flask and treated with β-mercaptoethanol for 24h when the cells were subconfluent. Afterwards, the cells were cultivated with all-trans-retinoic acid for 72h and then incubated with glial growth factors for two weeks. Then the morphology and phenotype of induced umbilical cord mesenchymal stem cells were identified through immunocytochemical staining and RT-PCR. **Results:** The primary umbilical cord mesenchymal stem cells were flat, platy, multipolar like Schwann cells, but shifted to spindle shape, bipolar or tripolar like fibroblast cells. Moreover, the specific markers for Schwann cells, S100 and P75, were positively expressed in the induced cells. The mRNA levels of S100 and P75 also significantly increased along with the expression GFAP. **Conclusion:** Schwann-like cells can be induced from umbilical cord mesenchymal stem cells by induction with chemical agents, which has the potential to be a novel cell source for neural tissue engineering.

Key words: Umbilical cord mesenchymal stem cells; Schwann-like cells; Inducen; Glial growth factors

Chinese Library Classification(CLC): Q813; R318 Document code: A

Article ID: 1673-6273(2015)05-834-05

前言

周围神经损伤，是一种常见疾病[1-4]。神经损伤后较难修复，易造成肢体的残疾[1-4]。长段神经缺损，需要神经移植治疗[5-9]。神经移植体来源有限，且给供体带来新的创伤，因此其应用受到限制。组织工程技术给周围神经损伤的治疗带来了新的希望[5-9]。制约组织工程神经发展的主要瓶颈，是种子细胞来源问题。组织工程神经的主要种子细胞，是许旺细胞[10-12]。许旺细胞同样

* 基金项目：国家自然科学基金项目(30872630)

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（收稿日期：2014-08-22 接受日期：2014-09-19）

存在来源有限,取材带来新的创伤等问题[13]。间充质干细胞具有强大的增殖分化潜能,可以诱导分化为类许旺细胞,解决了组织工程神经种子细胞来源不足的难题[14-21]。脐带间充质干细胞取材容易,来源广泛,且不给供体带来创伤,在增殖分化方面较其它干细胞具有明显优势[22,23],因此我们设计应用化学试剂诱导脐带间充质干细胞分化,以探讨脐带间充质干细胞诱导分化为许旺细胞的可行性。

1 材料和方法

1.1 标本与试剂

实验用脐带均由上海交通大学医学院附属第一人民医院妇产科提供。每例标本采集,均经孕妇知情同意。实验试剂:β-巯基乙醇(Sigma-Aldrich, UK),全反式维甲酸,碱性成纤维细胞生长因子(bFGF, PeproTech Ltd, UK),heregulin-β-1(HRG, Pepro Tech, USA),血小板源生长因子(PDGF,Pepro Tech Ltd, UK),福斯科林(FSK,Sigma, UK),兔抗 S100、P75 多克隆抗体(Abcam,UK),FiTC- 羊抗兔 IgG 多克隆抗体(Santa Cruz,USA),FLUOR555- 羊抗兔 IgG 多克隆抗体(Santa Cruz,USA),荧光封片剂(DAKO.USA)。

1.2 脐带间充质干细胞的分离及培养

无菌下取一段长约 10 cm 脐带,置于肝素钠处理过的试管中。肝素生理盐水溶液反复冲洗脐带,去除附着的血液。用血管钳钝性剥离脐带动静脉,去除血管。然后用眼科剪刀脐带组织剪成碎块,大小约 1 cm^3。将脐带组织碎块分装在 50 mL 的离心管中,加入含 0.1 %酵素酶(dispaseII),0.1% 透明质酸酶(hyaluronidase)和 0.1%复合胶原酶(collagenaseNB4)的混合酶溶液(组织块与酶溶液的体积比为 1:3),置于 37℃ 恒温摇床中。作用约 5 小时左右后,加入悬浮液体积 2 倍的 PBSA,摇匀后,室温下以 600× g 离心 5 min,吸除上清液。用含 10%胎牛血清的 DMEM 培养基重悬脐带组织细胞团块。血细胞计数仪计数所得细胞,按 1× $10^3/cm^3$ 密度接种于细胞培养瓶中,并置于细胞培养箱中培养。培养 72 小时后,通过换液,去除未贴壁的细胞成分。以后每隔 48 小时换液一次。待细胞长至亚融合后,用 0.25%的胰蛋白酶(Trypsin-EDTA)消化传代,按 1:2 接种传代,传至第 3 代(P3)后进行化学诱导实验。

1.3 诱导实验

参考 Dezawa 等方法[4]。即脐带间充质干细胞达到亚融合后,去除 培养液,加入 1 mM β- 巯基乙醇诱导 24 h。去除诱导液,用 PBS 清洗 3 次,加入 35 ng/mL 全反式维甲酸诱导 72 h。去除诱导液,用 PBS 液清洗 3 次,加入含有胶质细胞生长因子的诱导液(DMEM 培养基,10% FBS,55 μM Forskolin、10 ng/ml bFGF、5 ng/ ml PDGF、200 ng/ mL HRG)37℃ 、5 % CO_2 条件下诱导,每 72 h 半量换含胶质细胞生长因子的诱导液一次,诱导 2w。对照组:未经诱导的脐带间充质干细胞。

1.4 形态学观察

倒置相差显微镜下观察脐带间充质干细胞的生长情况,并在β- 巯基乙醇诱导 3 h、全反式维甲酸诱导 72 h 及含胶质细胞生长因子的诱导液诱导 2 w,3 个不同时间点观察细胞的形态变化并拍照。

1.5 免疫细胞化学染色并计数

脐带间充质干细胞经含胶质细胞生长因子的诱导液诱导两周,去除诱导液,PBS 清洗 3 次,加入 4%多聚甲醛溶液,室温固定 5 min;PBS 漂洗 3 次,每次 3 min;分别加入兔抗 S100 抗体、兔抗 P75 抗体,4℃ 孵育过夜;PBS 漂洗 3 次,每次 5 min;分别加入 FITC- 羊抗兔 IgG 抗体,37℃ 孵育 30 min;PBS 漂洗 3 次,每次 5 min。DAPI 核衬染 10 sec, PBS 漂洗 5 min 1 次;荧光显微镜下观察并拍照。

1.6 半定量 RT-PCR 检测

分别取经诱导后的脐带间充质细胞干细胞和 P3 脐带间充质干细胞 3× 10^6 个,用 Trizol 法提取其总 RNA[24]。用 Invitrogen 公司的试剂盒行 RT-PCR 检测。S100、GFAP 及 P75 引物序列利用 PUMBED 在线 primer3 设计,由上海博亚生物公司合成。引物序列分别如下,GFAP 为 Forward primer 5'-TGAGGGAGATTGGATTCTGG-3',Reverse primer 5'CCAGGCTCTTCTGAGGACAC-3',PCR 产物序列长度 526 bp;S100β 为 Forward primer 5'-CAGGAATTCATGGCCTTTGT-3',Reverse primer 5'-TGCGAGTTCTGATGGAGTTG-3',PCR 产物序列长度 417 bp;P75 为 Forward primer 5'-CCAGCTCTAGACAACCCTGC-3',Reverse primer 5'-ACACCCAGACTCTGTCCCAC-3',PCR 产物序列长度 337 bp.PCR 反应条件为 GFAP:95 ℃,43 s;55 ℃,40 s;72 ℃,1 min,共 35 个循环;S100β:95 ℃,43 s;58 ℃,1 min;72 ℃,1 min,共 35 个循环;P 75:95 ℃,43 s;58 ℃,1 min;72 ℃,1 min,共 35 循环。扩增产物在 4 ℃ 下,以 2 %的琼脂糖凝胶电泳 30 min,紫外灯观察并照相。

1.7 统计学处理

计数值以均数± 标准差表示,率用%表示,组间比较用 t 检验。用 SPSS10.0 统计软件分析,P<0.05 认为差异有统计学意义。

2 结果

2.1 形态学变化情况

细胞种植 3 h,就见少量细胞贴壁,24 h 见大量形态各异的细胞散在分布。约一周左右,贴壁的细胞有集落形成。集落周围细胞形态较散在细胞形态均一,呈梭形,双极或三极。经传至,第 3 代时细胞形态变的均一,为梭形或扁平形的成纤维细胞样细胞,核大居中。经β- 巯基乙醇诱导 3 h,大部分细胞胞体发生收缩,体积变小,胞体折光性增强,少部分细胞飘起。经全反式维甲酸诱导 72 h,细胞形态大多呈梭形,双极或三极,胞体折光性好,少量胞体收缩成较细长突起,同时细胞开始大量增殖,细胞数目增多。经含胶质细胞生长因子的诱导液诱导 2 w 时,细胞生长达到融合状态,排列密集呈栅栏状,胞体瘦长呈梭形,多为双极,类似许旺细胞。对照组细胞排列紧密,呈片状,形态基本无变化。

2.2 免疫细胞化学染色结果

经过上述方案诱导 2w,80%左右的细胞呈长梭形类似许旺细胞,P75 阳性率达到 90.5± 1.5%,S100 阳性率达到 60.4± 3.7%。对照组虽然有部分细胞呈长梭形,P75 阳性,阳性率约 3± 0.3%,但均不表达 S100,S100 阳性率为 0.诱导组的 P75 和 S100 的阳性率都显著高于对照组,差异有统计学意义(P< 0.05)。

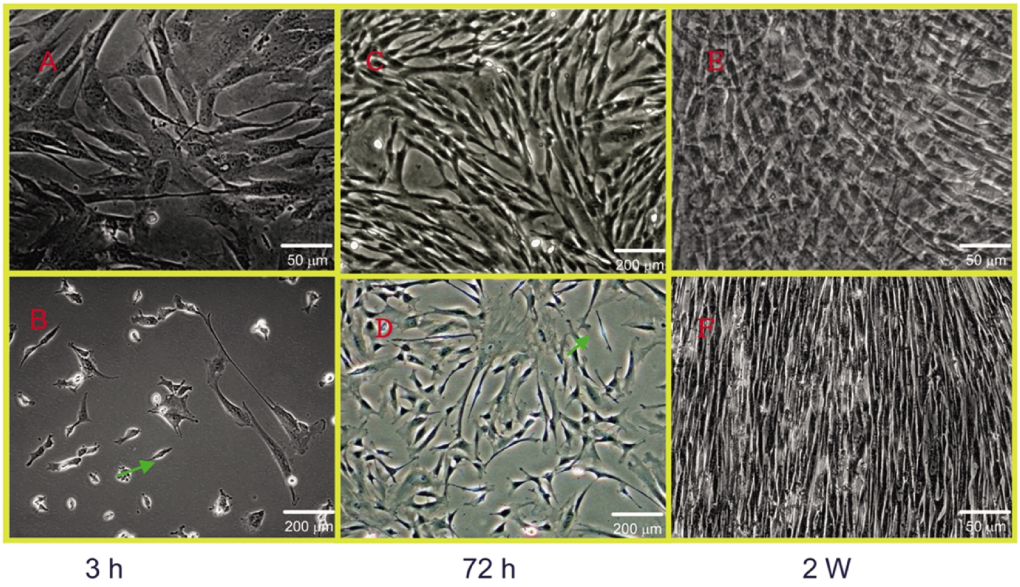

图 1 化学试剂诱导后细胞形态变化

Fig. 1 Morphological changes after chemicals induction

A: Control; B: the majority of cell body constricted and the halos were appeared at nucelus as green arrow pointed at after inducted by β-mercaptoethanol for 3h (x10); C: Control. D: Some of the cells were spindle-shaped with better refractivity after indution by all-trans retinoic acid for 72h as the green arrow pointed at; E: Control (x40) F: The cells were arranged in palisade-like polarity and most of the cell bodies were slender and spindle-shaped (× 40).

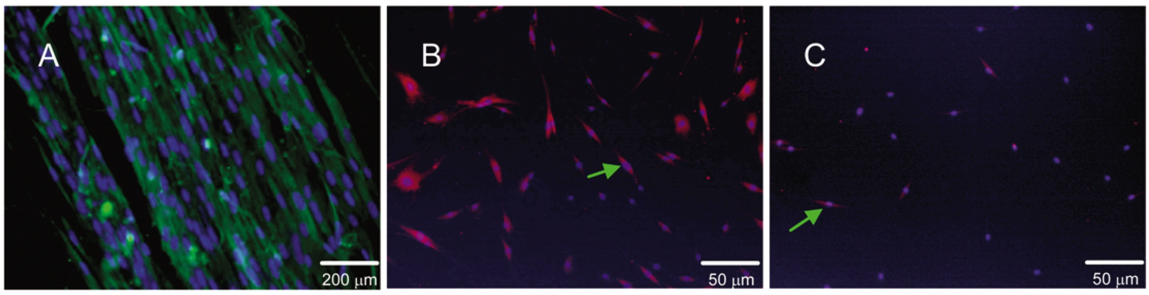

图 2 许旺细胞特异性标记鉴定

Fig. 2 Identification of Schwann cell by specific markers

A: When induced 2w, P75 marker, more than 90% cells were spindle-like, palisade-like arrangement as Schwann cells. B:After induction of 2w, digested from the Transwell membrane and re-planted in 6-well, cells marked by p75 were red, spindle-like, bipolar (as green arrows);C:After induction of 2w, digested from the Transwell membrane S100 were detected, some cell bodies were red (S100 positive), bipolar and spindle-like. Cell Nucleus were staining by DAPI. (× 40).

2.3 半定量 RT-PCR 检测结果

诱导前后 RT-PCR 检测示第 3 代脐带间充质干细胞 S100,P75 均有表达,但是诱导后表达明显升高。诱导前 GFAP 不表达,诱导后表达 GFAP。

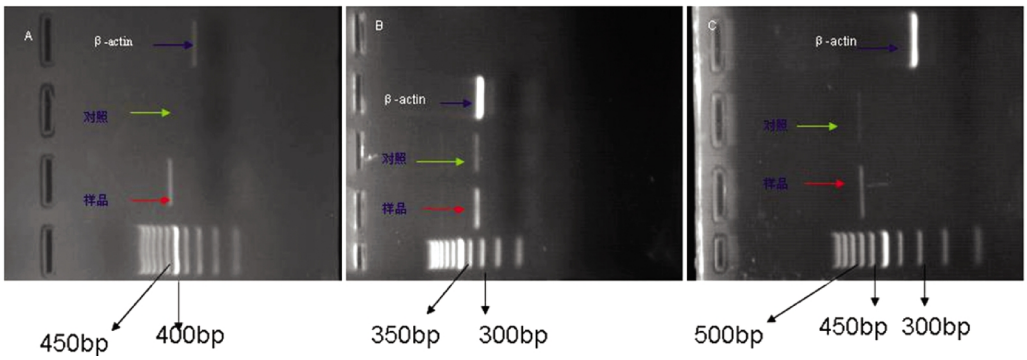

图 3 化学诱导后许旺细胞特异 RNA 水平表达情况

Fig. 3 Schwann cell specific expressions at RNA level after chemical agent treatment

A: Although S100 were positive before and after induction, but significantly increased after induction. B: The cells expressed P75 before and after induction, but increased signicantly after chemical agent treatment .C: Before induction, GFAP was not detected, but after induction, it was detected. The size ladder is 50bp.

3 讨论

骨髓、脂肪及毛囊等组织中含有大量成体间充质干细胞[25-27]，可以在体外通过化学诱导的方法分化为类许旺细胞，促进神经细胞轴突的增长[27-31]。成体干细胞取材时给病人带来痛苦、易造成供体部位感染，而且成体间充质干细胞随着供着年龄的增加其增殖分化潜能不断降低，所以成体干细胞作为周围神经种子细胞仍然存在着极大局限性[32,33]。为寻找新的种子细胞来源，我参考 Dezawa 等报道的诱导方案诱导脐带间充质干细胞向类许旺细胞分化[34]。诱导两周后，脐带间充质干细胞由成纤维细胞样细胞转变为瘦长双极，极性分布栅栏样排列的许旺细胞样细胞。免疫组织化学染色，表达 P75，S100。RT-PCR 鉴定发现，经过诱导后，S100，P75 表达明显上调。从蛋白和分子 mRNA 两种水平验证了脐带间充质干细胞诱导分化为许旺细胞的可行性。避免了 LU 等提出的诱导细胞的蛋白标记上调是由于化学试剂破坏了细胞的蛋白结构引起的争议[35]。

脐带间充质干细胞来源充足，并且已有大量文献报道，脐带间充质干细胞表达间充质干细胞特异性标记，可以诱导分化为脂肪，骨、软骨、神经元及胶质等细胞。但是其是否可以诱导分化为许旺细胞尚未见诸报道。因脐带间充质干细胞较之成体干细胞具有更加强大的增殖分化潜能，且免疫原性比较低，有成为组织工程神经种子细胞的巨大潜力。另外，我们在研究中发现，脐带间充质干细胞诱导前在 mRNA 水平上也表达 S100，P75 基因，而 P75 和 S100 是许旺细胞特异性的标记，所以脐带间充质干细胞中有可能存在许旺细胞的前体。所以脐带有望成为组织工程神经种子的新来源。

综上所述，我们的研究表明脐带间充质干细胞可以诱导分化为类许旺细胞，表达许旺细胞的特异性标记。Tohill 等报道经过诱导的骨髓间充质干细胞可以促进周围神经的再生，而未经诱导的间充质干细胞而不具有促进周围神经再生的作用[36]。而 Chen 等报道未经诱导分化的脐带间充质干细胞也能够促进周围神经再生，提高运动神经的功能。这可能是在体内损伤的许旺细胞和神经轴突分泌的因子或信号促使间充质干细胞分化为许旺细胞，促进了周围神经的再生[37]。但是经 / 未经诱导的脐带间充质干细胞对神经轴突的长期作用与转归还不确定，因此我们将继续研究脐带间充质干细胞的分化机制，以期为未来脐带间充质干细胞应用于临床治疗周围神经损伤，提供理论依据，为周围神经组织工程提供新的种子细胞来源。

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