Combined transplantation of mesenchymal stem cell and bFGF contained nanospheres into the injured rat spinal cord

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Directed by Professor Do Heum Yoon

The Master's Thesis
submitted to the Department of Medical Science
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical science

Sung Young Lee

December 2007

This certifies that Master's Thesis of Sungyoung Lee is approved.

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December 2007

ACKNOWLEDGEMENTS

군대에서의 시간이 여기서 석사를 했었던 시간만큼만 빨리 지나 갔었으면 얼마나 좋았을 까라는 생각이 들 만큼 정말 2년이라는 시간이 빨리 지나 간 것 같습니다. 많이 부족하고 저이지만 교수님을 포함한 많은 분들의 도움이 있었기에 이렇게 졸업논문을 완성 할 수 있었습니다.

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같은 실험실에서 오랜 시간을 함께 지내면서 많은 조언과 힘이 되어주신 새침 윤희샘, 80년대 개그 재형이형, 카리스마 용섭이형 저 오고 나서 금방 떠나셨던 후덕한 웃음 진환이형 그리고 동갑내기 친구들인 무뚝뚝 진수, 꽈당 영은 지금은 떠났지만 이쁜동생 선영이, 세대교체를 감행하고 있는 어린 것들인 컴 진영, 정답게 정다운, 차이니스 희진 에게도 감사의 맘을 보냅니다. 저희 실험실과 뗄레야 뗄 수 없는 관계에 있는 전기 생리학 실험실에 만물박사 김은정샘, 헬로 세정누나, 거친입담 명훈이형, 쾌활 발랄 경하누나, 돌아온 아기 엄마 나영 그리고 완전 어린 것 오므라이스 보미 전기생리학방은 아니지만 마치 그런 것처럼 보인 동갑내기

들인 완선 지은, 동방신기 예림, 총무 수진 그리고 은희 누님 아! 지금은 떠나고 없는 은진이와 지수형 이제 저 졸업해요! 그동안 정말 감사 했습니다. 세포치료실에서 항상 챙겨 주느라 고생하셨던 최유정 선생님과 지금은 아주대에 가 있는 경운이, 세포치료실에서 만날 때마다 반갑게 인사 해주시면서 많은 조언을 해주신 김일선샘, 318호에 경민이 형과 용광이형 그리고 인옥누나, 상헌이, 연정이 그리고 추운 고시원 방에서 갈 곳 없는 날 받아준 룸메이트 민호 고맙다. 마지막으로 대학교 때부터 거의 10년 째 집 나가 사는 막내아들 때문에 맘고생 몸고생 하신 우리 어머니와 막내동생 챙겨주느라 고생한 첫째형, 둘째형, 셋째형, 첫째 형수님, 둘째 형수님 모두 정말 진심으로 감사드립니다.

처음 고등학교 때 좋아했던 생물이란 과목이 이제는 단순히 좋아하는 과목이 아닌 제가 평생 공부를 해야되는 학문이 되었습니다. 뛰어나진 않지만 서두르지 않고 천천히 최선을 다하여서 우리나라 과학발전에 밑거름이 될 수 있는 사람이 되도록 노력을 하겠습니다.

석사 생활 뿐만 아니라 저의 모든 생활을 보호 해주시고 인도해 주신 하나님께 감사드리며 글을 마칩니다.

2008년 2월 이성영 드림

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Combined transplantation of mesenchymal stem cell and bFGF contained nanospheres into the injured rat spinal cord

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Currently, there has been no effective regenerative therapy for spinal cord injury. One of the emerging strategies for regenerating damaged neural tissue is the implantation of stem cells. The use of bone marrow stem cells(MSCs) in cell therapies may have some advantages over the use of other sources of cells including autologous transplantation. But the nave MSCs transplantation has some limitations including cell survival, migration and differentiation. The aim of this study was to investigate whether the transplantation of human MSCs with PLGA nanospheres that secrete bFGF into the contused spinal cord reconstitutes an axonal growth promotion and tissue sparing after spinal cord injury.

We transplanted human mesenchymal stem cell(hMSC) and bFGF-PLGA nanospheres into the moderate contused SCI model simultaneously.

bFGF-HCPNs have stimulating proliferation effect of hMSCs. hMSCs transplantation with bFGF-HCPNs increased neurofilament postive fibers in epicenter of injured spinal cord, survival of transplanted hMSCs and inhibited growth of the astrocytes.

Key words: spinal cord injury, mesenchymal stem cell, bFGF, nanosphere

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Ⅰ. INTRODUCTION

Because spinal cord injury(SCI), one of the central nervous system(CNS) injury, is irreversible, it has known hardly to regenerate injured spinal cord[1]. Frequently, primary injury of the acute spinal cord injury caused by trauma was induced by physical force. For example, out of joint, acute spinal compression or laceration in human. In animal, primary injury induced by impactor rod or pressure. Secondary injury is known that it causes pathological, morphological and neurophysiological damages in spinal cord such as hemorrhage, edema, cell death, demyelination and, decrease of the ATP level[2].

The CNS injury has limited capacity to regenerate injured neurons. Although remarkable development of treatment and rehabilitation of SCI has achieved, it has been impossible to treat perfectly as neuronal tissue could not be regenerated. Recently, operative and medical treatment is applied to prevent secondary injury rather than fundamental treatment.

Cell transplantation can be a very effective method to treat SCI. During past few years, many study for the `regeneration of the neural tissue' have accomplished

through the cell transplantation method[3-5]. Although stem-cell therapy has been thought as a method that it has possible to treat SCI in compliance with more fundamental treat than cell therapy, it also has limited to treat SCI.

Therefore SCI treatment need to variety methods such as combined treatment. Basic fibroblast growth factor(bFGF) can be use for treatment of the injured axon as one of the complex treatment elements. In vivo, bFGF has neuroprotective effect to motor neurons from death following spinal cord contusion injury[6,7]. And bFGF promotes axonal regeneration(Romero et al.,2001) in the injured adult spinal cord.

In previous study of our group, intrathecal infusion of bFGF to the subarachnoid space of injured spinal cord showed new protective effect[8]. So this study was performed for investigating the effect of combined transplantation (hMSCs and bFGF- HCPNs) to the injured spinal cord.

Ⅱ. MATERIALS AND METHODS

1. Separation and culture of hMSCs

Human bone marrow blood was obtained from the iliac crest of patients during spinal fusion surgery using sterile syringes aspiration. All samples were collected after written informed consent was procured.

MSCs were isolated by non-selective flask adherent method following collection of the layer fraction coat from a ficoll gradient. Brefly, 10ml of bone marrow blood aspirate was combined with 1XHBSS(ratio 1:1), gently onto a ficoll plaque and centrifuged at 800g for 30min. White layer(the low density mononuclear layer) was collected, washed twice with 1XPBS, then resuspended in DMEM culture medium, 10%FBS. When the cultures reach confluency, approximately after 2-3weeks, the cells are harvested with 0.05% w/v trypsin and 0.02% w/v EDTA in PBS(pH;7.4) for 5min at 37℃, replate and cultured once again for 2weeks, then harvested.

2. Preparation of bFGF conjugated HCPNs (heparin- conjugated PLGA nanospheres)

PLGA copolymer was prepared by ring-opening polymerization of L-lactide and glycolide. L-lactide(100mmol), glycolide(100 mmol), stannous octoate(1mmol), and 1,6-hexanediol(0.5mmol) were weighed into a glass ampoule equipped with a magnetic stirring bar. To synthesize PLGA-6OH, dipentaerythritol(0.5mmol) was used instead of 1,6-hexanediol. The ampoule was sealed under vacuum after purging three times with nitrogen at 90℃, and was immersed in an oil bath at 150℃ for 24 h with stirring. After the reaction, the polymer was dissolved in chloroform, filtered through a 0.45㎛ pore membrane filter, precipitated by pouring the polymer solution into an excess of methanol, and dried under a vacuum. Various types of PLGA were synthesized. The ^{1}H-NMR spectra of the polymers

were recorded using Varian Unity-300(300MHz) and Unity-600(600MHz) NMR spectrometers. The trimethlysilane signal at δ = 0.0 ppm was used as a reference line.

Amino-terminated PLGA was synthesized in a two-step reaction utilizing standard carbodiimide chemistry. PLGA(1mmol) was reacted with *N-t*-Boc-glycine(10mmol) and 4-dimethylamino pyridine (DMAP, 3mmol) in methylene chloride(CH_2Cl_2, 20ml). Subsequently, 10mmol of N,N0- dicyclohexylcarbodiimide(DCC) was added, and the reaction mixture was stirred at 4℃ for 24h. The dicyclohexylurea(DCU) was filtered, and the filtrate was concentrated in vacuum at room temperature. The product was dissolved in acetone(20ml) and cooled overnight. The precipitated DCU was filtered off. For the removal of the *t-Boc* group, the PLGA–*t-Boc*-amino acid diester was dissolved in a mixture of CH2Cl2/trifluoroacetic acid(10ml/10mlA). The reaction mixture was stirred for 3h and then precipitated with ethanol. The polymer was collected, dissolved in methylene chloride, and reprecipitated with ethanol. The chemical structure and the completeness of deprotection were verified with 1H-NMR. The disappearance of the chemical shift at δ = 1.4 ppm in the NMR spectra suggested the complete removal of the *t-Boc* group.

Amino-terminated PLGA nanospheres were prepared using the oil/water emulsion and solvent evaporation–.extraction method. In brief, 6g of amino-terminated PLGA in 60ml methylene chloride was added dropwise to 500ml of aqueous 3%(w/v) polyvinyl alcohol (M_w30,000–70,000) and emulsified for 10 min using a sonicator(60W,Sonic Dismembrator 550). After overnight evaporation at room temperature, the nanospheres were collected by centrifugation at 14,000rpm for 10min, washed five times with distilled water, and lyophilized for 2days.

Heparin, having two different molecular weights(Mw 3000, activity < 60 USPU/mg; M_w 15,000, activity: 170USPU/mg; Sigma), was covalently reacted with amino-terminated PLGA nanospheres using a general procedure employing standard

carbodiimide chemistry. Heparin(1g) was dissolved in a buffer solution(pH5.5) of 0.1M 2-morpholinoethanesulfonic acid containing 0.5M NaCl, and amino-terminated PLGA nanospheres(3g) were dispersed in the buffer solution. The solution was filtered through a 0.45㎛ filter to remove aggregates. N-hydroxysuccin imide(6mmol) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodii mide hydrochloride (10mmol) were added into the solution to activate the carboxylic acid groups of the heparin. After overnight reaction at 4℃, the nanospheres were collected by centrifugation at 14,000rpm for 10min, washed three times with sonication in distilled water to remove unreacted material and low molecular weight byproducts, and then lyophilized for 2days.

HCPNs and carrier free bFGF(25㎍/ml) were combined in 0.9% (w/v) saline solution. After 1h, HCPNs were collected by centrifugation at 14,000rpm for 10min[9]. Then resuspended in 200㎕ of the 0.9% saline(25㎍/200㎕).the amounts of the binding bFGF with HCPNs were determined by enzyme linked immunosortion assay kit.

3. Spinal cord injury

Adult male Sprague-Dawley rats(300-350g; Orient bio, kyounggi-do ,Korea) were handled according to US national institutes of health and USDA guidelines. All animal experiments were approved by the institutional Animal Care and Use Committeeof the Yonsei University College of Medicine.

Acute spinal cord injury(SCI) model was made by using Infinite Horiaontal impactor (Precision Systems, Lexington, KY). Adult male Sprague-Dawley rats were anesthetized with pentobarbital (50mg/kg,i.p) and opened at T9 by laminectomy. The opened site was received a contusion injury with a 225kdyne impact power. After SCI, everyday care was applied including bladder squeeze twice per day. Cefazolin (50mg/1kg) was injected for a week after SCI for preventing infection.

4. Transplantation of hMSCs, bFGF-HCPNs.

At 1week after SCI, the rats were anesthetized with pentobarbital (25mg/kg,i.p). $5*10^5$cells($1*10^5$cells/$\mu\ell$ * $5\mu\ell$) of the cultured hMSC, bFGF conjugated HCPNs (bFGF-HCPNs) $5\mu\ell$($25\mu g/200\mu\ell$), combined of bFGF-HCPNs $2.5\mu\ell$ and hMSC $2.5*10^5$cells($1*10^5$cells/$\mu\ell$ * $2.5\mu\ell$) or 1XPBS were injected into the injury site by capillary glass tube. CyclosporineA (1mg/100g) was infused from 2days before trans -plantation to reduce immune response.

5. Immunohistochemistry

Rats were anesthetized with pentobarbital (50mg/kg,i.p) and perfused transcardially with 300ml of cold saline followed 300ml of cold 4% paraformaldehyde in PBS. The spinal cords were removed and immerged for 3h in 4% paraformaldehyde in PBS at 4℃ followed by overnight immersion in 30% sucrose in PBS. The spinal cord was embedded and frozen in Tissue-Tek O.C.T compound at -80℃ for overnight. The spinal cord cut serial longitudinal($10\mu m$) using cryostat and then mounted onto coating slides. the sections stored at 4℃.

The sections were fixed with 50%ethanol for 10min, rinsed with buffer(0.1%BSA, 0.3%triton X-100 in PBS) 3times for 5min each time. the sections were blocked by blocking buffer(10% Normal donkey serum) for 30min to prevent non-specific staining and incubated with primary antibodies for overnight at 4℃. The following primary antibodies were used: anti human nuclei(1:250), anti neurofilament(1:200), anti vimentine(1:150), anti GFAP(1:500), anti p75(1:200) and anti bFGF(1:100). The sections were rinsed again with buffer 3times for 5min each time followed by 15min blocking with blocking buffer(2%normal donkey buffer). and the sections were incubated with secondary antibodies for 3h at 37℃. The following secondary antibodies: Cy3 conjugated anti mouse IgG, FITC conjugated anti Rabbit IgG and FITC conjugated anti goat igG. And the sections were washed with buffer and then with D.W 3times for 5min each time. The sections were mounted on slide glass with a fluorescent mounting medium and observed under a fluorescence microscope

Ⅲ. RESULT

1. hMSCs culture with bFGF-HCPNs

hMSCs were cultured in a medium with bFGF-HCPNs to determine whether bFGF -HCPNs had toxicity to hMSCs and bFGF affects to hMSCs. Because P3hMSCs vigorously proliferate, there are no different between bFGF-HCPNs group and control group at P3hMSCs. From P4, usually hMSCs tend to spread out instead of proliferation. so, we used P4hMSCs and then the result showed that cultured cells with bFGF conjugated HCNPs were more proliferative than with out it.(Fig.1)

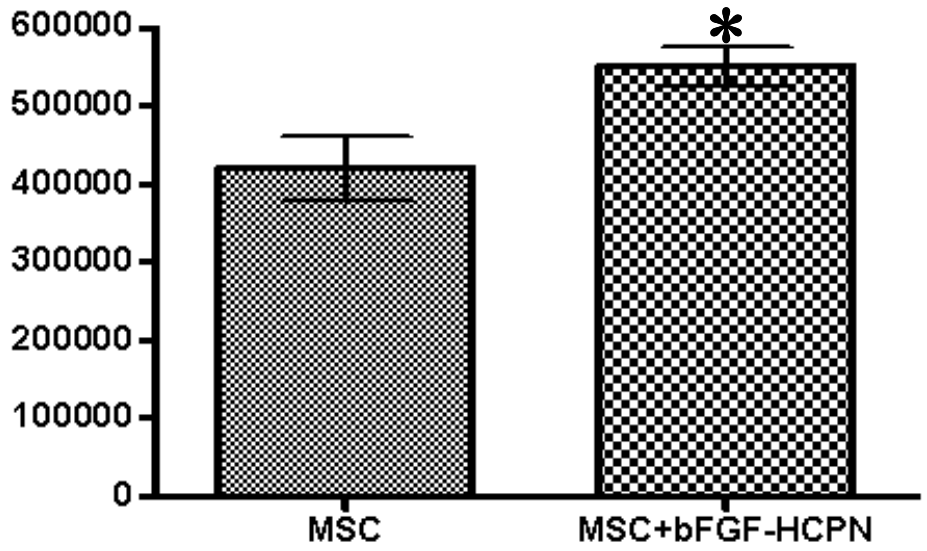

Figure 1. P4 hMSCs cultured with bFGF-HCPN for 1week. Seeding cell number was $5*10^4$ cells. With bFGF-HCPN cultured cells were more proliferative compared to MSC without it. (* : $p<0.05$).

2. Immunohistochemistry

2-1

Using immunohistochemical analysis, we observed the bFGF-HCPNs in the tissue that hMSC transplanted tissue. bFGF positive reactions were along with human nuclei positive cells(Fig.2).

2-2

We thought that bFGF support survival of hMSCs so hMSCs may survive longer time than without it in injured spinal cord. Usually, 2weeks after transplantation hMSCs hardly find, although cyclosporine is injected everyday. But many hMSCs in bFGF-HCPNs and hMSCs were observed in transplanted tissue(Fig.3). At both groups, many hMSCs were observed at 1week after transplantation. However several times more hMSCs were observed at hMSCs transplanted with bFGF-HCPNs group at 2weeks after transplantation.

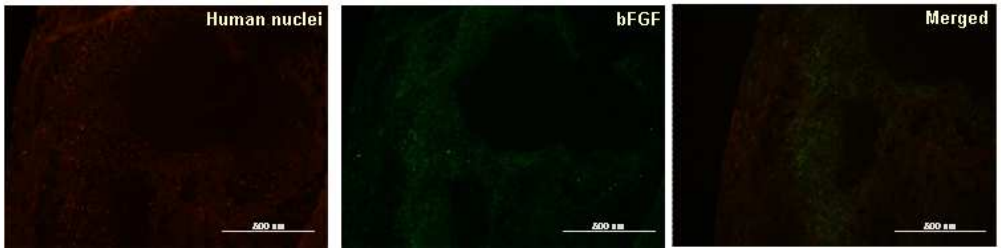

Figure 2. Immunoreactivity of human nuclei and bFGF antibody were observed by the fluorescence microscope in injured site, which indicates that hMSC and bFGF-HCPNs have successfully been grafted into the lesion site.

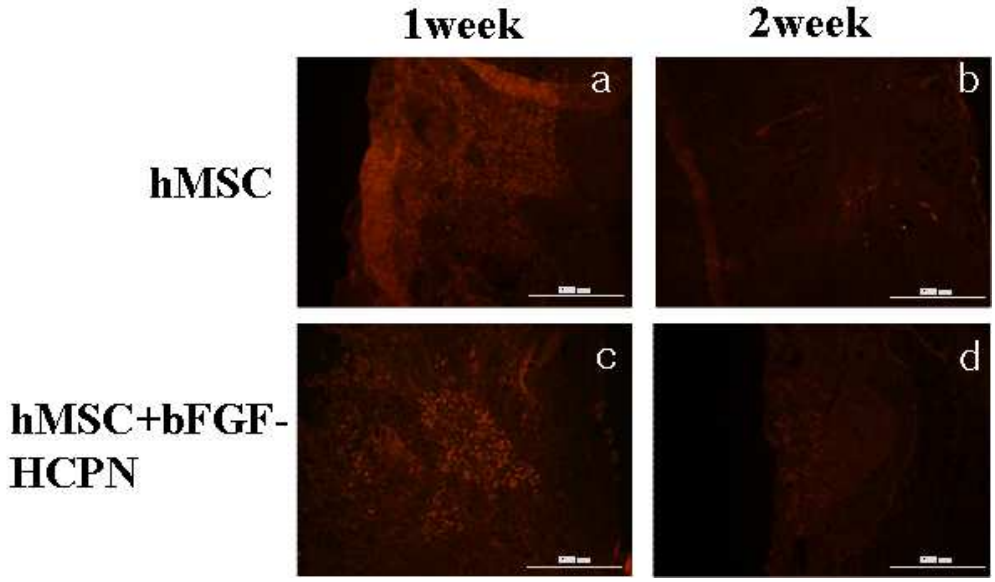

Figure 3. Staining of human nuclei analyzed by fluorescence microscope at 1week and 2weeks after transplantation. There were few human nuclei positive cells in hMSC transplanted tissue at 2weeks (b) but hMSC + bFGF-HCPN transplanted tissue contained many human nuclei positive cells (d). bFGF-HCPN may increase the survival of transplanted cells in injured spinal cord

2-3

To investigated, what are different between hMSCs transplanted with bFGF-HCPNs tisseu and only hMSCs transplanted tissue at 1week post transplantation. The reason why we chose the tissue at 1week post transplantation was that we want to know effect of the combined transplantation. Because hMSCs were usually many survive untill 1week post transplantation, we used 1week tissue for comparing with combined transplantation tissue.

Double staining of human nuclei and neurofilament antibody analyzed by fluorescence microscope. hMSCs transplanted tissue and combined transplantation tissue showed that both of groups many human nuclei positive cells were observed(Fig.3. a,g). But location of the neurofilament(NF) positive fibers were different. the hMSCs transplantation tissue, NF positive fibers were noted at outside of transplanted cells(Fig.3.b). The combined plantation tissue, NF positive fibers were observed along with transplanted cells(Fig.3.h). Double staining of human nuclei and GFAP antibody, the hMSCs transplantation tissue, GFAP positive cells were surrounded at a close of the transplated cells(Fig.3.e). The combined plantation tissue, GFAP positive cells were located at apart from transplanted cells(Fig.3.k). Also we used Vimentin antibody and p75 antibody but those were no different between two groups(data not shown).

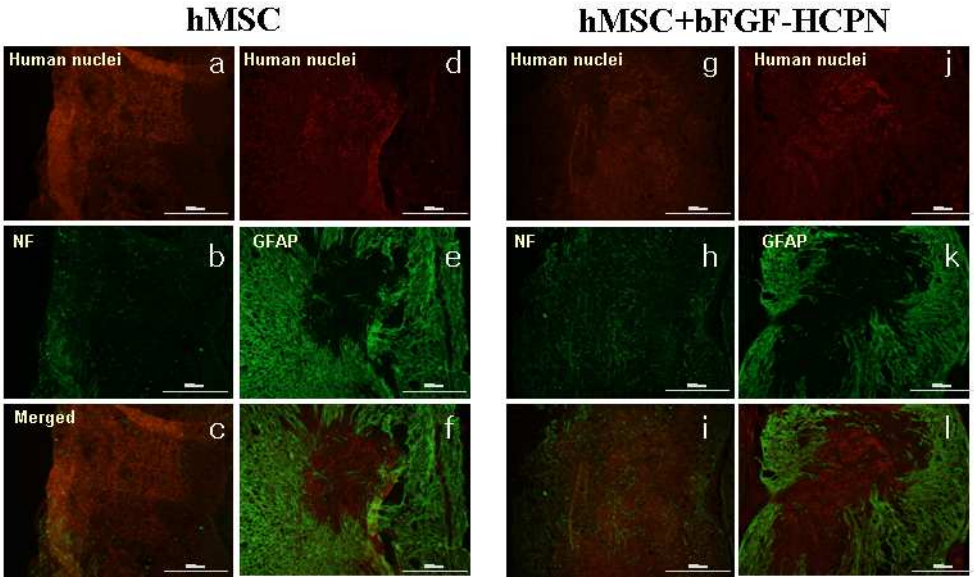

Figure 4. Double staining of human nuclei and neurofilament antibody analyzed by fluorescence microscope at 1week after transplantation. hMSC transplant ed tissue showed that neurofilament positive fibers were noted at outside of transplanted cells (c). But hMSC+bFGF- HCPN transplanted tissue showed that neurofilament positive fibers were observed along with transplanted cells (i). GFAP positive cells surrounded the transplated cells at a close in the hMSC transplanted tissue(f). But hMSC+bFGF- HCPN transplanted tissue showed that GFAP positive cells surrounded the transplated cells at a distance. In both group, p75 positive cells were noted in surrounding area of transplanted cells (data not shown).

Ⅳ. DISCUSSION

Several studies have shown improvement following transplanting of MSCs into injured spinal cord[10-13] and increasing the survival following treating growth factors[14-16]. So in this study combined transplanted hMSCs and bFGF-HCPNs.

Because HCPNs was much small (100nm - 250nm)[8], we thought that those may have toxicity through uptake. We cultured hMSCs with HCPNs to determined whether had toxicity and then hMSCs did not death(data not shown). And we cultured hMSCs with bFGF-HCPNs for knowing effect of the bFGF. At the result, cultured cells with bFGF conjugated HCNPs were more proliferation than with out it.(Fig.1) this suggest bFGF released from HCPNs support survival of the hMSCs and do not have toxicity to hMSCs in vitro. In vivo, more many transplanted cells were observed in the combined transplantation tissue at 2weeks after transplantation than in the hMSCs transplanted tissue (Fig.3). From this result, we know that also bFGF released from HCPNs support hMSCs survival in vivo and bFGF well release from HCPNs in vivo.

Double staining of human nuclei and NF show that bFGF-HCPNs have effect to axonal regeneration. Although NF positive fibers were observed in the hMSCs transplanted tissue, those did not along with transplanted cells. But in the combined transplantated tissue, NF positive fibers were noted along with transplanted cells(Fig.4.). GFAP positive cells were apart from transplanted cells in the combined transplanted tissue. From this result, we suppose that bFGF-HCPNs inhibit astrocyte's proliferation and scar formation.

Ⅴ. CONCLUSION

bFGF-HCPNs have stimulating effect to proliferate of hMSCs and they are nontoxic to the hMSCs in vitro. Positive immuno-reactivity of bFGF at bFGF-HCPNs injection site suggests that bFGF can be released at injured spinal cord after bFGF-HCPNs transplantation. Combined transplantation of bFGF-HCPNs may help survival of transplanted hMSCs. Combined transplantation of hMSC with bFGF-HCPNs increased neurofilament postive fibers in epicenter of injured spinal cord and bFGF-HCPNs may inhibit growth of the astrocytes.

Growth factor containing nanospheres can be a useful component of combination therapy for repair of injured spinal cord.

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골수 간엽줄기 세포와 신경 성장인자 함유 나노 입자를 이용한 척수신경 손상의 복합 치료

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척수 손상은 비가역적인 것으로 손상 후 재생은 매우 어려운 것으로 알려져 있다. 중추신경에 손상이 생기면, 손상된 신경의 자발적인 재생은 극히 제한적이다. 세포이식은 척수손상을 치료하기 위한 매우효과적인 방법으로 여겨지고 있지만 .척수손상은 병리학적 관점에서 매우 복잡하므로 다양한 복합적 치료법을 이용한 접근이 필요 되어 진다. 이러한 면에서 성장촉진인자는 복합적인 치료의 한 요소로써 사용가능성이 알려지고 있다.

따라서 본 연구는 아급성 척수 신경손상 시 척수 신경기능의 회복을 위하여 골수 간엽 줄기세포 또는 신경재생 등에 관여하는 성장촉진인자(bFGF)를 분비하는 생분해성 물질(PLGA nanospheres)을 골수 간엽 줄기세포와 함께 척수손상모델에 이식한 후 이식된 조직 내에서 이식세포의 생존, 세포의 분화여부 및 이식한 생분해성 물질(PLGA nanospheres)과 골수 간엽줄기 세포가 조직 내에서의 신경세포 보호 효과를 확인하고, 성장촉진인자(bFGF)와 생분해성물질(PLGA nanospheres)이 손상된 척수 조직과 이식한 세포의 생존분화에 어떠한 영향을 주는지 확인하고자 하였다.

생분해성 물질(PLGA nanospheres)이 in vitro실험상에서 골수 간엽 줄기세포의 증식을 도와주는 것을 확인 하였고 생분해성 물질(PLGA nanospheres)과 골수 간엽 줄기 세포를 함께 이식하였을 때 골수 간엽줄기만의 생존력이 증가하였으며 신경섬유가 증가 하였다 이식한 곳 주변으로 성상세포가 많이 모여 들지 않는 것을 확인하였다. 하지만 다른 세포로의 분화는 관찰 되지 않았다.

핵심되는 말 : 척추 손상, 골수 간엽줄기세포, 성장촉진인자(bFGF), 생분해성 물질(PLGA nanosphere)