Table 1 Profiles of patients with treated with bevacizumab

Patient	Age/sex	Duration	Previous therapy	Combined therapy	Outcome
A. Our patients treated with bevacizumab					
1	64 F	40 months	PSL, Thal+Dex	Thal+Dex, PBSCT	Improved. PBSCT in 2 months
2[9]	49 M	26 months	VAD, CPA, MP	Thal+Dex	Improved in effusion. No response in neuropathy
3	58 M	74 months	PSL	Thal+Dex	No response
4	43 M	61 months	PSL, CPA, MP	Thal+Dex, PSL	No response. Intractable ulcer/cellulitis in the leg
5	71 M	10 months	Thal+Dex	Thal+Dex, PSL	Worsened. Died in 6 months
6	66 F	68 months	MP, PSL	MP, PSL	Worsened. Died in 2 months

Patient	Reference	Age/sex	Duration	Previous therapy	Combined therapy	Outcome
B. Literature review of POEMS syndrome patients treated with bevacizumab						
1	Dietrich and Duchosal[11]	45 F	A few months	–	–	Improved. Treated with PBSCT
2	Buxhofer-Ausch *et al*[12]	44 F	NA	Radiation, CPA	–	Improved
3		57 M	5 months	Radiation, CPA	–	Improved
4	Badros *et al*[5]	60 F	2 years	–	CPA, Dex	Improved
5	Chahin *et al*[13]	59 M	13 months	–	PBSCT	Improved
6	Badros[4]	52 F	2 years	IVIg, steroids, Mel+Dex	Mel+Dex	Improved
7	Ropper *et al*[14]	49 M	4 years	IVIg, PP, AZP	Radiation	Improved. Treated with PBSCT
8	D'Souza *et al*[15]	NA	NA	PBSCT	Steroids, len, CPA	No improvement. Died
9	Straume *et al*[6]	41 M	NA	CPA	Radiation	Worsened. Died of multiorgan failure
10	Samaras *et al*[16]	57 M	8 years	MP, radiation, Dex, IVIg, PP, PBSCT	CPA, steroids	Worsened. Died of multiorgan failure
11	Chong *et al*[17]	48 M	NA	IVIg, Dex, PSL, MMF	Dex, MMF	Worsened. Died in a few months

AZP, azathioprine; CPA, cyclophosphamide; Dex, dexamethasone; IVIg, intravenous immunoglobulin; len, lenalidomide; Mel, melphalan; MMF, mycophenolate mofetil; MP, melphalan, prednisolone; NA, not available; PBSCT, peripheral blood cell transplantation; POEMS, polyneuropathy, organomegaly, endocrinopathy, M-protein and skin changes; PP, plasmapheresis; PSL, prednisolone; Thal, thalidomide; VAD, vincristine.

pretreatment level at Week 12 in two patients, and at Week 16 in four patients. The treatment resulted in entirely no response in four (Patients 3–6 in table 1), and two of them died of multiorgan failure due to massive and intractable pleural effusion, 6 and 2 months later respectively. Patients 1 and 2 showed a gradual decrease in peripheral oedema and pleural effusion over months, but the effects were likely to be explained by thalidomide/dexamethasone therapy because the changes in clinical and laboratory findings started after the treatment and 8 weeks after bevacizumab injection. The scores of overall neuropathy limitation scale and inflammatory neuropathy cause and treatment did not change in five patients (figure 1B). No serious adverse effects by bevacizumab were observed.

Literature review

We found 11 cases of bevacizumab treatment for POEMS syndrome (table 1B).[4–6 11–17] Of these, seven patients eventually had improvement in neuropathy and systemic symptoms, but six of them received previous or combined treatments. In only one patient with subacute onset of polyneuropathy (Patient 1 in table 1B)[11] bevacizumab was administered as an initial and single therapy, resulting in improvement in neuropathic symptoms within 4 weeks. Bevacizumab appeared to be effective in this patient. In Patients 2 and 3 (table 1B), serum VEGF levels were normalised after irradiation before bevacizumab injection.[12] In Patient 4 (table 1B), haematological remission was achieved by melphalan treatment, before bevacizumab administration. Therefore, it is difficult to conclude that bevacizumab had beneficial effects in these patients. The remaining patients (nos 5–11; table 1B) received combined treatments, and three of them died without any response to bevacizumab.[6 16 17]

DISCUSSION

Both our experience and the literature suggest ambiguous effects of bevacizumab on POEMS syndrome. Bevacizumab therapy results in a rapid decrease in the serum VEGF levels, but this was not necessarily associated with clinical improvement. The large biological and clinical heterogeneities of POEMS syndrome could explain variable sensitivities to anti-VEGF therapy. Furthermore, because of the lack of standard treatment regimen for POEMS syndrome, many patients had received multiple treatments as shown in table 1.

Of our six patients, bevacizumab was obviously ineffective in four, and in the remaining two it was difficult to determine the effects because of multiple treatments. In the literature, only one of the 11 patients had a single treatment with bevacizumab in the very early phase of the disease,[11] and the drug appeared to have positive effects in this patient. However, the efficacy was not justified in the remaining patients. It should be noted that two of our six patients, and four of the 11 reported patients died several months after bevacizumab injection.

There are several hypotheses for the failure of bevacizumab treatment. First, in POEMS patients, VEGF and several other cytokines, such as interleukin-6 (IL-6), IL-12, tumour necrosis factor-α and hepatocyte growth factor, are elevated,[18–20] and inhibition of VEGF alone is not sufficient to suppress the disease activity.

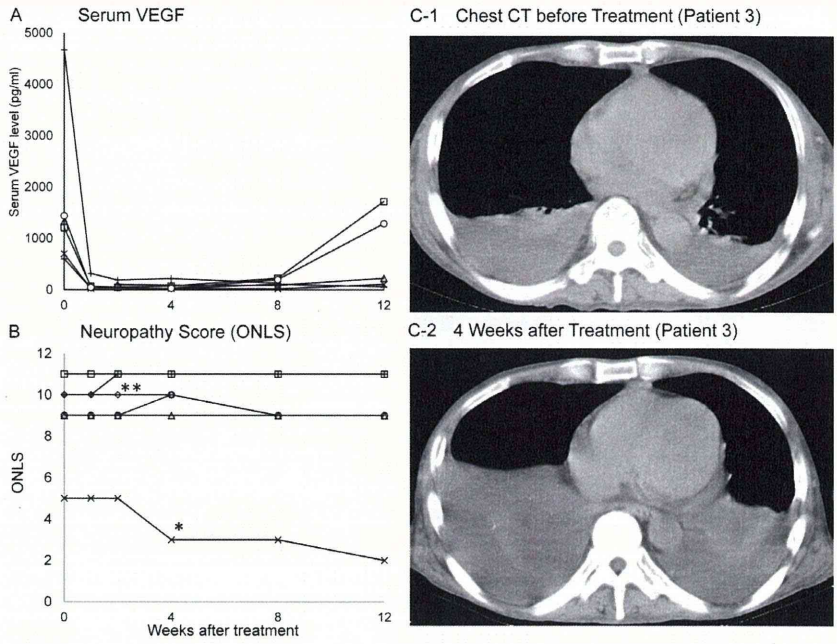

Figure 1 Serial changes in serum levels of vascular endothelial growth factor (VEGF; A), overall neuropathy limitation scale (ONLS; B) and chest CT (C) after bevacizumab administration. The serum VEGF levels dramatically decreased. However, ONLS score did not change entirely in five of the six patients and pleural effusion rather increased on chest CT in Patient 3 (table 1A). *Patient 1 underwent auto blood stem cell transplantation 47 days after bevacizumab. **Patient 6 died 43 days after the treatment. The ONLS score in Patient 3 was 11 at the baseline and 12 weeks later.

Second, during the long course of the disease, aberrant angiogenesis has already systemically developed, and the structural changes may result in permanently leaky vessels. Under these conditions, reduction of VEGF may not be enough to induce obvious clinical improvement. Some researchers also suggest that sudden VEGF removal may cause sudden collapse of newly formed fragile vessels because VEGF is an important factor for new vessels, and may lead to an increase capillary leakage.[6]

Buxhofer-Ausch *et al*[12] proposed that the short disease duration before bevacizumab initiation may be an important factor for a good outcome. We agree with that bevacizumab may be efficacious in the very early phase of the disorder when abnormal neovasculisation has not been fully developed. We also suggest that the standard or first-line treatment for POEMS syndrome should target plasma cell dyscrasia, and that temporary relief of symptoms by VEGF-targeted therapy would not be effective in many of the patients with long-standing POEMS syndrome.

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II. 各　　論

クロウ・フカセ(POEMS)症候群の病態と新規治療

桑原　聡

Crow-Fukase (POEMS) syndrome: pathophysiology and treatments

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Abstract

POEMS (polyneuropathy, organomegaly, endocrinopathy, M-protein, and skin changes) syndrome is a rare multiorgan disorder associated with plasma cell dyscrasia and overproduction of vascular endothelial growth factor (VEGF). VEGF presumably plays an important role in the pathogenesis of the syndrome by its strong action on neo-vasculization and increased vascular permeability. POEMS syndrome is potentially fatal disease, and patients' quality of life deteriorates because of progressive neuropathy and/or massive peripheral edema or pleural effusion/ascites. There is no established treatment regimen. In appropriate candidates, high-dose chemotherapies with autologous peripheral blood stem cell transplantation is recommended, because this treatment could result in obvious improvement in neuropathy as well as other symptoms, with a significant decrease in serum VEGF levels. However, from pooled data, the transplant-related mortality is reported to be 5%, and there is a risk of relapse several years later. Treatments that should be considered as future therapy include thalidomide or lenalidomide, and anti-VEGF monoclonal antibody (bevacizumab).

Key words: POEMS syndrome, Crow-Fukase syndrome, autologous peripheral blood stem cell transplantation, thalidomide, bevacizumab

はじめに

クロウ・フカセ症候群は末梢神経障害(多発ニューロパチー)を必発とし，形質細胞の単クローン性増殖(plasma cell dyscrasia)と血清中の血管内皮増殖因子(vascular endothelial growth factor: VEGF)の高値を基盤に，浮腫，胸・腹水，皮膚症状(剛毛・色素沈着，血管腫)，骨硬化病変，Mタンパク血症などを呈するまれな全身性疾患である[1]．我が国では報告者の名前をとってクロウ・フカセ症候群と呼ばれるが[2]，欧米では主要症状の頭文字をとってPOEMS (polyneuropathy, organomegaly, endocriopathy, M-protein, and skin changes)症候群と呼ばれている[1]．本稿ではクロウ・フカセ症候群で統一する．本症候群は稀少疾患であり2003年に行われた厚生労働省免疫性神経疾患に関する調査研究班の全国調査によると全国の患者数

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表1　クロウ・フカセ症候群の診断基準

大基準
　多発ニューロパチー(必須項目)
　血清VEGF高値
　Mタンパク
小基準
　骨硬化性病変
　キャッスルマン病
　臓器腫大
　浮腫，胸・腹水，心嚢水
　内分泌異常*
　皮膚異常
　乳頭浮腫
　血小板増多
definite: 大基準3項目＋小基準1項目以上
probable: ニューロパチーと血清VEGF高値＋小基準1項目以上
possible: 大基準のうちニューロパチー＋小基準2項目以上

*甲状腺機能異常，糖尿病については有病率が高いため単独の異常では小基準の1項目として採用しない．
(文献[1]より改変)

は約340人と推定されているが[3]，診断されていない患者も多いとみられることから有病率はより高い可能性がある．

本症候群の約半数は多発ニューロパチーで発症するが，残りの半数は浮腫，胸・腹水，男性の場合には女性化乳房での発症があり初診する診療科は多岐にわたっている．早期診断・治療のためには各内科系診療科においてこの疾患の可能性が常に考慮される必要がある．欧米からの報告は少なく，日本においてより頻度の高い疾患であるとされていることも含めて‘治療可能な見逃してはならない疾患’として認識されるべきである．

1996年に本症候群患者血清中においてVEGFが著明高値を示すことが報告された[4]．VEGFは強力な血管新生，血管透過性亢進作用をもつことから，本症候群における浮腫，臓器腫大，血管腫などの臨床症状を説明しやすく，病態と深く関連すると考えられている[1,4]．

1. 診断基準

約半数の患者において初発症状は多発ニューロパチーであり，下肢に始まるしびれ感と脱力が次第に上肢に進展する．残りの半数における初発症状としては浮腫，皮膚症状(色素沈着，剛毛，血管腫)，男性においては女性化乳房の頻度が高い．検診あるいは他疾患のための受診時に胸・腹水，Mタンパク，クレアチニン高値が発見されることもある[2]．疾患の進行に伴い複数の症状が出現してくるが，早期診断のためには初診時に本症を念頭に置いた体系的検索を行う必要がある．

表1に現在提唱されている血清VEGF値を含めた診断基準を示す．大基準である多発ニューロパチーと血清VEGF高値は全例に存在する．また90％以上の患者にはMタンパクが認められる．多発ニューロパチーで発症し，初診の際に浮腫，皮膚症状が認められることが多く，この場合には比較的診断は容易であるが，小基準に含まれる多彩な症状のどれかで初発した際に本症の可能性を念頭に置くことが重要であり，神経症状の評価(多発ニューロパチーの有無)，血清VEGF・Mタンパクの測定を行う．自覚症状に挙げられていなくても浮腫・皮膚症状は存在することが多い．

2. 病　　態

本症候群の病態の基盤にあるのが形質細胞の

単クローン性増殖であり，VEGFを中心とする各種サイトカインの過剰産生が多彩な臨床症状を惹起していることが想定されている．VEGFは強力な血管透過性亢進および血管新生作用を有するため，浮腫，胸・腹水，皮膚血管腫，臓器腫大などの臨床症状を説明しやすい[1]．しかし全例に認められる末梢神経障害(多発ニューロパチー)の発症機序については明らかにされていない．血管透過性亢進により血液神経関門が破綻し，神経毒性をもつ血清タンパクが神経実質に移行することや神経血管内皮の変化を介して神経の虚血が起こることなどが推定されている．神経生検における基本的な病理学的変化は脱髄(myelin uncompaction)であるが，下肢遠位部では軸索変性が認められる．

血清VEGFの異常高値は本症候群を特徴づける所見であり，診断とともに病勢のマーカーとして非常に有用であるが，VEGFの産生部位については，いまだ明らかにされていない．一つの可能性は単クローン性増殖をしている形質細胞が挙げられる．他の可能性として，単クローン性増殖している形質細胞からの何らかのシグナルにより，血小板や血管内皮細胞によりVEGFが過剰産生されることはありうると思われる．

本症候群で認められるMタンパクの軽鎖はほとんどがλ鎖であることから，2008年，Abeらはλ鎖の可変領域のgerm-lineを検索し，免疫グロブリン軽鎖は特定のVλ subfamily遺伝子をもつことを明らかにした[5]．すなわちクロウ・フカセ症候群における免疫グロブリン軽鎖は特定のVλ subfamily遺伝子を有しており，この配列をもつMタンパクが産生された場合に本症候群が発症することになる．この配列が特定のシグナルとなりサイトカイン産生が誘導される可能性があり，今後の分子メカニズムの更なる解明が望まれる．以上の病態から，本症候群に対する治療の標的は異常増殖している形質細胞であり，次にVEGFを標的とした対症療法がオプションとなると考えられる．

3. 従来治療と新規治療

1980年代までは本症候群に対して主に副腎皮質ステロイド剤が治療として用いられていたが，平均生存期間は約3年と生命予後は不良であることが報告されていた[2]．1990年代には長期メルファランによる化学療法が導入され生存期間は5-10年に延長した[6]．本症候群の治療法は，基本的には同じく形質細胞の増殖性疾患である多発性骨髄腫の治療を応用する形で進められてきた．多発性骨髄腫の標準的治療が自己末梢血幹細胞移植(auto-PBSCT)を伴う大量化学療法，サリドマイド・レナリドマイド，プロテアソーム阻害薬に移行しつつあることを受けて，本症候群に対する治療もそれを応用する試みがなされてきた[7]．特に2000年代に入って行われ始めたauto-PBSCTを伴う大量化学療法は長期寛解を目指す新規治療法として，本症候群の第一選択となる可能性がある．しかし治療関連死のリスクがあり，再発率を含めた長期予後は確立しておらず，今後の検討課題である．移植療法は高齢者や多臓器病変(特に腎障害)を有する患者には施行できないため，移植適応にならない場合の治療法としてサリドマイド療法が期待されている．

1) 自己末梢血幹細胞移植療法

多発性骨髄腫の治療として1980年代からauto-PBSCTを併用したメルファラン超大量療法が行われるようになり，現在では骨髄腫に対する標準的治療の一つとして位置づけられている．この治療法の原理は，前もって患者自身の造血幹細胞(CD34陽性細胞)を採取・保存し，超大量のメルファラン静脈内投与後に，幹細胞を輸注して造血を救済することである．

本症候群に対してのauto-PBSCTの第1例目は1998年にイスラエルで行われたが，この症例は残念ながら多臓器不全を合併して死亡している[8]．しかし，2000年代に入ってから報告が相つぎ，現在(2012年10月)までに，約50例の施行例が報告されている[9-11]．移植後にほとんどの症例では諸症状の劇的な回復が認められている．著者らの施設では2003年から，auto-PBSCTを併用した大量化学療法を開始しており，現在までに24人が移植を終了している．1例に治療関連死がみられこの治療法の大きな問

題点と思われる．既報告をまとめると本症候群におけるauto-PBSCTに伴う治療関連死は約5％である．しかし治療後の症状改善は従来のメルファラン療法より明らかに良好である[12]．

現在，自験23例において移植後15-105カ月が経過しているが，治療後3-5年での再発が4例に認められており，この治療法の適応を再検討する段階に入っている．移植療法の最も優れる点は神経症状の改善であることから，著者は末梢神経障害によるADL障害が高度な場合には積極的に移植療法を行うべきと考えている．ADL障害が軽い場合には症例の状態に応じて移植可能な状態であっても後述するサリドマイド療法などの他の治療法で経過をみるという選択も行われるようになっている．

auto-PBSCTの適応としては移植時の年齢と多臓器障害の程度が最も大きい因子である．年齢に関しては‘適応は65歳以下’が暫定的なコンセンサスである．更に‘重篤な臓器障害をもたないこと’が適応の条件とされる．66歳以上である場合には移植の適応にならないが，65歳以下であっても臓器不全，特に腎機能障害や大量の胸・腹水のために治療関連死のリスクが高いと考えられる場合には適応とはならない．

根治を目指す治療としては同種造血幹細胞移植または同種骨髄移植が挙げられるが，これらの治療法では多発性骨髄腫においては治療関連死が約10％にみられるとされており，また移植成功後にも長期にgraft-versus-host disease (GVHD)の問題があるため，本症候群への適応は，まずauto-PBSCTや下記に述べる新規治療の効果，安全性，長期予後についての知見を蓄積した後に検討すべきであると思われる．

2) サリドマイド療法

サリドマイドは睡眠・抗不安薬として我が国では1958年に発売され，その催奇形性によって300人以上の短肢症児を誘発する薬害に至り，製造は中止された．しかしその後サリドマイドのもつ血管新生抑制作用，抗サイトカイン(TNF-αなど)作用などが明らかになり，各種悪性腫瘍での治療効果が検討され，ついで多発性骨髄腫における有効性が明らかにされた．本症候群におけるサリドマイド治療は，これまで2例の症例報告と9症例におけるオープン試験が報告されている[13]．いずれの報告においても腹水，呼吸不全，末梢神経障害の改善がみられている．サリドマイドは形質細胞増殖抑制とともにVEGF産生を直接抑制すると考えられており，本症に対して今後期待の大きい治療法といえる．

サリドマイドの副作用は，一般的に200mg/日を超えると投与量に応じて発現率が高くなるとされている．便秘，眠気が主なものであり，重篤なものとして深部静脈血栓症が挙げられるが，日本人では少ないとされる．また蓄積毒性として，末梢神経障害があり，本症候群では末梢神経障害は主症状であるため，その発現には十分注意する必要がある．

また，多発性骨髄腫ではサリドマイド導入後でも，auto-PBSCTに十分な量のCD34陽性細胞採取が可能であることが報告されており，サリドマイド療法を導入し全身状態の改善が得られた後にauto-PBSCTを施行する方法も十分考慮に値すると考えられる．また移植適応例であっても症状が軽度の場合にサリドマイド療法が第一選択になる可能性も考えられる．現在サリドマイドのアミノ酸置換誘導体であるレナリドマイドも試みられている．この薬剤は，末梢神経障害の副作用が少ないことが特徴であり，本症候群に対して有効であった1例が2007年に米国から報告された[14]．

サリドマイド療法に関しては厚生労働省・免疫性神経疾患に関する調査研究班員施設を中心に，プラセボ対照・多施設共同群間比較試験が医師主導治験として2010年9月から開始され，現在進行中である(Japan POEMS syndrome thalidomide trial: J-POST trial)．詳細については[http://www.m.chiba-u.jp/class/neurol/kenkyu/ishisyudou/index.html]を参照頂きたい．

3) 抗VEGFモノクローナル抗体

ベバシズマブは抗VEGFモノクローナル抗体で，血管新生阻害作用による抗腫瘍効果を有し，我が国では2007年に‘治癒切除不能な進行・再

発の結腸・直腸癌'の治療薬として製造販売承認を受けた．ベバシズマブの本症候群患者への使用の報告は11例でなされているが，有効例と無効例が存在し，その有効性について結論は得られていない[15]．本症候群においては多種のサイトカイン血中濃度が上昇して病態を形成しているために，VEGFを単独で低下させた場合の治療効果については不明であるといわざるをえない．ただしこの治療によりVEGFの低下は非常に急速に認められるため，胸・腹水や腎機能障害の進行が亜急性にみられた場合に，救済的に併用する価値はある可能性がある．

おわりに

クロウ・フカセ症候群の病態解明において1996年の患者血清中VEGF高値の発見はブレイクスルーであったが，その後の解明はあまり進んでいない．しかし新規治療としての移植療法やサリドマイド療法は国際的にも国内的にも年々広まりつつある．今後，VEGF産生の部位や分子メカニズムに関する解明が進み，病態に応じた分子標的治療の進展することが期待される．治療に関しては長期予後を含めた移植療法，サリドマイド療法の治療効果と有用性についての知見を蓄積していくことが必要であると思われる．現在全国において実際にこれらの治療が行われており，特に移植療法後の長期予後調査とサリドマイド医師主導治験の結果が重要であると思われる．

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Crow-Fukase症候群
——病態と治療
Crow-Fukase syndrome：Pathophysiology and new treatments

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◎Crow-Fukase症候群は形質細胞の単クローン性増殖を基盤として多発ニューロパチー，浮腫・胸腹水，皮膚変化(色素沈着，剛毛)，M蛋白血症，骨硬化性病変など多彩な症状を呈する全身性疾患である．病態の基盤にはおそらく，形質細胞から分泌される血管内皮増殖因子(VEGF)を中心としたサイトカインの過剰産生がある．1980年代までは副腎皮質ステロイドを中心とした治療が行われてきたが，難治性胸腹水，腎不全から多臓器不全に至り平均33カ月で死亡する予後不良の疾患であった．1990年代からメルファランによる化学療法が導入され，生存期間は延長したが，神経症状の十分な回復は得られないことや再発の問題点があった．2000年代に入り自己末梢血幹細胞移植を伴う大量化学療法が導入されて，生命予後・機能予後は飛躍的に改善した．しかし移植療法は，66歳以上である場合や，多臓器不全が進行している場合には行えず，サリドマイド療法が2006年ごろから試みられている．移植，サリドマイド療法の有効性は確立されつつあるが，長期的には移植後の再発が問題となる．現在は患者の年齢，重症度，全身状態を考慮しつつ，移植療法と新規薬物治療を組み合わせて長期寛解をめざす治療計画を考える時代に入っている．

Key word：Crow-Fukase症候群，血管内皮増殖因子，自己末梢血幹細胞移植，サリドマイド

疾患の概要

Crow-Fukase症候群は形質細胞の単クローン性増殖(plasma cell dyscrasia)を基盤として，末梢神経障害を必発とし，浮腫・胸腹水，皮膚症状(剛毛・色素沈着，血管腫)，骨硬化病変，M蛋白血症などを呈するまれな全身性疾患である[1-3]．1956年，Crowは多発性骨髄腫に末梢神経障害を合併した2例を報告した．わが国からは1968年に，深瀬らにより“多発性神経炎および内分泌異常を惹起した孤立性骨髄腫”として報告されたが，その後あいついで同様の多発性神経炎，内分泌異常を伴うplasma cell dyscrasiaの症例が報告されて疾患概念が確立した．わが国では報告者の名前をとってCrow-Fukase症候群と呼ばれるが，欧米では主要症状の頭文字をとってPOEMS(Polyneuropathy，Organomegaly，Endocriopathy，M-protein，and Skin changes)症候群といわれることが多い[1]．

1996年に，本症候群患者血清中において血管内皮増殖因子(vascular endothelial growth factor：VEGF)が著明高値を示すことが報告された[4]．VEGFは強力な血管新生，血管透過性亢進などの生理的作用をもつことから，病態と深く関連すると考えられており，現在VEGFを含めた診断基準が提唱されている(表1)．

疫学と病態

男女比は約1.5：1であり，平均発症年齢は男女ともに48歳であるが，発症は20～80歳代と広く分布している．発症に地域特異性はなく，全国に広く分布している．2003年に行われた全国調査では国内に約340名の患者がいると推定された[5]．しかし，診断されずに見逃されている症例もあることが予想され，実際の患者数はより多いと推定

表 1　Crow-Fukase症候群の診断基準[1)]

大基準	多発ニューロパチー(必須項目) 血清 VEGF 高値 M 蛋白
小基準	骨硬化性病変 Castleman 病 臓器腫大 浮腫，胸水，腹水，心嚢水 内分泌異常* 皮膚異常 乳頭浮腫 血小板増多
Definite：大基準 3 項目＋小基準 1 項目以上 Probable：ニューロパチーと血清 VEGF 上昇＋小基準 1 項目以上 Possible：大基準のうちニューロパチー＋小基準を 2 項目以上	

*甲状腺機能異常，糖尿病については有病率が高いため，単独の異常では小基準の 1 項目として採用しない．

される．欧米より日本において頻度の高い疾患であるとされている．

本症候群の多彩な病像の根底にあるのが形質細胞の単クローン性増殖であり，VEGF を中心とする各種サイトカインの過剰産生が多彩な臨床症状を惹起していることが想定されている．VEGF は強力な血管透過性亢進および血管新生作用を有するため，浮腫，胸・腹水，皮膚血管腫，臓器腫大などの臨床症状を説明しやすい．

症状・診断

約半数の患者において初発症状は多発ニューロパチーである．残りの半数における初発症状としては浮腫，皮膚症状(色素沈着，剛毛，血管腫)，男性においては女性化乳房の頻度が高い[1)]．検診あるいは他疾患のための受診時に胸水・腹水，M 蛋白，クレアチニン高値が発見されることもある．疾患の進行に伴い複数の症状が出現してくるが，早期診断のためには初診時に本症を念頭に入れた体系的検索を行う必要がある．

表 1 に現在提唱されている診断基準を示す[1,5)]．大基準である多発ニューロパチーと血清 VEGF 高値はほぼ全例に存在すると考えてよい．また，90%以上の患者には M 蛋白が認められる．多発ニューロパチーで発症し，初診の際に浮腫，皮膚症状が認められることが多く，この場合には比較的診断は容易であるが，小基準に含まれる多彩な症状のどれかで初発した際にも，本症の可能性を念頭におくことが重要である[6)]．

治療法の変遷

1980 年代までは本症候群に対しておもに副腎皮質ステロイド剤が治療として用いられていたが，平均生存期間 33 カ月と，生命予後は非常に不良であった[7)]．進行の遅い症例は存在するが，適切な治療が行われない場合には多臓器不全により数年で死亡する重篤な疾患として認識すべきである．

1990 年代には長期メルファラン化学療法とステロイド剤の併用療法が導入され，生存期間は数年に延長したが，死亡率はいぜん高く，満足すべき治療効果は得られなかった[1)]．本症候群は稀少疾患であるために標準的治療は確立されていないが，2000 年代に入って行われはじめた自己末梢血幹細胞移植を伴う大量化学療法が長期寛解をめざす新規治療法として期待されている．しかし，高齢者や多臓器病変(とくに腎障害と多量の胸腹水)を有する患者には移植療法は施行できないため，非移植適応例の治療法としてサリドマイド療法が期待されている[1)]．

自己末梢血幹細胞移植を伴う大量化学療法

本症候群に対して 2000 年代に入ってから移植療法の報告が多くなされている[8-11)]．移植後には，ほとんどの症例は諸症状の劇的な回復が認められている．2012 年までにわが国で約 50 名がこの治療を受けていると推定される．多発性骨髄腫で行われている方法と同様であり，前もって自己末梢血幹細胞を採取し，骨髄破壊的といわれる超大量メルファランを投与した後に幹細胞を静脈内投与して造血を回復させるものである．この治療法によって神経症状を含めた諸症状の劇的な改善が得られることが明らかになっている[9-12)]．図 1 に移植療法による血清 VEGF 値の低下と末梢神経伝導速度の改善を示す．とくに末梢神経障害による筋力低下が高度な場合には，移植療法による改善が著明に認められる[12)]．

しかし，約 5%で治療関連死がみられることが

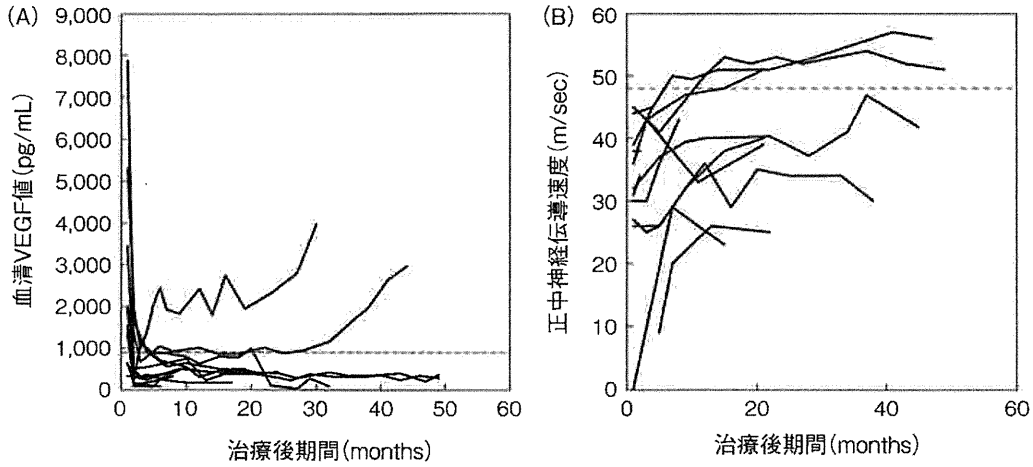

図 1　自己末梢血幹細胞移植後の血清VEGF値(A)および神経伝導速度の変化(B)[12]
破線は正常値を示す.

この治療法の大きな問題点であり，また移植後数年での再発例が散発的に報告されていることから，長期的な予後は明らかになっていない．再発の可能性を考慮する場合には，若年例でADL(日常生活動作)障害の軽い症例には初期治療として，後述するサリドマイド療法などの他の治療法による寛解導入が試みられている．

移植療法の適応を決める因子としては移植時の年齢と多臓器障害があげられる．年齢に関しては“65歳以下”が暫定的なコンセンサスである[1]．さらに，“重篤な臓器障害を有さないこと”が適応の条件とされる．すなわち，65歳以下であっても臓器不全のために治療関連死のリスクが高いと考えられる場合には適応とはならない．

サリドマイド療法

サリドマイドのもつ血管新生抑制作用，抗サイトカイン(TNF-αなど)作用などが明らかになり，各種悪性腫瘍での治療効果が検討され，多発性骨髄腫における有効性が明らかにされた．本症候群におけるサリドマイド治療はこれまで2例の症例報告と9症例におけるオープン試験が報告されている[13]．いずれの報告においても腹水，呼吸不全，末梢神経障害の改善がみられ，今後期待される治療法といえる．

サリドマイドの副作用は一般的に200 mgを超えると投与量に応じて発現率が高くなるとされている．便秘，眠気がおもなものであり，重篤なものとして深部静脈血栓症があげられるが，日本人では少ないとされる．また，蓄積毒性として末梢神経障害があり，本症候群では末梢神経障害は主症状であるため，その発現には十分注意する必要がある．図2にサリドマイド療法による血清VEGFと末梢神経伝導速度の改善を示す．その効果は移植療法ほど著明ではないものの確実な改善が認められている．サリドマイド療法に関しては医師主導多施設共同群間比較試験が2010年9月から開始され，2013年現在進行中である．

抗VEGFモノクローナル抗体

ベバシズマブは抗VEGFモノクローナル抗体で，本来は血管新生阻害作用による抗腫瘍剤であるが，本症候群患者で使用された報告では血清VEGF値は劇的に低下している．しかし，臨床的有効性について結論は得られていない[14]．本症候群にいては多種のサイトカインが上昇して病態を形成しているために，VEGFを単独で低下させた場合の治療効果については不明であるといわざるを得ない．ただしこの治療によりVEGFの低下は非常に急速に認められるため，胸・腹水や腎機能障害の進行が亜急性にみられた場合に，救済的に併用する価値はある可能性がある．

今後の展望

Crow-Fukase症候群の治療戦略は，多発性骨髄腫領域における新規治療戦略のめざましい発展

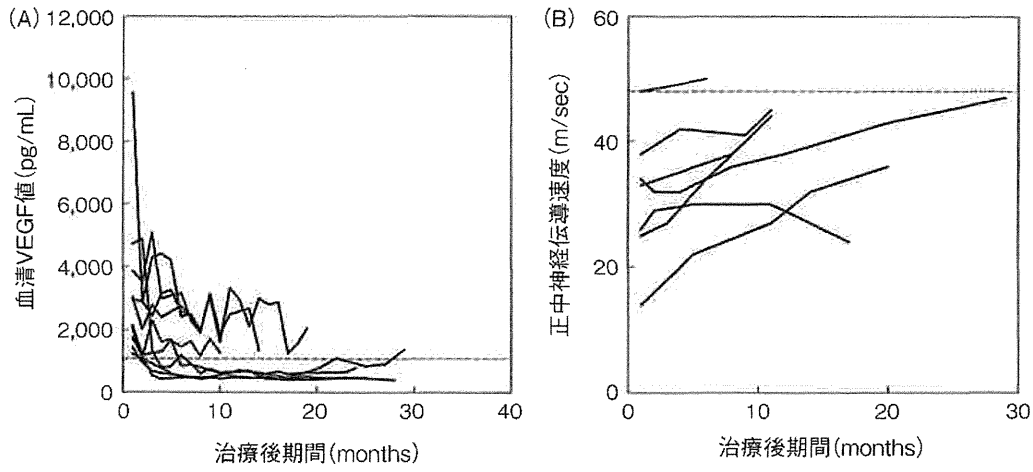

図 2　サリドマイド療法後の血清VEGF値(A)および神経伝導速度の変化(B)[13]
破線は正常値を示す.

により，国際的には移植療法・サリドマイドだけでなく，レナリドマイド，ボルテゾミブ，ベバシズマブ(抗 VEGF 抗体)へと発展している．一方で，日本国内ではいずれの治療も保険適応がないため，各医療機関の体制により提供できる治療の選択肢が異なり，治療の較差が生じているのが現状である．しかし，適応外使用の継続は事態の改善にはつながらない．

現在進行中のサリドマイドの有効性に関する治験は医師主導のプラセボ対照ランダム化群間比較試験であり，稀少疾病であってもエビデンスの構築をめざしている．本治験の達成により国内においても治療の選択肢が増え，本症候群の予後が改善することが望まれる．いずれにしろ Crow-Fukase 症候群に対する治療法はこの 10 年間で劇的に進歩しており，生命予後・機能予後の改善が得られている．今後さらなる進歩が期待されている．

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Case report

Granulocyte colony-stimulating factor reduced neuropathic pain associated with thoracic compression myelopathy: Report of two cases

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Context: A clinical trial was conducted to evaluate the safety and efficacy of neuroprotective therapy using granulocyte colony-stimulating factor (G-CSF) for patients with worsening symptoms of compression myelopathy. During this trial, we found that neuropathic pain associated with thoracic myelopathy was dramatically reduced after G-CSF administration in two cases.

Findings: A 32-year-old man with compression of the spinal cord at levels T7–T10 complained of spastic gait associated with spontaneous severe pain from his back to his chest. G-CSF 10 μg/kg/day was administered for 5 consecutive days; his pain was reduced 1 day after the initial G-CSF administration. One month after administration, he underwent spinal fusion surgery for decompression of the spinal cord. Six months after G-CSF administration, he showed recovery from myelopathy and no recurrence of pain. A 68-year-old man with spastic gait and bilateral thigh pain caused by ossified ligamentum flavum at T11–T12 was treated with G-CSF 10 μg/kg/day for 5 days; his pain was reduced 1 day after initial administration. One month later, he underwent a T10–T12 laminectomy. Three months after G-CSF administration, his thigh pain began to attenuate. At 6 months after administration, he showed recovery from myelopathy, and his pain was still improved compared with that before administration.

Conclusion: G-CSF may have a therapeutic effect on spinal neuropathic pain.

Keywords: Myelopathy, Spinal cord compression, Neuroprotective therapy, Granulocyte colony-stimulating factor, Thoracic myelopathy, Neuropathic pain, Spasticity, Clinical trial

Introduction

Granulocyte colony-stimulating factor (G-CSF) is a cytokine that promotes survival, proliferation, and differentiation of cells in the neutrophil lineage.[1] Recent studies have indicated that G-CSF also has non-hematopoietic functions and can potentially be used for the treatment of neuronal injury, including stroke and neurodegenerative diseases.[2] We previously demonstrated that G-CSF promoted the restoration of damaged spinal cord tissue and the recovery of neural function in experimental spinal cord injury (SCI) in both mice and rats.[3–5] On the basis of these findings, we initiated a clinical trial to evaluate the safety and efficacy of neuroprotective therapy using G-CSF for patients with worsening symptoms of compression myelopathy.[6] In phases I and IIa of the clinical trial, we recruited patients 20–75 years of age, in whom Japanese Orthopaedic Association (JOA) score for cervical and thoracic myelopathy decreased 2 points or more during a recent 1-month period.[6] In the first step of this trial, G-CSF 5 μg/kg/day was intravenously administered for 5 consecutive days in five patients. We then administered G-CSF 10 μg/kg/day for 5 consecutive days in 10 patients. No serious adverse events occurred during or after treatment, and all patients showed neurological improvement, although G-CSF 10 μg/kg/day resulted in better neurological recovery. Thus, we suggested that intravenous administration of G-CSF at a dosage of 10 μg/kg/day for 5 days is an appropriate protocol for G-CSF neuroprotective therapy.[6]

During this trial, we encountered an unexpected finding – two patients in whom neuropathic pain associated with thoracic myelopathy was dramatically reduced after G-CSF administration. Such a pain-relieving effect of G-CSF had not been included as an endpoint in this

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trial. However, the effect is a significant feature with implications for future clinical use of G-CSF for compression myelopathy.

Case reports

Case 1

A 32-year-old man was admitted to our hospital complaining of progressive motor weakness of his lower extremities and gait disturbance. On admission, his JOA score for thoracic myelopathy (motor function: 0–4 points, sensory function: 0–4 points, bladder function: 0–3 points, total possible score = 11 points)[7] was 4 points. He also showed spontaneous severe pain developing from his back to his chest.

Four years prior to this admission, he suffered from thoracic myelopathy because of postvertebral osseous spurs that compressed his spinal cord anteriorly at T7–T10 (Figs. 1A and B). He underwent surgical treatment for T7–T10 anterior decompression with spinal fusion. Before his first surgery, he had complained of gait disturbance and spontaneous pain from his back to his chest. After the surgery, his symptoms of myelopathy and pain were relieved. Three years after the surgery, however, his symptoms began to deteriorate.

Reconstruction images from a computed tomography (CT) myelogram showed that the grafted bone at the T7–T8, T8–T9, and T9–T10 intervertebral disc levels was absorbed, and spine fusion was not obtained (Fig. 1C). The CT images showed regrowth of osseous spurs that compressed his spinal cord anteriorly at T7–T8 and T9–T10 (Figs. 1C and D, arrows) and newly developed ossified ligamentum flavum (OLF) that compressed his spinal cord posteriorly at T9–T10 (Figs. 1C and D, arrowheads).

From the day of admission, he underwent administration of G-CSF (10 μg/kg/day) for 5 consecutive days. One day after the initial G-CSF administration, he felt relief of his back and chest pain. Visual analog scale (VAS) score of his pain was 80 mm before G-CSF administration, and it decreased to 50 mm 1 day after the initial G-CSF administration. At 1 week after the initial administration, his VAS score became 0 mm, and his pain was diminished. He also felt improved muscle strength of his legs, and his JOA score was increased to 6 points at 1 month after the administration.

According to the protocol for G-CSF neuroprotective therapy for worsening symptoms of compression myelopathy, we followed the patients without surgical treatment for 1 month after G-CSF administration.[6] At 1 month after the administration, he underwent surgery for decompression of the spinal cord using a posterior approach and T4–T12 posterior instrumented fusion. At 6 months after the administration, his recovery from myelopathy was maintained (JOA score = 6 points) with no recurrence of pain.

Case 2

A 68-year-old man was admitted to our hospital with a complaint of motor weakness of his lower extremities and gait disturbance. On admission, JOA score was 4 points. In addition to the symptoms of myelopathy, he complained of spontaneous severe bilateral pain at the level of his thigh.

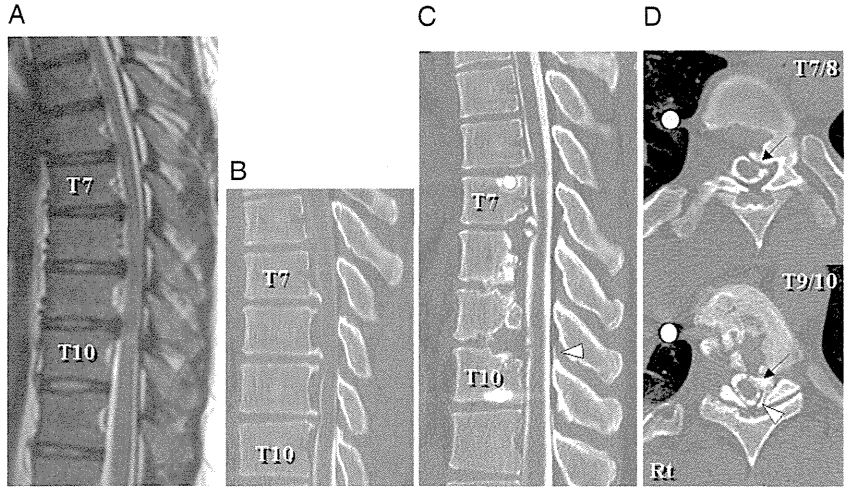

Figure 1 Case 1: T2-weighted midsagittal magnetic resonance image (A) and CT myelogram midsagittal reconstruction plane (B) 4 years prior to this admission showing anterior compression of the spinal cord by postvertebral osseous spurs at T7–T10. CT myelogram midsagittal reconstruction plane (C) and axial planes at T7–T8 and T9–T10 (D) on admission showing re-growth of the osseous spurs that compressed the spinal cord anteriorly at T7–T8 and T9–T10 (C, D, arrows) and a newly developed ossified ligamentum flavum (OLF) that compressed the spinal cord posteriorly at T9–T10 (C, D, arrowheads).

From 10 years earlier, his gait had become progressively unstable. Beginning 2 months previously, his gait disturbance progressed rapidly, and he could not walk without canes on admission. He had also felt severe bilateral thigh pain for the previous 2 months.

Sagittal magnetic resonance and reconstruction CT images showed that his spinal cord was severely compressed posteriorly by an OLF at T10–T11 (Figs. 2A–C).

From the day of admission, he underwent administration of G-CSF (10 μg/kg/day) for 5 consecutive days. One day after the initial G-CSF administration, he felt relief of his pain at his bilateral thigh. His VAS score for pain was 90 mm before the G-CSF administration, and it decreased to 40 mm 1 day after the initial G-CSF administration. His myelopathy also improved, and his JOA score became 6.5 points 1 month after G-CSF administration.

At 1 month after the administration, he underwent surgery for T10–T12 laminectomy. At 3 months after the administration, his pain recurred and the VAS score increased to 60 mm. After that, however, further aggravation of his pain did not occur, and the VAS score was 60 mm 6 months after the administration. The recovery from myelopathy was also maintained, and the JOA score was 6.5 points 6 months after G-CSF administration.

Discussion

Neuropathic pain has been defined as a type of pain arising from the direct consequence of a lesion affecting the somatosensory system such as in the brain, spinal cord, or peripheral nerves.[8,9] Among numerous diseases of the spinal cord, neuropathic pain following SCI has been studied most commonly. Previous studies have classified neuropathic pain from spinal cord lesions into two types: at-level pain and below-level pain.[1] At-level pain is characterized as pain located within two or three spinal segments below the neurological level of the spinal cord lesion. In contrast, below-level pain presents diffusely caudal to the level of the spinal cord lesion.

In case 1, the patient complained of spontaneous severe pain developing from his back to his chest. We suggest that his pain is a typical at-level pain originating from the spinal cord lesions at vertebral levels T7–T10. In case 2, the patient complained of spontaneous severe bilateral thigh pain corresponding to dermatome levels L2–L3. In this patient, the spinal cord was compressed by a T11–T12 OLF. Anatomically, the spinal cord level compressed by a T11–T12 OLF is considered to be the upper portion of the epiconus, where multiple spinal cord segments (usually L2–L5) are densely located.[10] Thus, we suggest that the thigh pain of this patient is also at-level pain. In the present two cases, G-CSF administration resulted not only in recovery from myelopathy, but also in reduction of neuropathic pain. In case 1, the VAS score was 80 mm before G-CSF administration, and it became 0 mm at 1 week after administration. In case 2, the pre-administration VAS score was 90 mm, and it decreased to 40 mm 1 day after G-CSF administration. In both cases, decompression surgery was performed 1 month after G-CSF administration. Thus, we suggest that the pain reduction observed in the present two cases during the 1 month after G-CSF administration was caused by the pharmacological effect of G-CSF and not by surgery. After surgery, however, the VAS score of both

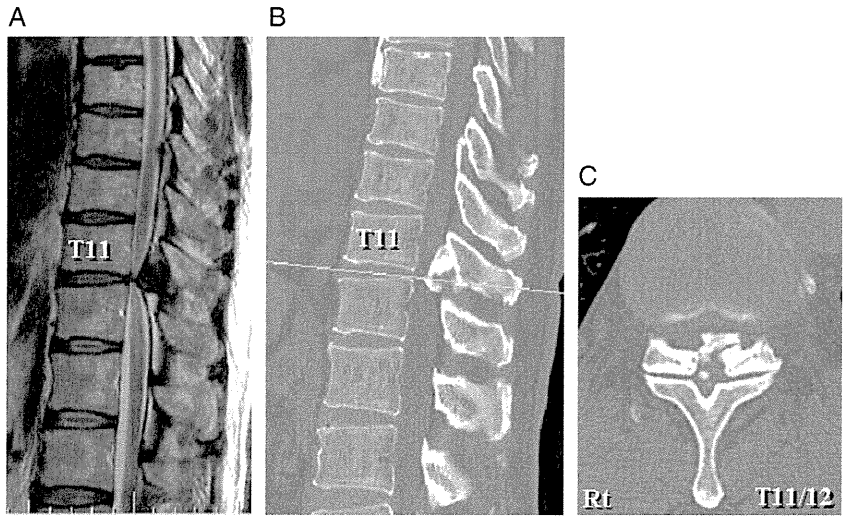

Figure 2 Case 2: T2-weighted midsagittal magnetic resonance image (A) and CT midsagittal reconstruction plane (B) and CT axial plane at T11–T12 (C) showing posterior compression of the spinal cord by an OLF at T11–T12.

cases did not necessarily reflect the neuroprotective effect of G-CSF. Despite the confounding factor of surgery, the present findings suggest that G-CSF may have a therapeutic effect on neuropathic pain in patients with thoracic compression myelopathy.

To the best of our knowledge, no reports of experimental studies of G-CSF administration in an animal model of spinal neuropathic pain have been published. In our studies using animal models of compression-induced and contusive SCI, intravenously administered G-CSF resulted in functional recovery by (1) promoting the migration of bone marrow-derived cells into the damaged spinal cord, (2) directly suppressing the neural apoptosis that occurs via G-CSF receptors at the injured spinal cord, and (3) decreasing the expression of inflammatory cytokines such as IL-1β and TNF-α.[3–5] Ro *et al.*[9] administered G-CSF to animal models of peripheral neuropathic pain, and demonstrated that G-CSF increased the number of opioid-contained polymorphonuclear cells and relieved neuropathic pain. We suggest that such mechanisms may participate in the pain-relieving effect of G-CSF on spinal neuropathic pain, although further studies are required to fully clarify all of the underlying mechanisms.

Among numerous diseases of the spinal cord, neuropathic pain following SCI has been studied most extensively.[8] Investigators have suggested that pregabalin, gabapentin, and tricyclic antidepressants are optimal first-line treatments for neuropathic pain associated with SCI. Furthermore, serotonin–norepinephrine reuptake inhibitors are considered to be second-line choices, and tramadol, opioids, and lamotrigine are used as third-line options. However, these researchers concluded that such oral pharmacological intervention is often inadequate, commonly resulting in a reduction of only 20–30% in pain intensity.[8] To date, therefore, no effective therapies for spinal neuropathic pain have been established.

Conclusion

To the best of our knowledge, this is the first report showing the therapeutic effect of G-CSF on neuropathic pain associated with compression myelopathy. We cannot deny the possibility that the placebo effect of injection and the surgical intervention contributed to the pain relief. On the basis of the experience of the present cases, however, we intend to advance to a clinical trial to verify the feasibility of using G-CSF for relief of spinal neuropathic pain. If the efficacy and safety of G-CSF treatment for spinal neuropathic pain is confirmed and clinical use of G-CSF therapy is approved, a novel and effective approach for the treatment of this disorder will be available.

Acknowledgement

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PLOS ONE

Granulocyte Colony-Stimulating Factor (G-CSF) Protects Oligpdendrocyte and Promotes Hindlimb Functional Recovery after Spinal Cord Injury in Rats

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Abstract

Background: Granulocyte colony-stimulating factor (G-CSF) is a protein that stimulates differentiation, proliferation, and survival of cells in the granulocytic lineage. Recently, a neuroprotective effect of G-CSF was reported in a model of cerebral infarction and we previously reported the same effect in studies of murine spinal cord injury (SCI). The aim of the present study was to elucidate the potential therapeutic effect of G-CSF for SCI in rats.

Methods: Adult female Sprague-Dawley rats were used in the present study. Contusive SCI was introduced using the Infinite Horizon Impactor (magnitude: 200 kilodyne). Recombinant human G-CSF (15.0 μg/kg) was administered by tail vein injection at 1 h after surgery and daily the next four days. The vehicle control rats received equal volumes of normal saline at the same time points.

Results: Using a contusive SCI model to examine the neuroprotective potential of G-CSF, we found that G-CSF suppressed the expression of pro-inflammatory cytokine (IL-1 beta and TNF- alpha) in mRNA and protein levels. Histological assessment with luxol fast blue staining revealed that the area of white matter spared in the injured spinal cord was significantly larger in G-CSF-treated rats. Immunohistochemical analysis showed that G-CSF promoted up-regulation of anti-apoptotic protein Bcl-Xl on oligpodendrocytes and suppressed apoptosis of oligodendrocytes after SCI. Moreover, administration of G-CSF promoted better functional recovery of hind limbs.

Conclusions: G-CSF protects oligodendrocyte from SCI-induced cell death via the suppression of inflammatory cytokines and up-regulation of anti-apoptotic protein. As a result, G-CSF attenuates white matter loss and promotes hindlimb functional recovery.

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Introduction

Acute spinal cord injury (SCI) is divided into two pathological phases termed primary and secondary injury [1]. The primary injury consists of focal tissue destruction caused by direct mechanical trauma. This physical insult then initiates the second phase of injury which is a pathophysiological reaction of spinal cord. Apoptosis of neurons and glial cells left intact by the initial trauma occurs during the secondary phase. In addition, oligodendrocytes distant from the immediate site of injury undergo apoptosis. Maximal cell death occurs one week after injury and leads directly to demyelination [2]. Several *in vivo* studies have demonstrated that the amount of spared white matter correlates to residual locomotor function [3,4]. Thus, protection of oligodendrocytes from apoptotic cell death might reduce demyelination and improve functional recovery. Many factors could exacerbate the secondary phase of injury, including vascular changes, increased concentrations of free radicals and free fatty acids, ionic mechanisms of axonal injury, glutamate excitotoxicity, and immune and inflammatory reactions [5]. Currently, high-dose methylprednisolone (MP) in acute SCI is an accepted treatment for attenuation of secondary injury [6]. However, it has become controversial in recent years due to the risk of serious adverse effects and its modest neurological benefits [7]. Therefore, development of new drug therapies which can substitute for high-dose MP is an area of intense study.

Granulocyte colony-stimulating factor (G-CSF) is a 19.6 kDa glycoprotein that was initially identified as a serum factor that induced differentiation of a murine myelomonocytic leukemic cell line [8]. It is widely known as a hematopoietic cytokine that promotes survival, proliferation and differentiation of cells of the

neutrophil lineage [8,9]. It is used clinically for patients with leukocytopenia and for donors of peripheral blood-derived hematopoietic progenitor cells prior to collection for transplantation [10].

Within the central nervous system (CNS), G-CSF has pleiotropic actions. In recent years, the beneficial effects of G-CSF have been demonstrated in rodent stroke models [11–15]. Moreover, clinical trials of G-CSF for stroke reported its safety and feasibility [16]. In the case of SCI, several research groups including us previously reported that G-CSF treatment promoted functional recovery in the mouse and rat SCI models [17–22].

Although the beneficial effects of G-CSF on neurons are partially understood, little is known about G-CSF-mediated reduction of apoptosis of oligodendrocytes after SCI. Therefore we hypothesized that G-CSF could attenuate apoptosis of oligodendrocytes and, as a result, improve white matter preservation and functional recovery. This may represent another mechanism by which G-CSF provides neuroprotection following SCI. In the present study, our aim was to assess the anti-apoptotic effects of G-CSF on oligodendrocytes and to elucidate the mechanism using the rat contusive SCI model.

Results

G-CSF Receptor (G-CSFR) Expression

To assess the expression of G-CSFR, we performed immunofluorescence analysis on histological sections of spinal cords. The data revealed that G-CSFR was expressed on neurons, astrocytes and oligodendrocytes in normal spinal cord (Fig. S1A–C). One week after injury, G-CSFR was expressed on glial fibrillary acidic protein (GFAP) -positive astrocytes and myelin oligodendrocyte-specific protein (MOSP) -positive oligodendrocytes (Fig. 1). Quantification of G-CSFR/MOSP double-positive cells showed significant increase of the number of MOSP-positive oligpodendrocytes in the G-CSF-treated group 2 mm rostral and caudal to the epicentre (Fig. S1D). Almost all of MOSP-positive oligodendrocytes expressed G-CSFR in both the vehicle and G-CSF groups (Fig. S1D).

Expression of Inflammatory Cytokines after SCI

To detect the anti-inflammatory effects of G-CSF in the SCI model, we performed Real-Time PCR for interleukin 1-beta (IL-1β), tumor necrosis factor-alpha (TNF-α), FAS, FAS ligand (FASL), interferon-gamma (IFN-γ), matrix metalloproteinase-2 (MMP2) and matrix metalloproteinase-9 (MMP9) (Fig. 2). The study revealed that 12 h after surgery, expression of IL-1β and TNF-α mRNAs was significantly suppressed in the G-CSF group (Fig. 2 A, B, closed columns) compared to the vehicle control group (Fig. 2 A, B, open columns). Specifically, expression of IL-1β mRNA in the G-CSF group was 3.36-fold lower than in the vehicle group (Fig. 2 A, $p<0.05$). For TNF-α mRNA in the G-CSF group, expression was 1.98-fold lower than in the vehicle group (Fig. 2 B, $p<0.05$). Twenty-four h and 72 h following surgery, expression of IL-1β and TNF-α mRNAs tended to be lower than controls; however, the differences were not statistically significant. The results of Real-Time PCR for the other factors showed no significant difference between the vehicle and G-CSF-treated groups.

To further confirm G-CSF-mediated attenuation of SCI-induced up-regulation of IL-1β and TNF-α, we performed western blot analysis for IL-1β and TNF-α on protein samples extracted from spinal cord with or without G-CSF treatment 24 and 72 h following spinal cord injury. Western blot analysis revealed that G-CSF suppressed protein expression of IL-1β and TNF-α 72 hours after the injury (Fig. 2C, D, $p<0.05$).

Enumeration of Early Inflammatory Leukocytes and Microglia/macrophages

Immunohistochemistry for IL-1β and myeloperoxidase (MPO, a marker for leukocyte) revealed that the frequencies of cells positive for MPO were not statistically different in the two groups 12 and 24 h after surgery (data not shown). In contrast, the frequencies of cells positive for both IL-1β and MPO was significantly smaller in the G-CSF group than in the vehicle group 12 and 24 h after surgery (Fig. 3D). Twelve hours after surgery, the mean numbers of double positive cells per section in the G-CSF group were 52 ± 3.8 in 2 mm rostral to the epicenter and 56 ± 2.5 in 2 mm caudal to the epicenter, respectively (Fig. 3D). In contrast, the frequencies of double positive cells in the vehicle group were considerably higher 12 hours after surgery (103 ± 4.0 per section in 2 mm rostral to the epicenter and 118 ± 5.3 per section in 2 mm caudal to the epicenter, respectively; Fig. 3D). Twenty-four hours after the injury, the G-CSF group showed decrease of the number of IL-1β and MPO double-positive cells as same as 12 hours after injury (Fig. 3D). Double immunofluorescence study for ionized calcium-binding adaptor molecule 1 (Iba-1, as a marker for activated microglia and macrophages) and inducible nitric oxide synthase (iNOS, as a marker for Th1-driven activation of microglia/macrophages) or arginase-1 (a marker for Th2-driven activation of microglia/macrophages) was performed to elucidate G-CSF-mediated reaction and phenotypic alteration of macrophage/microglia. The number of Iba-1-positive cells in the G-CSF group was significantly smaller than that in the vehicle group in the rostral and caudal segments (Fig. S3), whereas the ratios between iNOS and arginase-1 did not change in both the vehicle and G-CSF groups in lesioned spinal cord at any segments observed (Fig. S3).

Suppressed Apoptosis of Oligodendrocytes

Near the epicenter of the injury, apoptotic oligodendrocytes (adenomatous polyposis coli; APC^+ and caspase 3^+ cells) were observed (Fig. 4 A–C, arrowheads). The percentages of apoptotic oligodendrocytes were significantly smaller in the G-CSF group than that in the vehicle group both 72 h and 1week after surgery. Specifically, 72 hours after surgery, the mean percentages of apoptotic oligodendrocytes among the sections were 32.0% in the G-CSF group and 47.8% in the vehicle group (Fig. 4 D). The percentages of apoptotic oligodendrocytes were significantly suppressed in most sections: 4 mm and 6 mm rostral to the epicenter ($p<0.05$) and 6 mm caudal to the epicenter ($p<0.01$). Significance was not reached for the section 4 mm caudal to the epicenter. One week after surgery, the mean percentages of apoptotic oligodendrocytes among sections were 13.9% in the G-CSF group and 35.2% in the vehicle group (Fig. 4 E). The percentages of apoptotic oligodendrocytes were significantly suppressed in all of the sections: 6 mm rostral to the epicenter ($p<0.05$), and 4 mm rostral and 4 mm and 6 mm caudal to the epicenter ($p<0.01$). To further confirm the results of immunohistochemistry for apoptotic oligodendriocytes, we performed double immunofluorescence study for MOSP as another marker for oligodendrocytes and activated caspase-3 as a marker for apoptotic cells (Fig. S4). The staining pattern was similar to that of the double fluorescence study for APC and activated caspase-3, suggesting that the data of apoptotic oligodendrocytes were convincing.

There was no APC- and Bcl-Xl-double positive cells in vehicle control rats 1 week after injury (Fig. 4 I–K), whereas a part of

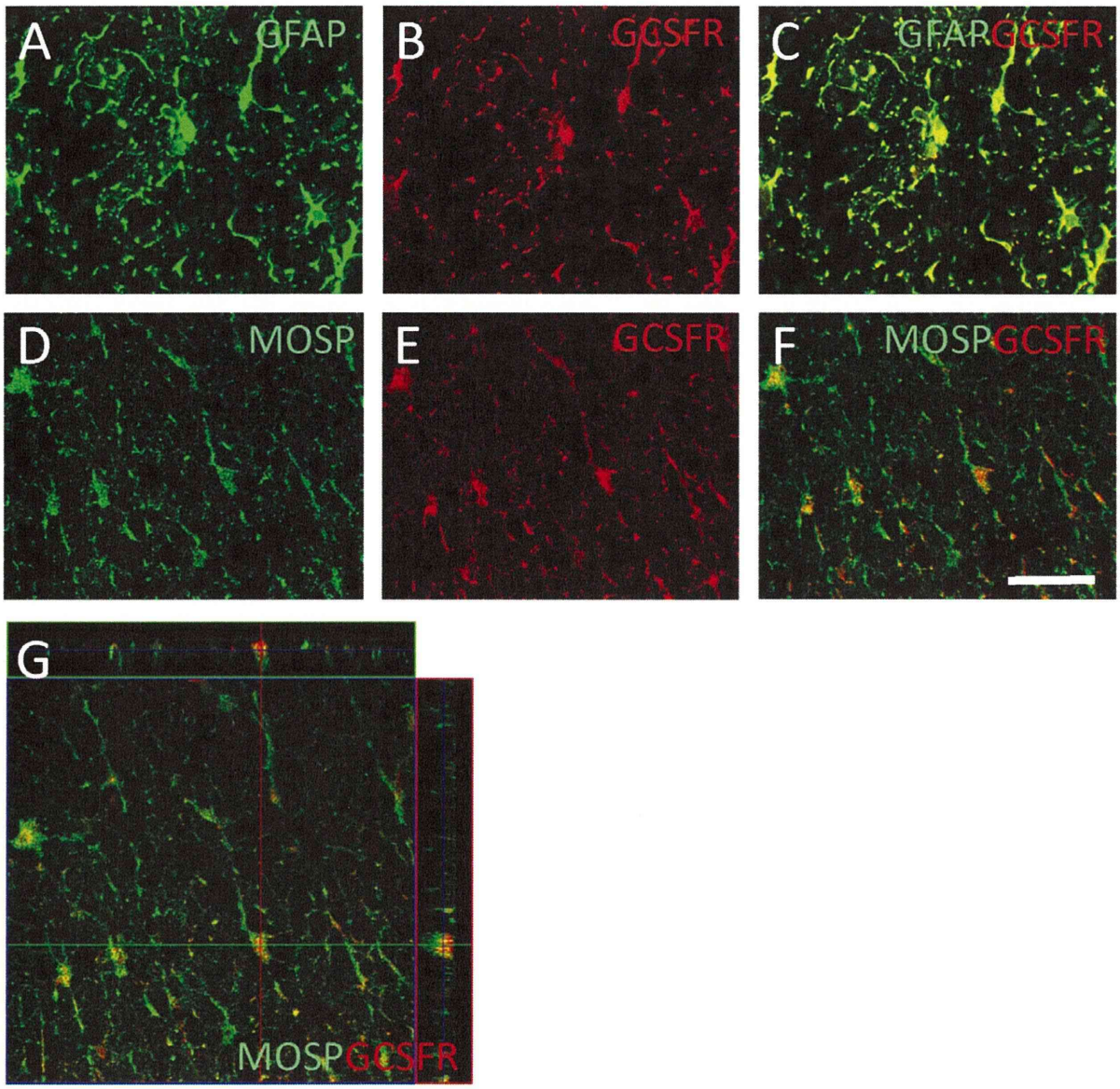

Figure 1. Granulocyte colony-stimulating factor receptor (G-CSFR) expression. Immunofluorescent double labeling for G-CSFR and cell-specific markers 1 week after surgery in vehicle-treated rats. Double-positive cells for G-CSFR and glial fibrillary acidic protein (GFAP, marker for astrocytes; A–C) and G-CSFR and myelin oligodendrocyte specific protein (MOSP, marker for oligodendrocytes; D–G) were detected. To show co-localization precisely, positive signal for G-CSFR/GFAP and G-CSFR/MOSP were detected using confocal laser microscopy (A–F) and 3-dimensional image was reconstructed (G). Bars = 50 μm.
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APC-positive cells simultaneously expressed Bcl-Xl in G-CSF-treated rat 1 week after injury (average 31.8% of APC-positive cells expressed Bcl-Xl, Fig. 4F–H).

The number of MAP-2-positive neurons was significantly larger in the G-CSF group than that in the vehicle group in the rostral and caudal segments to the lesion epicenter (Fig. S2).

White Matter Sparing after SCI

Luxol fast blue (LFB) staining 6 weeks after injury revealed better myelin integrity and preservation in G-CSF-administered rats in increased magnification of the section (Fig. 5C, D, 4 mm caudal to epicenter). The percentage of normal-appearing myelin in the G-CSF group (Fig. 5E, closed columns) was significantly higher than that in the vehicle group (Fig. 5E, open columns) in all the segments analyzed except for the lesion epicenter.

Immunohistochemistry for myelin basic protein (MBP) 6 weeks after injury showed preservation of myelin sheath in G-CSF-treated animals. The number of MBP-positive myelin sheath in the G-CSF group (Fig. 5H, K and Fig. 5L, closed columns) was significantly larger than that in the vehicle group (Fig. 5G, J and Fig. 5L, open columns).

Recovery of Hindlimb Motor Function

All rats had a full score (21 points) prior to surgery, and the score dropped to zero immediately after SCI. Hindlimb function showed a significant recovery in rats in the G-CSF group six weeks after surgery compared with that of the vehicle group (Fig. 6A).

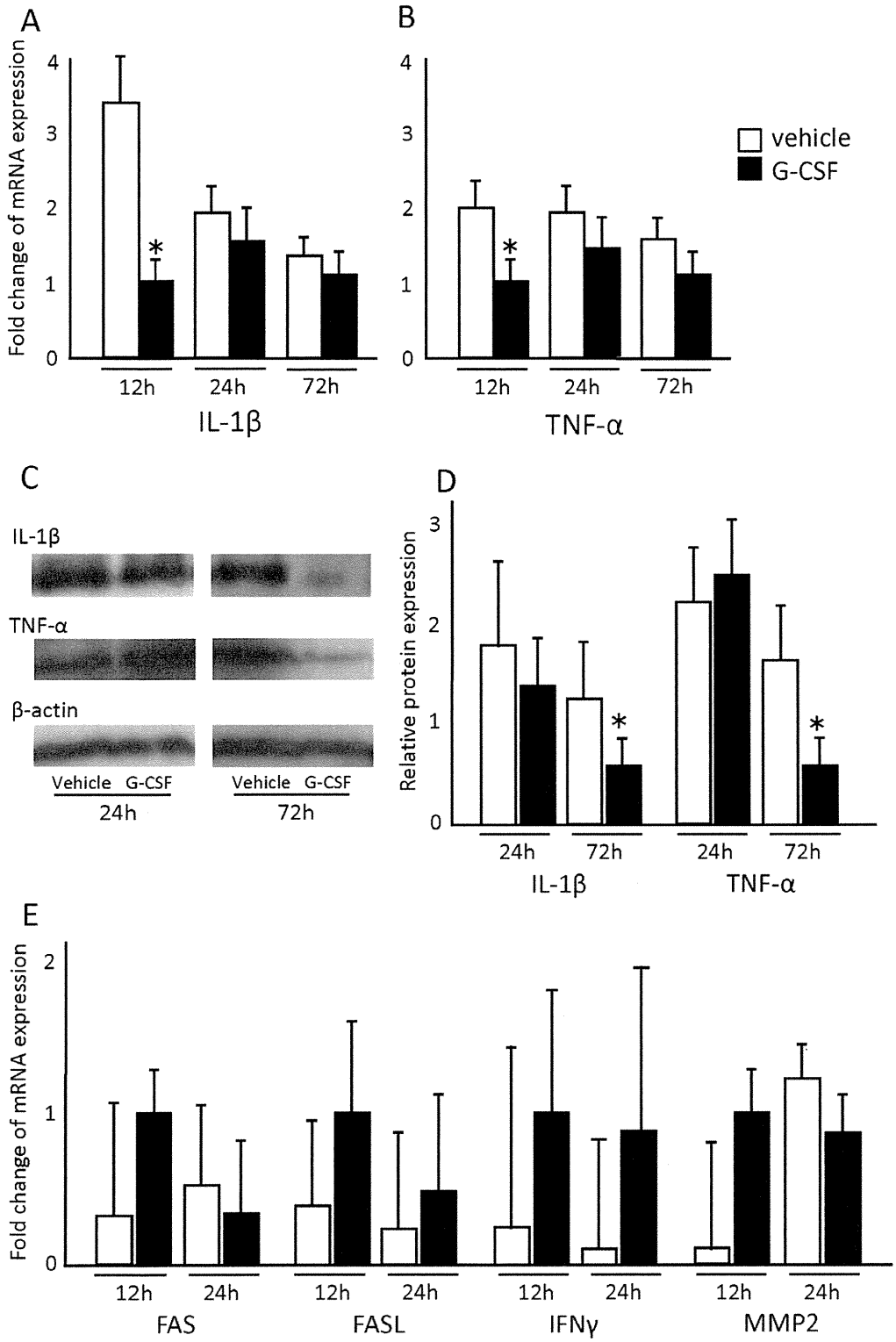

Figure 2. G-CSF suppressed pro-inflammatory cytokine expression. Real time quantitative PCR and western blot analysis were performed to quantify mRNA and protein expression of pro-inflammatory cytokines in the acute phase of spinal cord injury. Values obtained were normalized to value of 18S ribosomal RNA expression and were expressed as the fold-increase over values of the G-CSF group 12 hours after surgery. The expression of interleukin-1 beta (IL-1 β, A) and tumor necrosis factor-alpha (TNF-α, B) mRNA was significantly suppressed in the G-CSF group (closed column) compared with the vehicle group (open column) 12 hours after surgery. Western blot analysis revealed that G-CSF suppressed the expression level of IL-1 β (C, upper row right, D, closed column, $p<0.05$) and TNF-α (C, middle row right, D, closed column, $p<0.05$). There was no significant difference between the vehicle and G-CSF groups in mRNA expression of FAS, FASL, IFN-γ, MMP-2 and MMP-9. Values are mean±SEM. *$p<0.05$.
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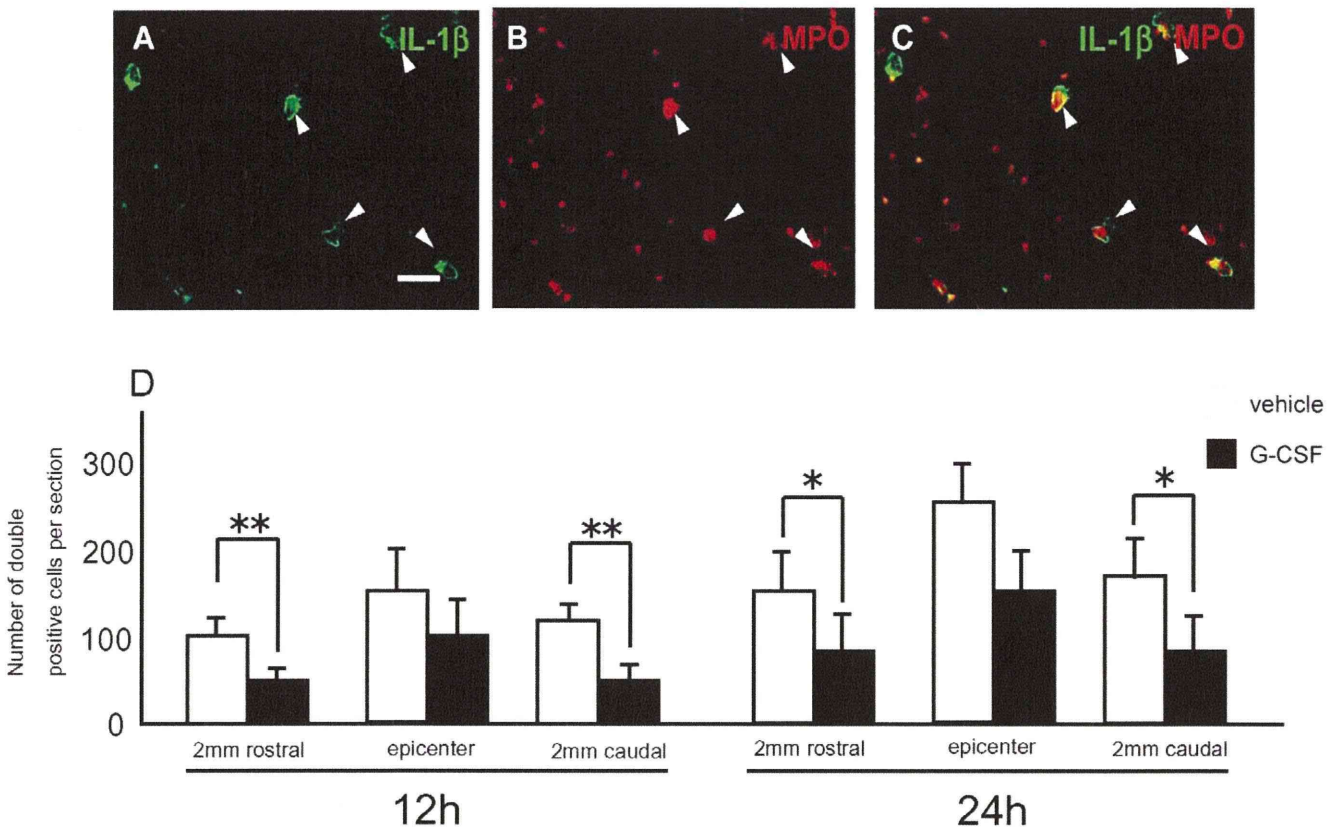

Figure 3. G-CSF decreased the number of IL- β expressing leukocytes. Immunohistochemistry for IL-1β and myeloperoxidase (MPO) in the acute phase of injury. Near the lesion epicenter, IL-1β-positive round cells were also positive for MPO, a marker for leukocytes (A–C, arrowheads). The number of double positive cells for IL-1β and MPO was significantly smaller in the G-CSF group (D, closed column) than in the vehicle group (D, open column). Bars = 50 μm Values are mean±SEM. *$p<0.05$.
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The average recovery score six weeks post-surgery was significantly higher in the G-CSF group (Fig. 6B, closed circle, $p<0.01$) than the vehicle group (Fig. 6B, open circle). The average final score in the G-CSF group was 12.5±0.9 (9 to 16), indicating frequent (51–94%) to consistent weight-supported plantar steps *and* occasional (<50%) forelimb-hindlimb coordination. In contrast, the score was 9.5±0.3 (7 to 13) in the control group, indicating plantar placement of the paw with weight support in stance only (i.e., when stationary) or occasional, frequent, or consistent (95–100%) weight-supported dorsal stepping and no plantar stepping. The mean value of the inclined plane test was significantly higher in the G-CSF group than in the vehicle group (50.0 to 31.7, Fig. 6C, $p<0.01$). Finally, there was good correlation between the percentage of normal myelin and the final motor function score ($r = 0.676$, $p<0.01$, not shown). Those results showed that five days treatment with G-CSF had an impact on the animal status six weeks later.

Discussion

The present results showed the beneficial effects of G-CSF in the setting of SCI. G-CSF significantly reduced injury-induced up-regulation of IL1β and TNF-α expression in mRNA and protein levels and the number of infiltrating leukocytes and activated microglia/macrophages which potentially involve in SCI-induced inflammatory reactions. Furthermore, early treatment with G-CSF significantly attenuated subsequent apoptosis of oligodendrocytes and white matter degeneration, and promoted better long-term functional recovery of the hindlimbs.

It is believed that the injury-induced up-regulation of inflammatory cytokines substantially contributes to secondary injury following SCI. Exogenous administration of IL1β has been shown to exacerbate ischemic damage, whereas administration of an endogenous IL-1β receptor antagonist [23] or a neutralizing antibody [24] reduced brain damage and edema when administered before a stroke in rats. Previous reports showed that G-CSF mediates anti-inflammatory effects after a variety of infections [25]. G-CSF decreases monocytic production of pro-inflammatory cytokines such as IL-1β and TNF-α *in vitro* [25,26]. And, *in vivo* studies showed that G-CSF also suppresses the expression of IL-1β in a cerebral infarct model [11] and TNF-α in experimental encephalomyelitis [24]. In the current study, G-CSF significantly reduced the expression of IL-1β and TNF-α mRNA in the acute phase after SCI. Moreover, results of immunofluorescence double staining for IL-1β and MPO suggest that G-CSF reduced inflammatory cytokine expression by neutrophils, whereas G-CSF had no influence on the extent of neutrophil infiltration. The discrepancy between the results of real-time PCR and immunofluorescence may be caused by the time lag between transcription and translation. The results obtained here conflict with a report that G-CSF reduced neutrophil infiltration in a model of splanchnic ischemia and reperfusion [27]. This discrepancy might be caused by general differences between the experimental models and/or unique microenvironments within the organs. Furthermore, the present results showed that GCSF decreased the