V. *H. pylori* 感染症関連疾患と除菌治療の意義

特発性血小板減少性紫斑病（ITP）

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Idiopathic thrombocytopenic purpura

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Abstract

In many extragastric manifestations with *Helicobacter pylori*（HP）infection, the most convincing evidences were observed in idiopathic thrombocytopenic purpura（ITP）. The prevalence of HP infection in ITP was not different to general population. The high association of HP infection in ITP was reported in Italy and Japan but low in USA and UK. After eradication therapy, platelet counts response was found in over 50 % of ITP cases in Japan and Italy, and about 40 % in other countries. The platelet counts response following eradication was maintain for long time with drug free and the relapse case was rare.

The platelet elevation reaction by eradication was not influenced by previous treatment, sex and age, but the disease duration as ITP and the platelet count before eradication were controversial factors. The main side effects of eradication were diarrhea, vomiting and skin rash.

Based on these evidences, ITP guideline in Japan（2012）recommended that eradication therapy was the first choice treatment for HP positive ITP cases.

Key words: ITP, *Helicobacter pylori*, eradication therapy, platelet recovery

はじめに

特発性血小板減少性紫斑病（ITP）は，基礎疾患や原因の明らかでない血小板減少によって皮膚や粘膜に紫斑を主体とする出血症状をきたす後天性の出血性疾患である．

この中で，成人に多く緩慢に出血傾向を繰り返す慢性ITPは出血性疾患の中で日常最も遭遇する機会が多く，中高年齢者に多い傾向のある疾患で，厚生労働省の難治性疾患の一つに特定されている．約15年前に慢性ITPの中にヘリコバクター・ピロリ菌（*H. pylori*，以下HP）感染との関連を示す臨床治療成績が報告され[1]，以来本症とHPの関連について多くの臨床研究がなされている．

1. ITPの病態の概略

ITPの発症機序としては種々の原因で血小板膜特異糖タンパク（GPIIb/IIIa，GPIb/IX/V，GPVI，GPVなど）に対する自己抗体が産生され，これによって感作された血小板が主として脾臓を中心とする網内系細胞のFcレセプターを介

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して貪食破壊され，血小板減少が生じる．一方ではこの抗体は骨髄巨核球にも作用し，巨核球の成熟を抑制する結果，血小板産生低下を生じることが明らかにされた．したがって血小板減少の原因には，血小板の破壊亢進と産生低下が考えられている[2]．

このことから特発性(idiopathic)というよりむしろ免疫性血小板減少性紫斑病(immune thrombocytopenic purpura: ITP)としての概念が主流である．免疫性血小板減少性紫斑病には免疫学的機序の引き金が明らかである場合と原因が不明の場合があり，前者は続発性免疫性血小板減少性紫斑病，後者は原発性免疫性血小板減少性紫斑病と分類され，一般には特発性血小板減少性紫斑病を指している．

1998年イタリアのGasbarriniらは，この特発性血小板減少性紫斑病の中でHP感染陽性症例に対して除菌療法を行ったところ血小板増加反応を示した症例を報告した[1]．

以来HP陽性ITPに対する除菌療法により血小板増加反応を示す事例が多く報告され，HP感染が血小板減少を引き起こす可能性が示唆されている．その結果現在ではHP陽性で除菌療法により血小板増加反応が認められたITP症例は，ヘリコバクター・ピロリ菌関連ITP(HP関連ITP)と呼ばれ続発性免疫性血小板減少性紫斑病の範疇に入れられている[3]．

2. ITPにおけるHP感染頻度とHP感染ITPの臨床病態

HP感染と消化器系疾患以外との関連ではITPについて最も多くの報告がなされている．すなわち除菌療法が疾患改善に関係している客観的解析を基に，一部のITPの発症にHP感染が関係していることが明らかとなっている[4-6]．

1) HP感染頻度

ITPにおけるHP感染率は，イタリア，日本では一般人口の感染率と同様に70-90％と高く，他の先進国では36.7％と低い．特にITPに感染率が高いわけではなくHP感染者の極一部にITPが発症する．世界的にITPを含めてHP陽性率に性差は認められていない．

小児のHP感染率は成人に比し低く，成人と同様に社会背景，環境によって左右され，台湾，日本，イタリアでは20-40％が陽性であるが，北欧では0％である．除菌による血小板増加効果も成人と同様であるが，小児に多い急性ITPにおいてはHP感染との関連は有意ではなく，急性ITPの発症にHPはかかわっていない．

2) HP感染ITPの臨床病態

厚生労働省研究班におけるレトロスペクティブな共同研究や世界の文献レビューの結果から以下の点が明らかになっている[6,7]．

HP陽性ITP症例群とHP陰性ITP症例群との臨床的背景については，性差，ITP罹病期間，出血症状を中心とする臨床症状において両群間で差は認められない．ただし年齢はHP陽性群が陰性群に比し有意に高い．またHP陽性ITPでは有意に初診時血小板数が激減している症例は少なく，骨髄巨核球数は増加している症例が多く，ITPの病態として重症例は少ない傾向がある．

また我が国ではHP感染ITPにおける胃腸障害の頻度や程度は非感染ITPと同様であるが，米国ではdyspepsiaが多く認められる[6]．

3. HP除菌によるITP病態への影響

1) 血小板増加反応

1998年のGasbarriniらの報告以来，HP除菌療法による血小板増加効果を検討した数多くの報告がある[5-7]．その一覧を**表1**に示したが，除菌による血小板増加効果は国によって有効率に差があり，スペイン，英国，米国からの報告では除菌による血小板増加効果を示す症例が少ない．一方イタリアや日本からの報告では，一部を除き約50％以上の症例に血小板増加反応が認められている(33-100％)．

地域による除菌後の血小板増加効果の差に関して①対象者の免疫学的背景に差がある，②地域的に感染したHP菌株の違いによって発現している抗原性の程度が異なる(Cag，Vagがコードするタンパク抗原，Lewis(Le)抗原など)ことなどが推測されている．

このうち感染菌株について我が国の報告で

表1 成人慢性ITPにおけるHP陽性率と除菌による血小板増加効果

報告者(国)	報告年	症例数	HP陽性例数(%)	除菌成功症例数	血小板増加症例(%)	平均観察期間(月)
Gasbarrini, et al(伊)	1998	18	11(61)	8	8(100)	4
Emilina, et al(伊)	2001	30	13(43)	12	6(50)	8.3
Jarque, et al(スペイン)	**2001**	**56**	**40(71)**	**23**	**3(13)**	**24**
Kohda, et al(日)	2002	48	27(56)	19	12(63)	14.8
Hino, et al(日)	2003	30	21(70)	18	10(56)	15
Hashino, et al(日)	2003	22	14(64)	13	5(39)	15
Ando K, et al(日)	2003	61	50(82)	27	16(59)	11
Michel, et al(米)	**2004**	**76**	**16(21)**	**14**	**0(0)**	**11.5**
Takahashi, et al(日)	2004	20	15(75)	13	7(54)	4
Fujimura, et al(日)	2004	435	300(69)	155	88(57)	12<
Ando T, et al(日)	2004	20	17(85)	17	15(88)	24
Sato, et al(日)	2004	53	39(74)	27	15(56)	6
Inaba, et al(日)	2005	35	25(71)	25	11(44)	6
Veneri, et al(伊)	2005	43	43(100)	41	20(49)	31.2
Stasi, et al(英, 伊)	2005	137	64(47)	52	17(33)	12<
Suzuki, et al(日)	2005	36	25(69)	23	10(44)	6
Suvajdzic, et al(英)	**2006**	**54**	**39(72)**	**23**	**6(26)**	**18**
Satake, et al(日)	2007	38	26(68)	23	13(57)	25.8
Campuzano(南米)	2007	32	29(91)	26	21(81)	12.2
計		1,244	814(65.4)	559	282(50.4)	13.7<

は血小板減少を伴わない単なる胃・十二指腸潰瘍症例とITP症例は同様の菌株で，Cag A, Vac A, ice A, IL-8の発現なども他のHP感染消化器疾患と差はないとされている．したがって我が国では血小板減少を引き起こす特有の菌株や菌の性状は認められない[8]．

我が国のレトロスペクティブな解析では，除菌後12カ月以上経過観察可能な155例(除菌成功群122例，除菌不成功群33例)の長期予後は，除菌成功群のうち79例は血小板数増加を維持し，このうち28例は除菌後無治療で血小板数15万以上となり完全寛解の症例である(23%)．残り51例の多くは血小板数5万以上となりほとんどが無治療観察(PR)状態である(42%)．43例は除菌に成功したものの血小板数の増加が認められない症例で(35%)，除菌後の血小板数は除菌前値と変わりなく軽微な増減にとどまっている．すなわち除菌成功群では65%の症例が12カ月以上血小板増加を維持している．

この頻度は除菌不成功群(9/33，27%)に比し有意に高く，経過による血小板の自然回復よりもHPの除去が血小板増加に密接にかかわっていることが確認された[7]．

世界の報告をまとめたレビューによると696例中完全寛解は42.7%で，全体で50.3%が血小板増加反応を示した．これを日本とその他の国で比較すると，完全寛解率はそれぞれ43.5%，27.3%，全体の血小板増加反応はそれぞれ59.7%，38.3%で，いずれも日本の症例での有効率が高い．一般にHP感染率と除菌による血小板増加効果の間には正の相関があると報告している[6]．

除菌後の血小板増加反応時期は血小板数を測定する時期が報告によってまちまちであり，3日目～1カ月と様々である．イタリアからの報告ではほとんどが2週間以内に増加し，除菌による血小板増加反応は早期に現れる傾向がある．

また長期間の経過観察では血小板減少の再発例はほとんどない．平均4年の観察期間中23例中1例が再発した事例がイタリアから報告されているが，HP再感染によるもので再除菌により血小板増加が得られている[6]．

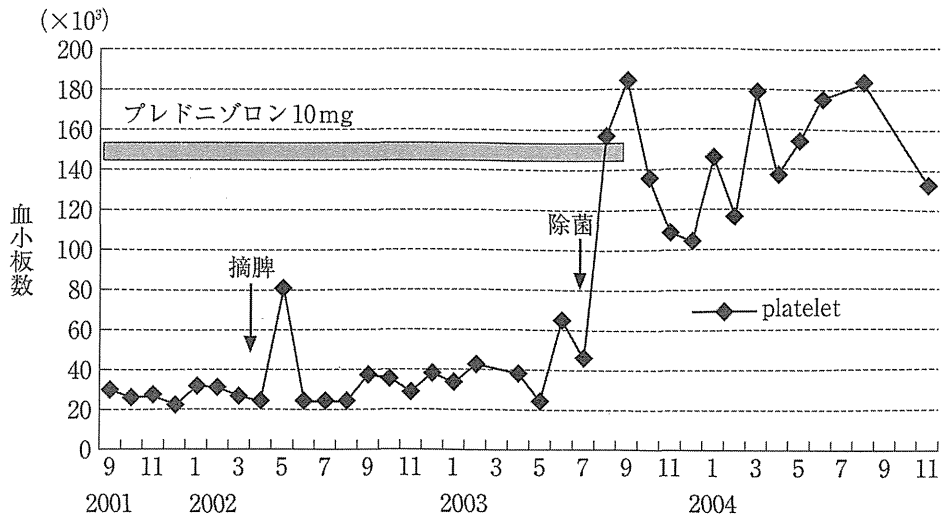

図1 プレドニゾロン，摘脾無効 除菌有効例
2001年1月診断，40歳女性.

以上除菌後の血小板増加効果は従来のITPに対する副腎皮質ステロイドホルモン療法や摘脾療法を凌駕し，これらの治療よりも副作用は少なく，除菌後2-4週後には効果が出始め，血小板増加は長期に持続し再発が非常に少ない特徴がある.

2）除菌療法後血小板増加反応を示す症例の特徴

結論的に除菌療法によって血小板が増加する症例群と，無反応群の間には臨床的背景に著明な差は認められない．すなわち性差，年齢，除菌直前の血小板数，除菌直前のITP治療の有無，治療歴，過去の治療経過などは除菌による血小板増加効果に影響しない．ステロイド療法や摘脾に対して不応性の症例に対しても除菌療法により血小板増加が認められる(**図1**)．しかし我が国では極度に血小板数が少ない(1万以下)症例では血小板増加の程度が軽度である傾向がある[7]．世界のレビューでも血小板数3万以下の症例では完全寛解率20.1％，血小板増加反応は35.2％で，血小板数が少ない症例での寛解率は低い傾向があるが，議論の余地が残されていると指摘している[6]．

我が国ではITPとしての罹病期間が短い群(6.52±4.67年)が長い群(9.85±7.77年)に比し有意に除菌後の有効率が高いが，Stasiらははっきりとした差を見いだしていない[6,7,9]．したがって除菌療法による血小板増加反応を予知する臨床的所見は世界的に一致をみていない.

3）HP除菌療法による血小板増加反応はHP陽性ITPに特異的か

当初除菌療法に使用される薬剤による非特異的な血小板増加反応が生じる可能性が指摘された．その後種々の臨床研究がなされ，除菌療法による血小板増加反応はHP陽性ITPに特異的な現象であることが以下の報告により支持されている.

(1) HP陽性SLEに伴う血小板減少においては，除菌による血小板増加反応は認められない[10].

(2) HP陰性ITP症例に無作為的に除菌を試みた報告では血小板増加反応を示した症例は認められず，除菌療法による非特異的血小板増加作用ではない[11].

(3) HP陽性ITP症例を無作為的に除菌療法群(13例)と非除菌群(12例)の2群に分け血小板増加反応を検討した報告では，除菌群のみに血小板増加が認められる[8].

などである.

以上よりHP陽性ITPの中には除菌治療によって特異的に血小板数が増加を示す症例が多いことから，このようなITP症例をHP関連ITPと呼称する根拠となった.

4）HP感染による血小板減少機序

HPの胃局所感染によるITPの発症機序につ

いては種々の臨床的研究がなされてきた.

除菌により血小板が増加したITP症例でTh1/Th2の比が上昇することは,HP感染が全身的な免疫反応の不均衡を引き起こしていることを示している.またHP陽性ITP症例においては,末梢血において特異的なT細胞のクローナルな増殖が認められ除菌効果に伴ってクローンが消失すると報告され,HPに対する全身的な免疫反応が血小板減少と関連していることが示された[12].

局所感染から血小板減少を引き起こす機序として,①Lewis抗体の関与,②分子相同性[13],③von Willebrand factor(vWF),抗HP抗体による血小板活性化反応による,④HP,HP抗体による血小板活性化に伴うPセレクチン誘導と,それに伴う血小板凝集とアポトーシスなどが報告されている[14].

これらの中では分子相同性を示唆する機序が理解しやすい.HP陽性ITP症例の血小板から誘出した抗体はHPのCag A抗原と反応することが免疫ブロッティング法で明らかにされた[13].すなわちCag A抗原と血小板膜抗原(この場合GPIIb/IIIaと考えられている)の間の分子相同性が推測されている.またCag A IgG抗体価が高い症例には除菌による血小板増加反応を示す症例が有意に多いとの報告は,Cag A抗原がITP発症の免疫反応にかかわっていることを示唆している[9].

感染からITP発症までに時間が経過する点については,自己抗原認識のためのT細胞のプライミングに時間が必要で,いわゆる抗原エピトープの拡大に要する時間と考えれば理解可能である.今後はこれらの仮定を証明することが必要である.

5) 除菌療法による血小板増加機序

除菌療法による効果は早期に認められ,しかも寛解状態が長期間続く特徴がある.この機序としてHP感染によって単球やマクロファージなどの血小板貪食細胞のFcγRのバランス(FcγRIIA/IIB)が変調をきたし,FcγRIIBが抑えられ貪食機能がより亢進し血小板減少に導いていることが推測された.除菌後1週間では既に貪食を抑制するFcγRIIBの発現が亢進することが示され,貪食能が低下する結果血小板増加反応が起こると考えられている[15].したがって早期の血小板増加反応は,FcγRのバランスの正常化が関与し,長期間の血小板増加反応の維持にはHPが除去され抗原刺激が消失することが推測されている.

6) 除菌療法の副作用

我が国の集計では222例中39例(17.6%)に,世界的には482例中56例(11.6%)に軽度から中等度の副作用が認められている.ほとんどが消化器症状で下痢,軟便,胃部不快感などである.我が国ではこのほか皮疹が9例に認められた.

おわりに

従来より成人の慢性ITPは完全寛解率が約30%程度であり,多くは何らかの治療継続や経過観察が必要であった.しかしこの中に多くのHP陽性ITP症例が含まれていることから,除菌療法が行われ臨床的効果を上げてきた.これに基づきHP感染頻度の高い我が国では,HP感染の検索と陽性例に対する除菌療法を2012年度のITPの診療ガイドでは推奨している[16].

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内科

Q 特発性血小板減少症で*H. pylori*陰性例の除菌治療

特発性血小板減少症（成人型）で*H. pylori*陽性の場合は除菌療法が60％程度奏効するとされるが，*H. pylori*陰性の症例に除菌治療を試みて奏効した例はあるか．文献も併せて．

（埼玉県　N）

A *H. pylori*陰性ITP症例に対する除菌療法について6つの報告を基にした系統的レビュー報告によると，血小板増加反応が認められた頻度は有意に*H. pylori*陽性ITP症例に高い

1998年にGasbarriniらは*H. pylori*陽性，特発性血小板減少性紫斑病（idiopathic thrombocytopenic purpura；ITP）症例に対して除菌療法を行ったところ，経時的に血小板増加反応が認められ，除菌療法を行わなかった症例では血小板増加反応が認められなかったと報告した．

以来，*H. pylori*陽性ITPにおける除菌療法の有用性が日本，イタリアを中心に世界各地から報告されてきた．その結果，ご指摘のように*H. pylori*陽性ITP症例においては除菌療法成功例の約40～60％に血小板増加反応が認められている．その結果，現在では除菌療法が成功し，血小板増加反応が認められる*H. pylori*陽性ITPは続発性（二次性）ITPの範疇に入れられ，特発性（原発性）血小板減少症とは区別されるようになってきた．

この間，除菌療法による血小板増加反応が*H. pylori*陽性ITPに特異な現象であるか否かについても，臨床研究が行われている．

その結果，以下のようなことが明らかになった．

(1) *H. pylori*陽性の膠原病や自己免疫性溶血性貧血などに伴う二次性の免疫性血小板減少症においては，除菌成功後の血小板増加反応が認められない．

(2) *H. pylori*陽性ITPにおいて除菌療法が成功しなければ，血小板増加反応は除菌成功例に比し有意に低頻度である．

(3) *H. pylori*陽性ITP症例を無作為に除菌療法群と無除菌療法群の2群に分け血小板増加反応を検討すると，除菌成功群のみに血小板増加反応が認められる．

(4) *H. pylori*陰性ITP症例に除菌療法を行った場合，血小板増加反応を示した症例が認められない．

以上により，除菌療法による血小板増加反応は*H. pylori*陽性ITPに特異的な所見であることが認められている．

ご質問の*H. pylori*陰性ITP症例に対する除菌療法については，いくつかのプロスペクティブな研究報告がある．Inabaら[1]は，*H. pylori*陽性ITP25例，陰性ITP10例にそれぞれ除菌療法を行い6カ月後の血小板増加症例の頻度を検討している．その結果，*H. pylori*陽性ITPでは除菌成功25例中11例（44％）が血小板数10万／μL以上となったのに対し，*H. pylori*陰性ITP症例では10例全例に血小板増加反応が認められていない．

2009年に6つの報告を基にした系統的レビューが報告されている．それによると除菌後6カ月の時点で血小板数が3万以上となった，いわゆる血小板増加反応が認められた頻度は*H. pylori*陽性ITPでは125例中64例（51.2％）

study	*H. pylori*陽性	*H. pylori*陰性	OR；95%CI
Emilia 2007	24/38	0/5	∞ [1.78, ∞]
Asahi 2006	16/26	0/11	∞ [3.95, ∞]
Inaba 2005	11/25	0/10	∞ [1.77, ∞]
Michel 2004	1/15	0/10	∞ [0.04, ∞]
Jackson 2008	3/4	3/18	15.00 [0.74, 837.60]
Kato 2004	9/17	2/3	0.56 [0.01, 13.17]
計	64/125	5/57	14.51 [4.17, 83.01]

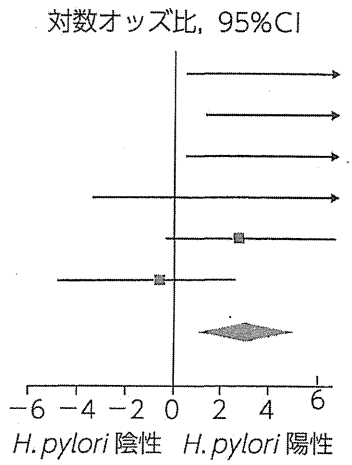

図1　*H. pylori*陽性，陰性ITPにおける*H. pylori*除菌療法による血小板増加反応の効果
除菌後6カ月の時点で血小板数3万/μL以上．
（文献[2]より改変）

であるのに対し，*H. pylori*陰性ITP症例では57例中5例（8.8%）で有意に*H. pylori*陽性ITP症例に有効性が高いとしている（図1）[2]．

*H. pylori*陰性ITP症例においても，わずかではあるが血小板増加反応が認められ，また除菌が成功しなくても血小板増加反応が認められる*H. pylori*陽性ITP症例が頻度は少ないが認められる．このような有効症例に関してはITP自体に自然寛解を来す症例が5～10%存在することから，自然寛解の可能性が考えられている．

一方，除菌療法による血小板増加機序は諸説があり一致を見ていない．

■文 献
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2) Arnold DM, et al：Haematologica 94：850, 2009.

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arthritis research&therapy

RESEARCH ARTICLE Open Access

Versican is upregulated in circulating monocytes in patients with systemic sclerosis and amplifies a CCL2-mediated pathogenic loop

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Abstract

Introduction: Altered phenotypes of circulating monocytes of patients with systemic sclerosis (SSc) have been reported, but the role of these alterations in the pathogenesis of SSc remains unclear. This study was undertaken to identify molecules that are preferentially expressed by SSc monocytes, and to investigate the roles of these molecules in the pathogenic process of SSc.

Methods: We analyzed circulating $CD14^+$ monocytes isolated from 36 patients with SSc and 32 healthy control subjects. The monocytes' gene expression profiles were assessed by Oligo GEArray® (SABiosciences, Frederic, MA, USA) and semiquantitative or quantitative PCR; their protein expression was evaluated in culture supernatants of unstimulated monocytes by immunoblotting or ELISA, and by immunocytostaining. Monocyte chemoattractant activity of CCL2 was assessed in a TransWell® system (Corning Incorporated, Corning, NY, USA) in the presence or absence of chondroitin sulfate (CS).

Results: A step-wise approach to profiling gene expression identified that versican and CCL2 were upregulated in SSc monocytes. Subsequent analysis of proteins expressed in monocyte culture supernatants confirmed enhanced production of versican and CCL2 in SSc monocytes compared with control monocytes. CCL2 bound to CS chains of versican and colocalized with versican in the monocytes' Golgi apparatus. Finally, CCL2 had a greater ability to mediate monocyte migration when bound to CS chains, because this binding provided efficient formation of CCL2 gradients and protection from protease attack.

Conclusion: Circulating monocytes with elevated versican and CCL2 levels may contribute to the fibrotic process in a subset of SSc patients by amplifying a positive feedback loop consisting of versican, CCL2, and the influx of monocytes.

Keywords: systemic sclerosis, monocytes, versican, CCL2

Introduction

Systemic sclerosis (SSc) is a multisystem disease characterized by microvascular abnormalities and excessive fibrosis [1]. Current research suggests that the pathogenic process of SSc damages endothelial cells and activates immune cells and fibroblasts, causing excessive accumulation of extracellular matrix (ECM) [2]. Mononuclear cell infiltration, consisting predominantly of macrophages and T cells, has been detected histopathologically in SSc lesions in the skin, lung, and other tissues, especially in the early phases of SSc [3,4]. Tissue macrophages in the perivascular skin express activation markers such as HLA-DR, platelet-derived growth factor B receptor [5], and CD163 [6]. Activated macrophages in the skin express CD204 [6], a marker for the M2 macrophages that are associated with wound repair and fibrotic conditions [7]. A recent study of lung tissue in SSc patients with interstitial lung disease found prominent infiltrates of fibrocytes expressing CD34, CD45, and collagen type I [8]; precursors of cells expressing these markers are found among circulating $CD14^+$ monocytes [9]. These findings indicate

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that monocytes and monocyte-lineage cells are actively involved in the pathophysiology of SSc.

Circulating $CD14^+$ monocytes derive from hematopoietic stem cells in the bone marrow and migrate to their ultimate sites of activity, and form a heterogeneous population in terms of surface markers, phagocytic capacity, and differentiation potential. Although circulating monocytes are committed precursors with the capacity to differentiate into a variety of phagocytes, including macrophages and dendritic cells, there is growing evidence that these monocytes can differentiate into other cell types as well, including cells with the typical characteristics of endothelial cells and fibroblasts [10-13]. Circulating monocytes are now recognized as multifunctional precursors, playing critical roles not only in immune and inflammatory responses but also in tissue regeneration and in pathologic tissue remodeling, such as excessive fibrosis and tumor development [13,14].

$CD14^+$ monocytes are increased in peripheral blood of SSc patients [6], and the molecular phenotypes and the proportions of cell types in the population are altered, with a larger proportion of type I collagen-producing monocytes [15], $CXCR4^+$ circulating cells with monocytic and endothelial features [16], monocytic proangiogenic hematopoietic cells [17], and $CD163^+CD204^+$ cells with a profibrotic M2 phenotype [6]. Moreover, recent microarray analyses of circulating monocytes identified several genes that are overexpressed in SSc monocytes, including type I interferon-regulated genes such as Siglec-1 [18,19]. The SSc pathogenic process thus probably recruits circulating monocytes to the affected sites, where they acquire profibrotic properties. Although the details are still unclear, there may be at least two distinct mechanisms underlying profibrotic properties of these monocytes - the production of a variety of profibrotic growth factors, cytokines, and chemokines, including transforming growth factor beta and platelet-derived growth factor [2], and their transdifferentiation into ECM-producing cells [10,11,20].

In this study, we evaluated the gene and protein expression profiles of circulating $CD14^+$ monocytes in patients with SSc, using a high-throughput platform. We were particularly interested in genes related to ECM metabolism, chemokines, and their receptors, or endothelial cell function.

Materials and methods

Patients and controls

This study included 36 patients (four men and 32 women) who met the preliminary SSc classification criteria proposed by the American College of Rheumatology [21]. Using the published criteria, 19 patients were classified as having diffuse cutaneous SSc (dcSSc) and 17 as having limited cutaneous SSc (lcSSc) [22]. The study included 32 healthy control subjects (16 men and 16 women). The average age at the time of examination was 55.3 ± 15.9 in SSc patients and 45.8 ± 19.3 in control subjects.

Organ involvement related to SSc was defined for each patient as described previously [23]. SSc-related autoantibodies were identified by indirect immunofluorescence using commercially prepared slides of monolayer HEp-2 cells (MBL, Nagano, Japan) and immunoprecipitation assays [23]. The mean disease duration from onset of Raynaud's phenomenon was 13.6 ± 10.4 years. In patients with dcSSc, 13 of 19 were in late phase, with disease duration >5 years from the onset of non-Raynaud's phenomenon symptoms. Table 1 presents patients' autoantibody profiles, SSc-related organ involvement, and medications reported at the time of blood collection. Before collecting blood samples, we obtained written, informed consent from both patients and control subjects in accord with the tenets of the Declaration of Helsinki, and as approved by the International Review Board of Keio University.

Table 1 Clinical characteristics of 36 patients with systemic sclerosis

Characteristic	*n* (%)
Organ involvement	
Joint contractures	16 (44%)
Esophageal hypomotility	24 (67%)
Cardiac involvement	1 (3%)
Renal involvement	1 (3%)
Interstitial lung disease	22 (62%)
Pulmonary arterial hypertension	1 (3%)
Digital pitting scars	18 (50%)
Systemic sclerosis-related autoantibodies[a]	
Anti-topoisomerase I	16 (44%)
Anticentromere	7 (19%)
Anti-RNA polymerase III	2 (6%)
Anti-U1 ribonucleoprotein	7 (19%)
Anti-Th/To	2 (6%)
Not identified	5 (14%)
Medications reported when blood samples collected	
Prednisolone (≤10 mg/day)	11 (31%)
Cyclophosphamide	1 (3%)
Nonsteroidal anti-inflammatory drug	5 (14%)
D-Penicillamine	3 (8%)
Oral prostanoid	19 (53%)
Calcium channel blocker	2 (6%)
Statin	6 (17%)
Anti-platelet aggregation	6 (17%)
Antacid	12 (33%)

[a]Three patients had two systemic sclerosis-related autoantibodies: one patient with anti-topoisomerase I and anticentromere; one patient with anti-topoisomerase I and anti-U1 ribonucleoprotein; and one patient with anticentromere and anti-U1 ribonucleoprotein.

Cell preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by Lymphoprep™ (Fresenius Kabi Norge, Halden, Norway) density-gradient centrifugation. $CD14^+$ monocytes were separated from PBMCs using an anti-CD14 mAb coupled to magnetic beads (CD14 MicroBeads; Miltenyi Biotech, Bergisch Gladbach, Germany) followed by magnetic cell sorting column separation according to the manufacturer's protocol [24]. Flow cytometric analysis showed that the sorted fraction consistently contained more than 95% $CD14^+$ cells.

Gene expression profiling

Total RNA was extracted from purified monocytes using the ArrayGrade™ Total RNA Isolation kit (SABiosciences, Frederic, MA, USA) according to the manufacturer's protocol. Pooled RNA was prepared by mixing equal amounts of total RNA from five patients with SSc or from five healthy control subjects. We prepared two different independent sets of RNA. The first SSc patient set was composed of RNA from four females and one male with dcSSc (mean age at examination 40.0 ± 12.3, and mean disease duration from onset of Raynaud's phenomenon 9.4 ± 7.1 years). The second set was derived from four females and one male (four dcSSc and one lcSSc, mean age at examination 50.4 ± 8.9, and mean disease duration 16.2 ± 10.6 years). We generated biotin-16-uridine-5'-triphosphate-labeled cRNA probes from pooled total RNA (3 μg) using reverse transcription and a TrueLabeling-AMP™ 2.0 kit (SABiosciences). Gene expression was profiled from pooled total RNA (3 μg) using Oligo GEArray® (SABiosciences) according to the manufacturer's instructions. This array covers 330 genes encoding ECM and adhesion molecules, chemokines and receptors, and proteins with endothelial cell functions.

The intensity of individual bands was measured by densitometry with National Institute of Health image software (Image J; National Institute of Mental Health, Bethesda, MD, USA). Relative gene expression levels were calculated as a ratio of the intensity of the target spot to that of glyceraldehyde-3-phosphate dehydrogenase. To identify genes that were upregulated in SSc monocytes, we compared the expression levels of individual genes in two independent sets of pooled monocyte RNA obtained from five SSc patients and from five healthy subjects. We selected candidate genes that met both of the following criteria: they were expressed at higher levels in SSc than in control monocytes in two independent sets, and they had 1.5-fold greater expression in SSc than in healthy monocytes in at least one set [25].

Semiquantitative and quantitative PCR

Total RNA was extracted from monocytes using an RNeasy® mini kit (Qiagen Inc, Valencia, CA, USA), first-strand cDNA was reverse-transcribed with an oligo $(dT)_{12\text{-}15}$ primer (Invitrogen, Carlsbad, CA, USA), and cDNA equivalent to 2 ng total RNA was used for PCR analysis. The primer sequences, annealing temperatures, and cycles used to amplify individual genes are summarized in Table 2. Individual band intensity was quantified by densitometry. Relative mRNA expression levels were calculated as a ratio of the band intensity of the target gene to that of glyceraldehyde-3-phosphate dehydrogenase.

Gene mRNA expression levels were further evaluated by quantitative PCR using the TaqMan® real-time PCR system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols. Each gene's expression was measured relative to glyceraldehyde-3-phosphate dehydrogenase. Specific primers and probes for amplifying genes encoding L-selectin (Hs00174151), versican (Hs00171642), CCL2 (Hs00234140), CXCL8 (Hs00174103), versican V0 isoform (Hs01007944), and versican V1 isoform (Hs01007937) were purchased from Applied Biosystems. In some experiments, high and low mRNA expression levels were defined according to the mean plus two standard deviations of the levels in healthy control samples.

Quantifying proteins in monocyte culture supernatants

Monocytes were plated and cultured in RPMI 1640 containing 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. To measure versican production, 5×10^6 monocytes were cultured in six-well plates without exogenous stimulation. Supernatants were harvested at 48 hours, concentrated with an Ultra-free MC 30K filter (Millipore, Billerica, MA, USA), and treated with chondroitinase ABC (Seikagaku Kogyo, Tokyo, Japan) to cleave chondroitin sulfate (CS) chains. The samples were then analyzed by SDS-PAGE, followed by immunoblotting with mouse mAb to human versican (clone 2B1; Seikagaku Kogyo) and horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Thermo Fisher Scientific, Rockford, IL, USA). Bound antibodies were detected with a chemiluminescence detection system (Perkin Elmer Life Sciences, Boston, MA, USA). The signal intensity of the band corresponding to the molecular weight of a truncated versican (250 kDa) was quantified by densitometry. To measure CCL2 production, 10^5 cells were cultured in 24-well plates without exogenous stimulation, culture supernatants were harvested at 24 hours, and CCL2 was measured in culture supernatants using a Quantikine® ELISA kit (R&D Systems, Abingdon, UK).

Immunocytostaining

The intracellular localization of versican and CCL2 was determined by immunofluorescence as reported previously [26]. Briefly, $CD14^+$ monocytes were cultured on BD BioCoat™ Poly-D-Lysine Cellware (BD Biosciences,

Table 2 Primer sequences, annealing temperatures, and cycles used for semiquantitative PCR

Gene	Sense primer (5′ → 3′)	Antisense primer (5′ → 3′)	Annealing temperature (°C)	Cycle
CCL2	agcaagtgtcccaaagaagc	gcaatttccccaagtcctg	66	32
Type I collagen α1	cctggatgccatcaaagtct	ccttcttgaggttgccagtc	66	33
Versican	tcattcaacgtcaccttcca	ggtccaaaaatccaaaccaa	66	34
L-selectin	tcagctgctctgaaggaaca	taaccatgactgccactgga	60	30
CCR1	tcctcacgaaagcctacgaggagagtccaagc	ccacggagaggagggagccatttaac	66	30
CXCL8	cagttttgccaaggagtgct	attgcatctggcaaccctac	63	27
MMP-2	ccaaggagagctgcaacct	ccaaggtccatagctcatcgtc	63	40
CCRL2	ctgggctcatgctggggg	tgcagcagtgggtggtgg	60	30
GAPDH	tgaacgggaagctcactgg	tccaccaccctgttgctgta	60	25
Versican variants				
Versican V0	tcaacatctcatgttctccc	ttcttcactgtgggtataggtcta	57	34
Versican V1	ggctttgaccagtgcgattac	ttcttcactgtgggtataggtcta	57	28
Versican V2	tcaacatctcatgttctccc	ccagccatagtcacatgtctc	65	38
Versican V3	ggctttgaccagtgcgattac	ccagccatagtcacatgtctc	61	32

San Diego, CA, USA) for 2 hours. The cells were fixed with acetone and incubated with goat anti-human versican polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in combination with rabbit anti-human CCL2 polyclonal antibodies (Santa Cruz Biotechnology) or a mouse anti-human goldin-97 mAb (clone CDF4; Invitrogen), followed by incubation with the appropriate secondary antibodies conjugated to Alexa Fluor-488 or Alexa Fluor-568 (Invitrogen). For negative controls, cells were incubated with an isotype-matched mouse or rat mAb against an irrelevant antigen instead of the primary antibody. TO-PRO3 (Invitrogen) was used to counterstain nuclei. Images were taken with a Fluoview FV1000 confocal laser fluorescence microscope (Olympus, Tokyo, Japan).

Assessing capacity of CCL2 for binding chondroitin sulfate

Carbonate buffer (15 mM Na_2CO_3, 10 mM $NaHCO_3$) alone or a solution of synthetic CS (Seikagaku Kogyo) dissolved in carbonate buffer (200 μg/ml) was incubated in 24-well plastic plates overnight at 4°C. Unbound CS was removed, and recombinant CCL2 (50 ng/ml; R&D Systems) was added to the wells and incubated for 2 hours at 37°C. Protein components attached to the plate were recovered with 2% SDS, applied to immunoblots with rabbit anti-CCL2 polyclonal antibody (Abcam, Cambridge, MA, USA), and visualized with a chemiluminescence detection system.

To assess how binding to CS affected CCL2's vulnerability to protease-mediated degradation, we incubated recombinant CCL2 in 24-well plastic plates in the presence or absence of CS at 37°C for 2 hours, and then left the wells untreated or treated them with elastase (2 mM), cathepsin G (1 ml; Calbiochem, San Diego, CA, USA), or trypsin (0.0005%; BD Biosciences) at 37°C for 1 hour. Protein components were recovered and analyzed by immunoblots probed with anti-CCL2 polyclonal antibody. The signal intensity of the band corresponding to intact CCL2 (10 kDa) was semi-quantified using densitometry. The percentage of intact CCL2 in individual samples was expressed as a percentage of that found on pretreated CS-coated wells that did not receive protease treatment.

Migration assay

Monocyte migration was evaluated as described previously [27], with some modifications. Briefly, the lower chambers of 24-well TransWell® plates with 5 μm pore filters (Corning Incorporated, Corning, NY, USA) were left untreated (vehicle) or coated with serial concentrations of CS (10, 50, and 250 μg/ml). The wells were incubated with recombinant CCL2 (50 ng/ml) for 2 hours at 37°C, after which monocytes (3×10^5) were placed in the upper chambers for 2 hours at 37°C with 5% carbon dioxide. Cells in the lower chambers were counted manually using a hemocytometer, and migration ratios were calculated as a percentage of the cells induced to migrate by vehicle alone. All experiments were carried out in duplicate. In some experiments, mouse anti-CCL2 mAb (R&D Systems) or mouse IgG (3.0 ng/ml; Dako, Glostrup, Demark) was added to the lower chamber. The relative monocyte migration in individual experiments was calculated as a percentage of the migration induced in vehicle-coated wells without CCL2.

Statistical analysis

All continuous variables were recorded as mean ± standard deviation, and statistical differences were compared using a nonparametric Mann-Whitney U test. Categorical variables were compared with Fisher's exact test or a chi-square test when appropriate. The correlation

coefficient (r) was determined using a single regression model.

Results

Identifying genes with altered expression in SSc monocytes

We used the Oligo GEarray™ system, which can screen 330 genes associated with ECM and adhesion molecules, chemokines and receptors, and endothelial cell biology, to compare gene expression profiles in circulating monocytes from SSc patients or healthy controls. We performed two independent analysis sets on mixed total RNA samples: one set from five SSc patients and the other from five healthy controls. Based on results of two independent sets of analysis, we selected collagen type I α1, versican, L-selectin, matrix metalloproteinase-2, CCL2, CXCL8, CCR1, and CCRL2 as candidates for genes preferentially overexpressed in SSc monocytes (Figure 1). Of these, only versican was confirmed by semi-quantitative PCR and quantitative TaqMan® real-time PCR to be significantly upregulated in SSc monocytes.

Figure 2 shows versican mRNA levels, quantified by TaqMan® real-time PCR, in monocytes from 24 SSc patients and 13 control subjects (219.9 ± 376.5 vs. 46.2 ± 31.1, $P = 0.002$). Although CCL2 expression tended to be higher in SSc patients, the difference was not statistically significant ($P = 0.06$). Since CCL2 levels in SSc monocytes varied widely, we increased the number of subjects sampled (36 patients with SSc, 32 control subjects) and the difference in CCL2 gene expression between the two groups reached statistical significance (0.37 ± 0.53 vs. 0.11 ± 0.07, $P = 0.04$) (Figure 2). The remaining six candidate genes were excluded because confirmatory analyses did not show a statistically significant difference between their expression levels in SSc and control monocytes.

Clinical features associated with high versican or CCL2 mRNA expression in monocytes

Versican and CCL2 mRNA levels varied considerably among SSc monocytes, and high expression levels were detected in a subgroup of patients. We examined clinical

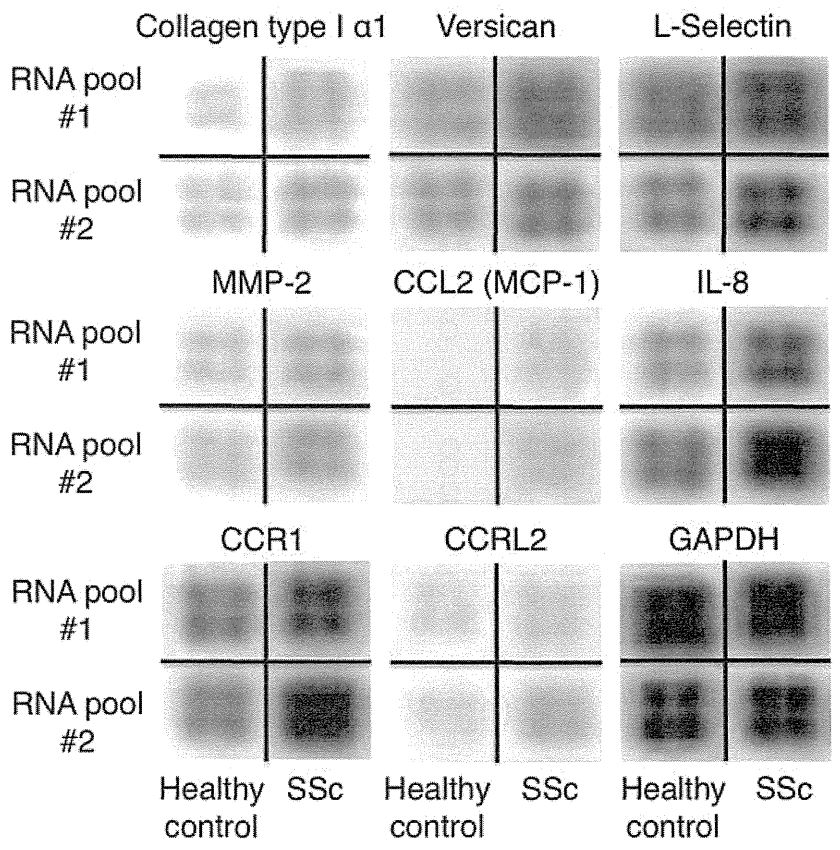

Figure 1 Gene expression profiles of circulating monocytes from patients with systemic sclerosis and from healthy controls. Two sets of genes, each set containing RNA from five subjects, were analyzed (RNA pool #1 and #2) with the Oligo GEArray® (SABiosciences, Frederic, MA, USA). The results for eight candidate genes for genes preferentially overexpressed in systemic sclerosis (SSc) monocytes are shown along with control (glycelaldehyde-3-phosphate dehydrogenase (GAPDH)).

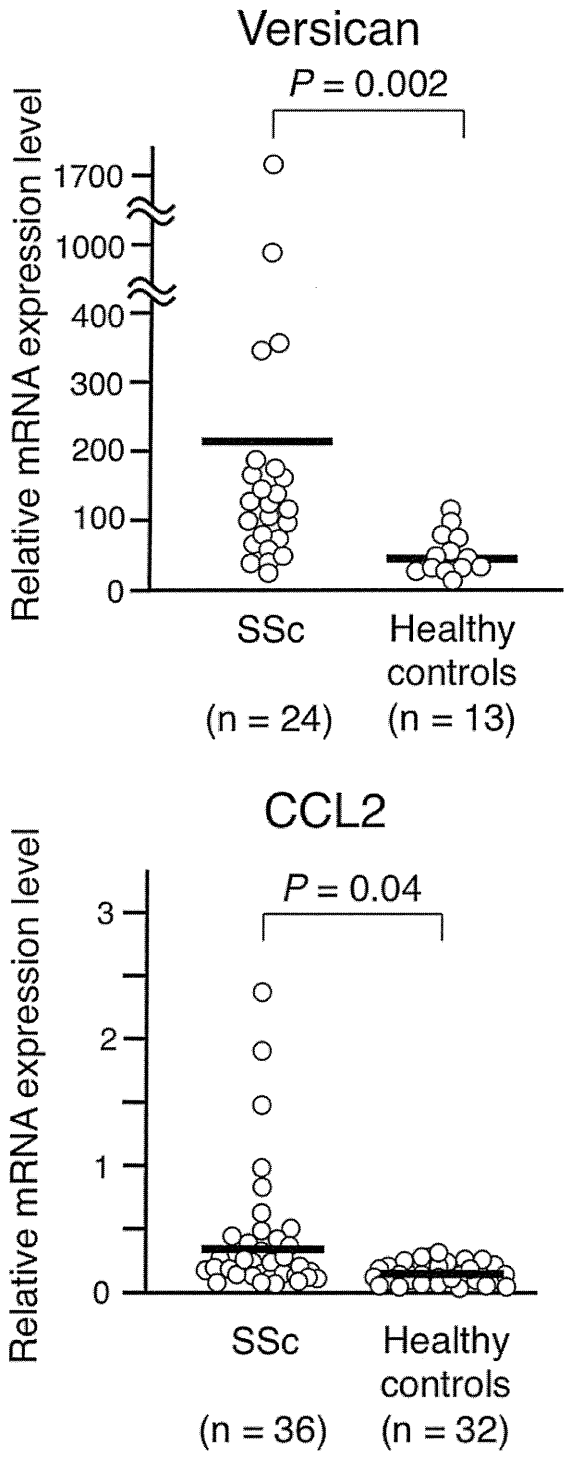

Figure 2 Versican and CCL2 mRNA levels in monocytes from patients with systemic sclerosis and healthy controls. Quantitative PCR analysis. Relative mRNA expression levels were calculated as a ratio of mRNA levels of the genes of interest to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Bars in the graph denote the mean. Differences between the groups were analyzed by Mann-Whitney *U* test. SSc, systemic sclerosis.

features associated with a high level of versican or CCL2 mRNA in circulating monocytes, which were defined based on above the mean plus two standard deviations of the levels in healthy control samples. We examined 24 patients with SSc, 11 with high levels and 13 with low levels of versican expression, and found differences in the frequencies of dcSSc (82% vs. 25%, $P = 0.02$), interstitial lung disease (82% vs. 46%, $P = 0.04$), positive anti-topoisomerase I antibody (64% vs. 15%, $P = 0.01$), and esophageal involvement (100% vs. 46%, $P = 0.006$). In fact, versican levels were significantly higher in patients with dcSSc than in those with lcSSc (413 ± 531 vs. 100 ± 92, $P = 0.03$), and in patients with esophageal involvement than those without (340 ± 459 vs. 54 ± 34, $P = 0.002$). In particular, all four patients with an extremely high mRNA expression level of versican (>300) had dcSSc. We did not find any correlation with clinical characteristics and the level of CCL2 mRNA expressed by circulating monocytes in SSc patients.

Upregulated mRNA expression of the versican isoforms V0 and V1 in SSc monocytes

Versican, or CS proteoglycan 2, is a large extracellular matrix proteoglycan (>1,000 kDa) that is present in a variety of human tissues, including skin and blood vessels [28]. Versican consists of an amino-terminal hyaluronan binding region, a glycosaminoglycan (GAG)-binding domain, and a C-type lectin-like domain. Numerous CS chains are attached to a GAG-binding domain (Figure 3A). In addition to full-length versican (V0), three short isoforms having GAG-binding domains of different sizes (V1, V2, and V3) are generated by alternative splicing (Figure 3B).

We designed PCR primers to detect each of the four versican isoforms separately, and assessed their mRNA levels in SSc and control monocytes. Semiquantitative PCR analysis of monocytes from 30 patients with SSc and 17 healthy controls showed significantly higher mRNA levels of both V0 and V1 in SSc than in control monocytes ($P = 0.01$ for both comparisons), while V2 and V3 levels were comparable in the two groups (Figure 3C).

TaqMan® real-time PCR confirmed that mRNA expression of the V0 and V1 isoforms, both of which have long GAG-binding domains, was upregulated in SSc patients as compared with healthy control subjects (Figure 4).

Versican and CCL2 proteins are upregulated in SSc monocytes

We cultured freshly isolated monocytes without any exogenous stimuli, and measured versican and CCL2 proteins spontaneously secreted into the supernatant during cultures. As shown in Figure 5A, the versican V0 isoform was concentrated and detected in supernatants by immunoblotting. Versican levels were significantly higher in culture supernatants from SSc monocytes than in those from healthy control monocytes ($P = 0.03$) (Figure 5B). SSc monocytes also produced more CCL2 than did control monocytes ($P = 0.01$) (Figure 5C). The mRNA and protein expression levels in a given patient were correlated with each other for versican ($r^2 = 0.66$, $P = 0.003$) and CCL2 ($r^2 = 0.51$, $P = 0.004$).

Capacity of CCL2 to bind chondroitin sulfate chains

Versican's negatively charged CS chains can bind to chemokines such as CCL2, CCL3, and CCL5 via ionic interactions, and can function as a chemokine reservoir [28,29]. The versican isoforms V0 and V1, which were both elevated in SSc monocytes, have numerous CS chains attached to the GAG-binding domain and thus have a large capacity for binding chemokines [28]. Both versican and CCL2 are upregulated in SSc monocytes; to determine whether these form a complex, we examined CCL2's binding capacity using plastic plates coated with or without synthetic CS (Figure 6A). As expected, CCL2 was able to bind to the plates only when CS was present. Immunocytostaining showed the cellular localization of versican and CCL2 in the Golgi apparatus of monocytes; the representative images of SSc monocytes in Figure 6B show versican and CCL2 colocalized in the Golgi apparatus. Additional experiments using monocytes derived from a patient with SSc and a healthy control subject produced concordant findings. Since the Golgi plays an important role in the synthesis of proteoglycans [30], these results suggest that versican forms a complex with CCL2 before secretion by monocytes.

Chondroitin sulfate chains enhance CCL2-mediated monocyte migration

The chemokine CCL2 induces monocytes, neutrophils, and lymphocytes to migrate [31]. To examine whether CCL2's capacity to induce migration is enhanced by binding to versican's CS chains, we performed migration assays in a TransWell® double-chamber system using $CD14^+$ monocytes derived from healthy controls and SSc patients. First, monocytes were cultured in the upper chamber, and the lower chamber was pre-coated with CS or vehicle alone in the presence or absence of CCL2 (Figure 7A,B). Monocyte migration was promoted only in the presence of CS-coated plates treated with CCL2, and the strength of the effect depended on the CS concentration in the coating (Figure 7C,D). This enhanced monocyte migration was completely blocked by adding an anti-CCL2 neutralizing antibody (Figure 7E,F). Monocytes derived from a healthy control and an SSc patient showed the similar behavior. Concordant results were obtained in additional experiments using monocytes derived from three healthy controls and three SSc patients. These findings indicate that CCL2 activity is augmented by binding to CS chains.

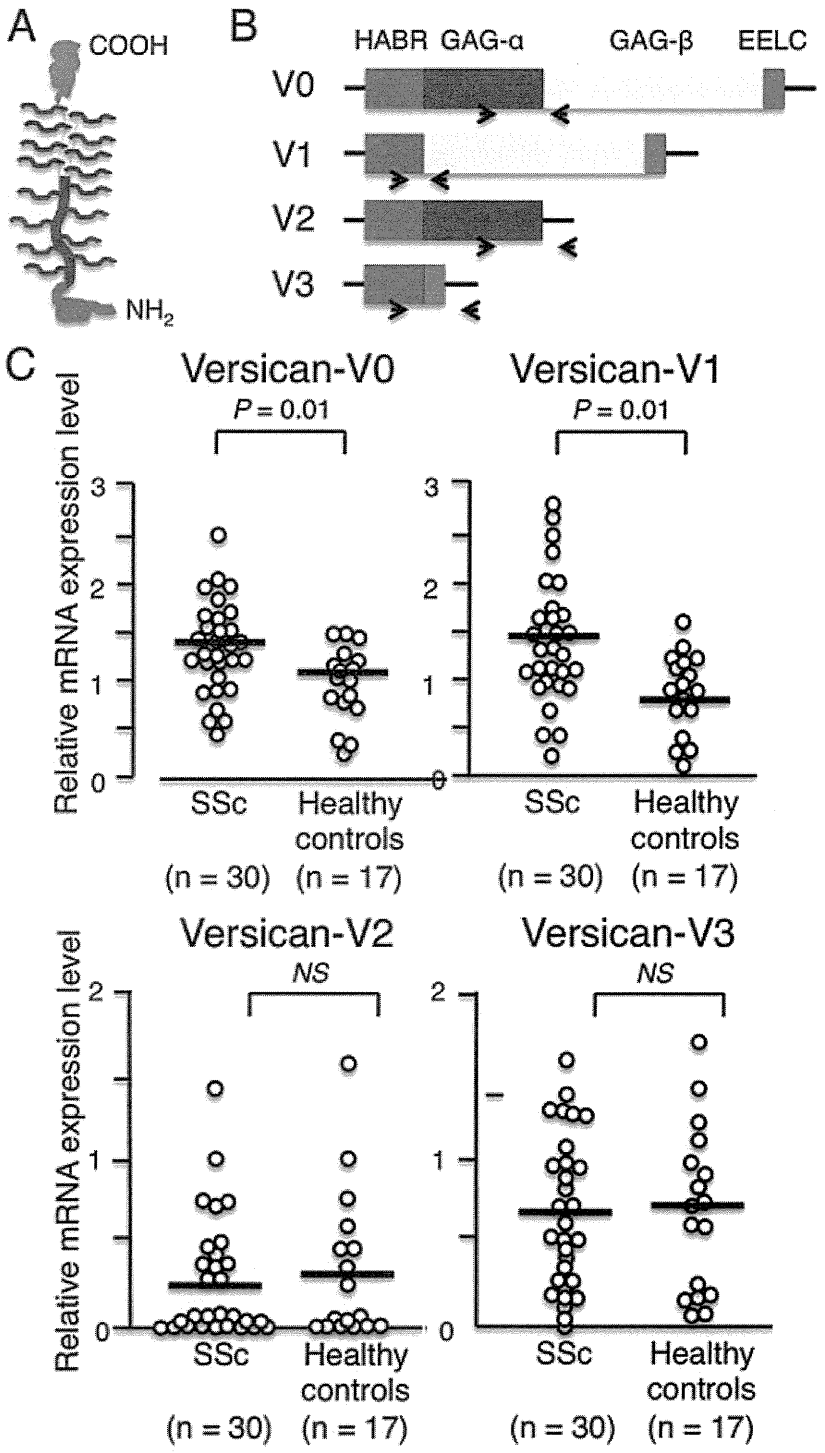

Figure 3 Versican splice variants and mRNA levels in monocytes from systemic sclerosis patients and healthy controls. **(A)** Molecular structure of full-length versican (V0), which has numerous chondroitin sulfate (CS) chains attached to its glycosaminoglycan (GAG)-binding domain. **(B)** mRNA structures of versican splice variants (V0, V1, V2 and V3). Versican is composed of a hyaluronan binding region (HABR, green), GAG-binding domains (blue and yellow), and epidermal growth factor-like, lectin-like, and complement-regulatory-like domains (EELC, red). Individual mRNA components are shown in the same color as their corresponding protein structures. Arrows denote primers used to amplify each splice variant. **(C)** Levels of versican V0, V1, V2, and V3 mRNA in systemic sclerosis (SSc) and control monocytes, analyzed using semi-quantitative PCR. Relative mRNA levels were calculated as a ratio of the expression level of the gene of interest to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each bar in the graph denotes the mean. Differences between the two groups were analyzed by Mann-Whitney *U* test. NS, not significant.

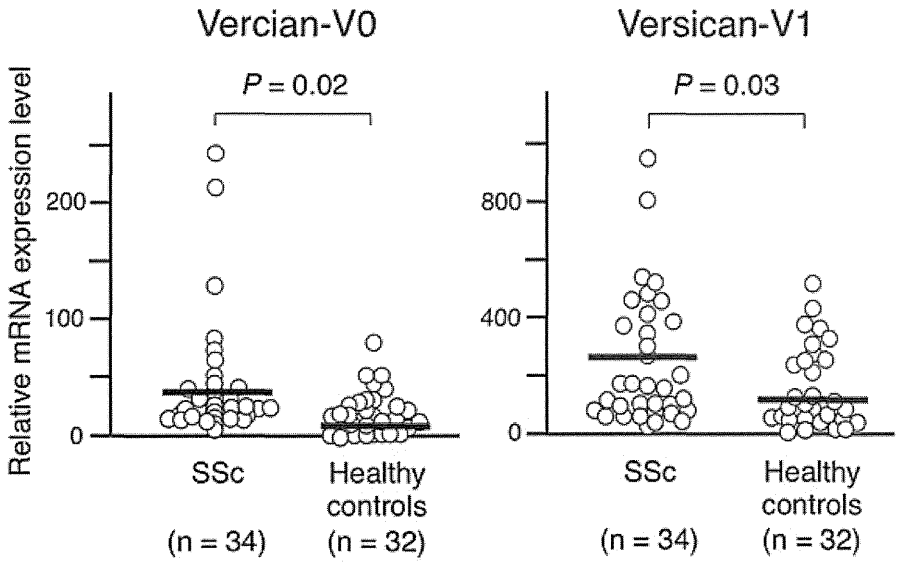

Figure 4 Versican V0 and V1 mRNA levels in systemic sclerosis and control monocytes. Quantitative PCR analysis. Relative mRNA levels were calculated as a ratio of the level of the gene of interest to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each bar in the graph denotes the mean. Differences between the groups were analyzed by Mann-Whitney *U* test. SSc, systemic sclerosis.

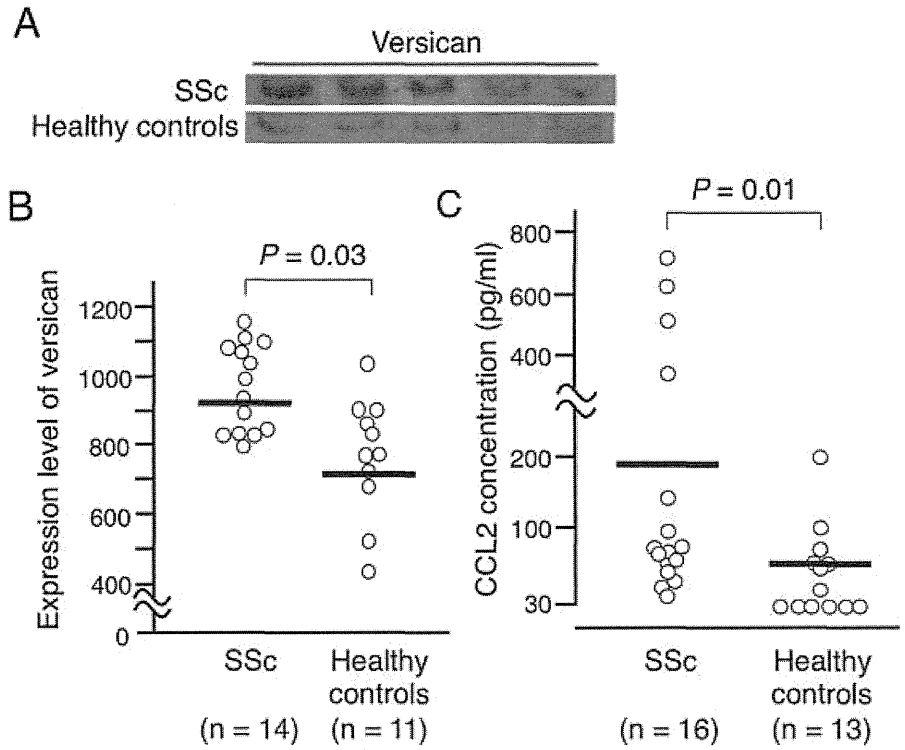

Figure 5 Versican V0 and CCL2 protein levels in monocyte culture supernatants. Versican V0 and CCL2 protein levels in monocyte culture supernatants derived from systemic sclerosis (SSc) patients and healthy controls. **(A)** Representative immunoblot evaluating versican V0 protein expression. Monocyte culture supernatants were concentrated, truncated, and applied to immunoblots. **(B)** Versican V0 protein levels in monocytes from 14 patients with SSc and 11 healthy controls, semiquantitatively measured by densitometry. **(C)** CCL2 protein levels in monocytes from 16 patients with SSc and 13 healthy controls. CCL concentration in culture supernatants was measured by an ELISA. Each bar in the graph denotes the mean. Results from the two groups were compared by Mann-Whitney *U* test.

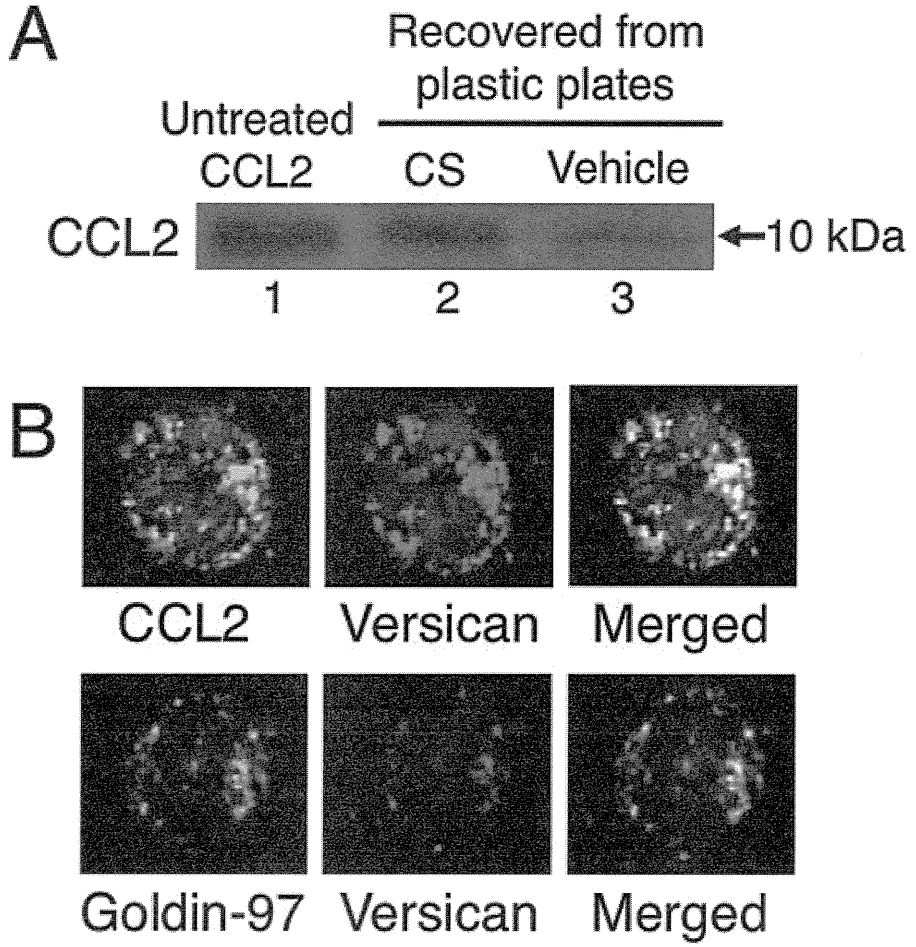

Figure 6 Formation of CCL2 and versican complex in circulating monocytes. **(A)** CCL2 was incubated with plastic plates coated with chondroitin sulfate (CS) or vehicle. Bound CCL2 was recovered and subjected to immunoblots (lanes 2 and 3, respectively). Lane 1, untreated CCL2 as a positive control. A representative result from three experiments is shown. **(B)** Versican and CCL2 cellular localization in monocytes from an systemic sclerosis (SSc) patient, assessed by multi-color immunocytostaining: upper panel, CCL2 (green), versican (red), and their merged image; lower panel, goldin-97 (green), versican (red), and their merged image. Nuclei were counterstained with TO-PRO3 (blue). A representative result from three independent experiments is shown. Original magnification, ×600.

Enhanced CCL2-mediated monocyte migration was probably due to the efficient formation of a chemotactic gradient, but it was also possible that CCL2 was protected from degradation when bound to a CS chain. To test this hypothesis, CCL2 was incubated with plates treated with CS or vehicle, and exposed to a variety of proteases that included elastase, cathepsin G, and trypsin. As shown in Figure 8A, CCL2 was degraded in the presence of proteases, but was protected from protease-mediated degradation when bound to the CS-coated plates. Concordant findings were obtained from three healthy controls (Figure 8B).

Discussion

This study has demonstrated that versican and CCL2 are upregulated in circulating $CD14^+$ monocytes in a subset of SSc patients. High versican levels in circulating monocytes were associated with fibrotic characteristics of SSc, such as diffuse cutaneous involvement. Interestingly, versican forms a reservoir for various CC chemokines that induce the migration of circulating monocytes, which produce additional versican after arriving at the versican-rich site. CCL2's ability to induce monocytes to migrate is enhanced by its binding to versican, due to the efficient formation of chemokine gradients and protection from proteolytic degradation. This positive feedback loop, consisting of versican, CCL2, and the influx of monocytes, may be enhanced at the affected sites of a subset of SSc patients with phenotypically altered circulating monocytes.

Versican is involved in many physiologic and pathologic processes, including neuronal development [32], atherosclerosis [33], and the invasive and metastatic

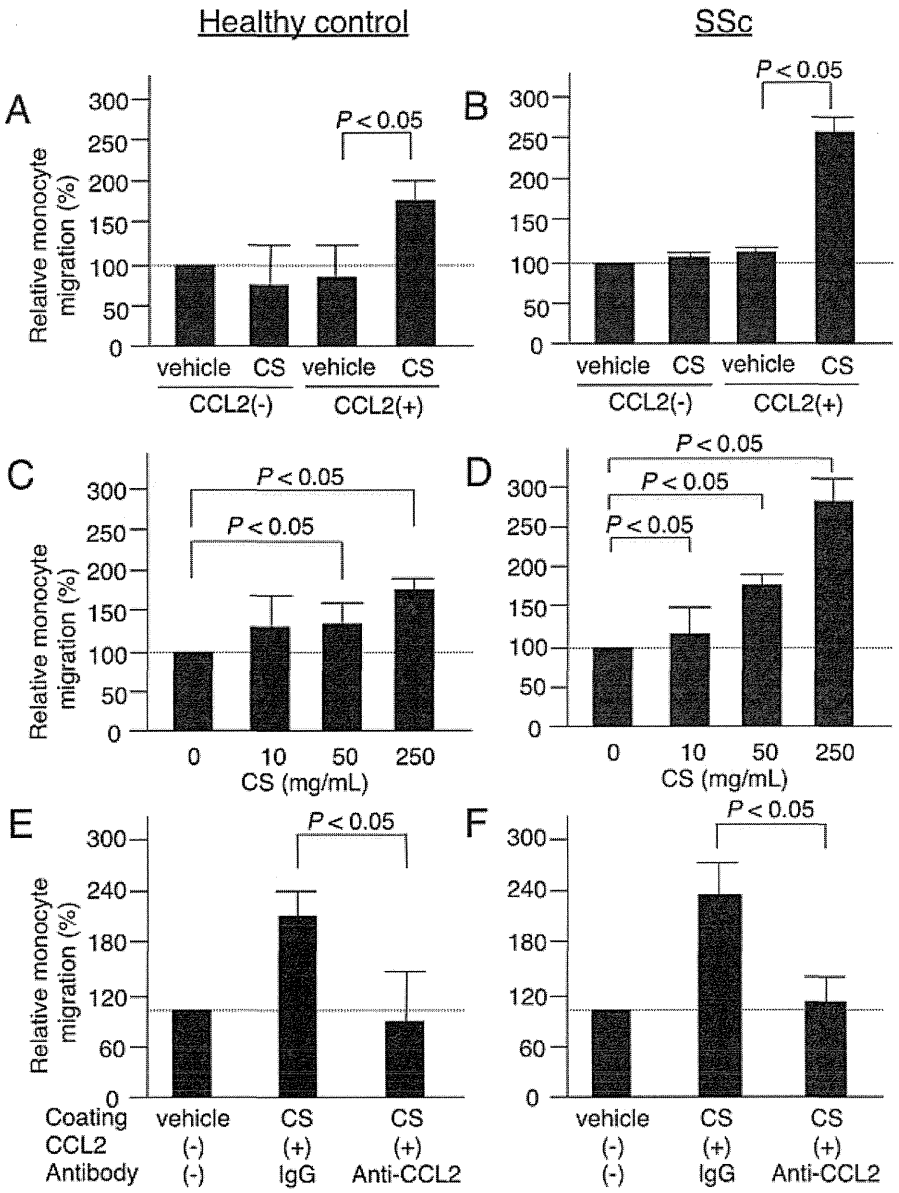

Figure 7 CCL2 chemoattractant activity promoting monocyte migration, with or without chondroitin sulfate binding. Circulating monocytes derived from healthy controls (A, C, E) and systemic sclerosis (SSc) patients (B, D, F). **(A, B)** CCL2-induced monocyte migration in the presence or absence of chondroitin sulfate (CS) coating. Lower chambers of a TransWell® double-chamber system (Corning Incorporated, Corning, NY, USA) were coated with CS or vehicle, and $CD14^+$ monocytes were applied to the upper chambers. **(C, D)** CCL2-induced monocyte migration on plastic plates precoated with serial concentrations of CS. **(E, F)** CCL2-induced monocyte migration on CS-coated plastic plates in the presence of anti-CCL2 mAb or control IgG. Relative monocyte migration was calculated as a percentage of migration in a control experiment using vehicle-coated wells without CCL2. All experiments were carried out in duplicate; the mean and standard deviation of three measurements is shown. Results from the two groups were compared using a Mann-Whitney *U* test. A representative result from four independent experiments is shown.

signatures of many cancers [34]. As with other ECM components, versican is enriched in the skin of patients with SSc [35], although little is known about versican's role in SSc pathogenesis. Versican is able to bind type I collagen and hyaluronic acid to maintain the integrity of the ECM [28], which may be important in forming the stiff fibrotic tissue seen with SSc. Versican also functions as a unique reservoir for a variety of growth factors, chemokines, and cytokines, which it gathers via numerous CS chains attached to its GAG-binding domain [28]. Chemokines known to bind versican include CCL2, CCL3, CCL5, CCL21, CXCL10, and CXCL12. It is

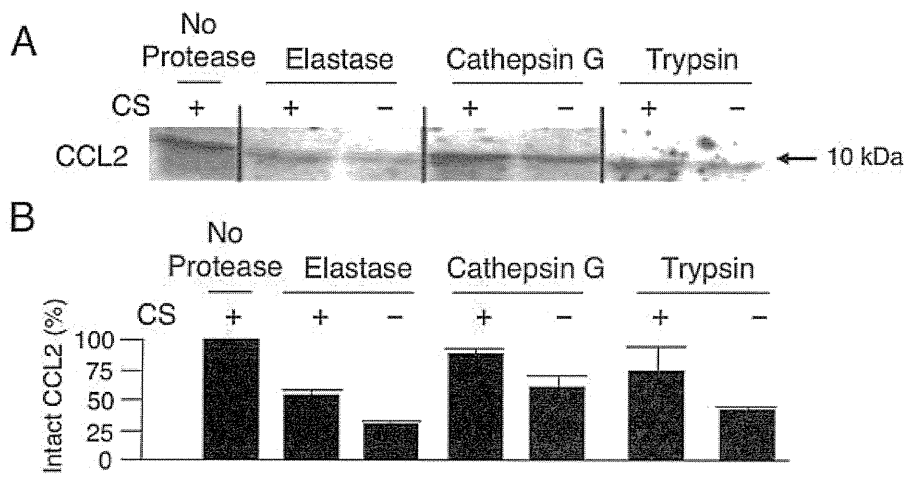

Figure 8 Binding to chondroitin sulfate chains protects CCL2 from protease-mediated degradation. **(A)** CCL2 was pre-incubated with chondroitin sulfate (CS) or vehicle, and treated with a series of proteases including elastase, cathepsin G, and trypsin. A representative immunoblot from three different experiments shows a band corresponding to intact CCL2 (10 kDa). **(B)** Amount of intact CCL2 in individual wells precoated with CS or vehicle, and subsequently treated with a series of proteases. Mean and standard deviation of three independent measurements is shown. The quantity of intact CCL2 in individual samples was expressed as a percentage of the quantity of CCL2 on wells precoated with CS and not treated with protease.

particularly noteworthy that versican generates a chemotactic gradient of CC chemokines that attracts circulating monocytes and T cells to versican-rich sites [36]. Versican subsequently promotes the adhesion and activation of recruited monocytes through binding to adhesion receptors such as integrins and CD44 on cell surfaces [37]. Through these processes, versican generates a complex set of environmental cues for infiltrating mononuclear cells and resident cells [28].

Interestingly, monocytes and monocyte-derived cells are not only recruited by versican, but are also major producers of versican [38,39]. Therefore, after migrating to versican-rich sites, these monocytes amplify the tissue response by producing more versican, which in turn promotes the influx of more monocytes [40]. In this regard, recent studies indicate that the positive feedback loop formed by versican and monocyte-lineage cells is critical for inducing certain pathologic conditions, such as tumor invasion and metastasis [41] and the formation of atherosclerotic plaques [38]. In patients with SSc, this versican-mediated positive feedback loop probably contributes to the fibrotic process by recruiting certain subsets of monocytes that acquire profibrotic properties [6,15]. The mechanisms that initially stimulate the release of versican in the early stages of SSc remain elusive, but once this positive feedback loop is established the profibrotic response would, theoretically, be amplified endlessly.

CCL2, a chemokine known to be involved in pathogenic process of SSc [42], is also elevated in circulating monocytes from SSc patients. Levels of circulating CCL2 were elevated in SSc patients, especially in those with early dcSSc [43,44] or interstitial lung disease [43,45,46]. A recent longitudinal analysis in patients with dcSSc found that circulating CCL2 decreases year after year, along with improvements in skin sclerosis [47], suggesting CCL2 as an indicator of profibrotic activity in patients with SSc. Several animal models of tissue fibrosis have demonstrated CCL2's crucial role in the fibrotic process, in which attenuating CCL2 activity prevents tissue fibrosis. Mice lacking CCL2 are protected from bleomycin-induced dermal fibrosis [48], while mice lacking the CCL2 receptor CCR2 are protected from bleomycin-induced lung fibrosis [49,50]. In these models, monocyte infiltration and collagen deposition were remarkably lower than in wild-type mice. The present study raises the question of which cell type producing CCL2 is more likely to be important for SSc pathogenesis. In this regard, abundant expression of CCL2 was observed in fibroblasts and mononuclear cells in the skin of SSc patients [45,51,52]. CCL2 has also been reported to be expressed mainly by infiltrating monocytes early in the disease, whereas fibroblasts become the major source for CCL2 in the skin later in the disease [42]. Unfortunately, our *ex vivo* analysis failed to demonstrate which cell type is the primary source of CCL2 involved in the pathogenic process of SSc.

Despite this definitive role of CCL2 in the development of excessive fibrosis *in vivo*, the details of the profibrotic mechanisms remain unclear. Yamamoto and colleagues reported that CCL2 significantly increased the levels of collagen mRNA expressed in cultured dermal fibroblasts [53], but another study failed to