suppressed the increased gene expression of both α-SMA and HSP47. Furthermore, in preliminary *in vitro* experiments, vitamin B_{12} protected rat primary hepatocytes from hepatotoxin-induced cell death. Therefore, vitamin B_{12} might suppress liver inflammation and the subsequent fibrogenesis by protecting hepatocytes from liver injury.

During liver injury, hepatic stellate cells (HSCs) are activated to transdifferentiate into myofibroblasts and overproduce extracellular matrix, which leads to fibrosis.[12] Oxidative stress stimulates the activation of HSCs, and substances with antioxidative activity, such as vitamin E,[13] glutathione,[14] and L-cysteine,[15] inhibit HSC activation, thus suppressing liver fibrosis. However, we have not found any antioxidative activity by vitamin B_{12} (data not shown). It has been reported that the activity of glutathione reductase was found to be significantly lower in B_{12}-deficient liver.[16] Recently, it was reported that the interaction between vitamin B_{12} and glutathione could protect against disease related to vitamin B_{12} deficiency.[17] Although vitamin B_{12} itself does not have radical scavenging ability, it might play an important role to maintain the sulfhydryl level under oxidative conditions. Vitamin B_{12} contains a cobalt complex and is widely used to describe compounds of the cobalamin group. It is possible that the cobalt complex of vitamin B_{12} is involved in the inhibition of liver inflammation and fibrogenesis, but further studies are necessary to clarify vitamin B_{12}'s mechanism of action.

We previously reported that $Zn(Mal)_2$ suppresses cytotoxin-induced apoptotic and necrotic cell death in isolated hepatocytes.[18] This zinc complex has free-radical scavenging activity. Several manganic porphyrins mimicked superoxide dismutase and had protective effect against oxidative stress.[19,20] Finally, here we found that the increase in α-SMA and HSP47 gene expression caused by DMN treatment was suppressed by vitamin B_{12}. Thus, a variety of metal complexes seem to have therapeutic potential.

In conclusion, we found that vitamin B_{12} is potent a hepatoprotective agent. This report is the first to demonstrate the hepatoprotective effect of vitamin B_{12} on liver fibrosis.

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肝再生医療における間葉系幹細胞の有用性

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Application of Mesenchymal Stem Cells to Liver Regenerative Medicine

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Stem cell-based therapy has received attention as a possible alternative to organ transplantation, owing to the ability of stem cells to repopulate and differentiate at the engrafted site. We transplanted bone marrow-derived mesenchymal stem cells (BMSCs) into liver-injured rats to test the therapeutic effect. Rat bone marrow cells were cultured in the presence of hepatocyte growth factor (HGF). RT-PCR and immunocytochemical analysis indicated that the BMSCs expressed the albumin mRNA and the production of protein after cultivation with HGF for 2 weeks. The BMSCs appeared to differentiate into hepatocyte-like cells in response to the culture with HGF. After labeling with a fluorescent marker, the BMSCs were transplanted into CCl_4-injured rats by injection through the caudal vein. The liver was excised and blood samples were collected 4 weeks later. Engraftment of the transplanted BMSCs was seen with significant fluorescence in the injured liver. Transplantation of the BMSCs into liver-injured rats restored their serum albumin level and suppressed transaminase activity and liver fibrosis. Therefore, BMSCs were shown to have a therapeutic effect on liver injury. Recently, we have been trying to use mesenchymal stem cells isolated from dental papilla of discarded human wisdom teeth. Autologous transplantation of mesenchymal stem cells from bone marrow and dental papilla could be ethically and functionally promising for stem cell-based therapy.

Key words——mesenchymal stem cell; regenerative medicine; bone marrow; wisdom teeth

1. 緒言

現在，重篤な肝不全患者に対する有効な治療法は肝臓移植とされているが臓器移植はドナー不足，拒絶反応，高額医療という極めて深刻な問題を抱えている．そこで肝臓移植に替わる新たな肝疾患治療法の開発が期待されている．細胞移植はレシピエントに対する負担が臓器移植に比べ軽く，免疫学的な問題も少ないと言われている．肝臓移植用のドナー肝の約50%がなんらかの理由で使用されず廃棄されている現状を考慮すると，未使用肝を凍結保存後複数のレシピエントに細胞移植を行うことが可能であ

り，肝疾患治療への適用が期待されている．現在まで肝細胞移植は劇症肝炎，肝酵素疾患の治療に臨床で試験的に試みられている．[1-3] さらには肝細胞に治療用遺伝子を導入後，移植した例も報告されている．[4] しかし成熟肝細胞は生体外で増殖させることは困難であり，臓器移植と同様ドナー不足の問題は解消し得ない．そこでわれわれは生体に存在する組織幹細胞に着目し in vitro で増殖させ治療に必要な細胞数を確保したのちに，目的の細胞へ分化させ移植を行うことを試みている．この方法は患者自身の細胞を増殖させて移植用の細胞とするため拒絶反応はなく，理想的な肝疾患治療法となる可能性を秘めている．Terai らは増殖はさせていないが肝硬変患者に対して自己の骨髄細胞を移植する臨床試験を開始し，肝機能が向上したことを報告している．[5] 本稿においては有望な組織幹細胞の候補として間葉系

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幹細胞を取り上げ，その特性，肝細胞への分化能，肝傷害モデル動物に対する治癒効果について紹介する．

2. 間葉系幹細胞とは

これまで様々な幹細胞が再生医療用の細胞源として検討されてきた．胚性幹細胞（Embryonic stem cells; ES 細胞）は無限増殖能を有し，あらゆる細胞に分化が可能であるため注目を集めてきた．[6] また成体に存在する組織幹細胞は多分化能と有限ではあるが増殖能を有し，自己細胞を使用することもできることから有用であると考えられている．骨髄由来の造血幹細胞（hematopoietic stem cells; HSC），[7] 多能性体性前駆細胞（multipotent adult progenitor cells; MAPC），[8] 間葉系幹細胞（mesenchymal stem cells; MSC）[9]の多分化能がこれまで多数報告され，テラトーマ形成や倫理的な問題が懸念される ES 細胞に比べ臨床での使用がより早期に実現するものと期待されている．

原腸陥入において上皮性の原始外胚葉細胞から上皮-間葉移行により移動性の高い非上皮性の中胚葉細胞が分化してくる．MSC は中胚葉細胞に由来する間葉系組織前駆細胞へ分化する幹細胞であり，胎生期において組織と組織の間を充填する間充織と呼ばれる間葉系細胞群の中に存在し，骨，軟骨，脂肪，筋に分化するものとして認識されていたが，最近胚葉を超えて，外胚葉系の神経細胞，内胚葉系の肝細胞へと分化することが報告された．[8,10,11] MSC は成体においては骨髄中で造血細胞の生存，分化を支持する間質細胞中に存在しており培養により容易に細胞数を増やすことが可能である．最近では骨髄以外に脂肪組織，歯髄，臍帯血などから MSC が単離され注目を集めている．[12-15] 産業技術総合研究所の大串らは既に変形性膝関節症の患者に生体外で増殖させた自己の MSC を移植しその有効性を報告している．[16] 本稿においては骨髄及び親知らず由来の MSC の肝細胞への分化と細胞移植の細胞源としての有用性について筆者らの結果を中心に紹介する．

3. 骨髄由来間葉系幹細胞（bone marrow-derived MSC; BMSC）

3-1. BMSC の肝細胞への分化誘導　肝臓には肝幹細胞が存在すると予想され卵形細胞，[17] 肝上皮細胞，[18] 小型肝細胞[19]などが報告されている．しかし肝疾患を持つ患者から肝幹細胞を分離することは現実的ではないことからほかの組織由来の幹細胞を用いることが望ましいと考えられる．そこで肝不全時にも採取が可能である骨髄に着目し，分離後培養により細胞数を増加させ移植を行うことを目指し基礎的な検討を行った．筆者らは培養した BMSC をそのまま移植するのではなく in vitro で肝細胞への分化誘導を行い肝細胞への"方向付け"を行ったのちに移植する方が効果が出易いと考えた．

フィッシャー 344 系雄性ラットの大腿骨より骨髄細胞を採取し，培養用プレートに 2×10^4 cells/cm^2 の密度で播種した．接着性細胞に MSC が含まれて入るか否かを検討するため骨分化能の評価を行った．骨分化に必要とされる β-グリセロリン酸，アスコルビン酸，デキサメタゾンを添加して培養を行い，骨分化による石灰化を観察した．その結果，カルシウムと親和性のある蛍光色素カルセインを添加すると石灰化部に蛍光が観察され骨基質の形成が確認されたことから，MSC の存在と培養系で増幅が可能であることが示された．続いて肝細胞への分化誘導条件の検討を行った．基本培地に ITS（insulin-transferrin-selenium），デキサメタゾン，そして肝細胞への分化に重要と考えられている肝細胞増殖因子（hepatocyte growth factor; HGF）[20]を添加し培養を行った．HGF，デキサメタゾンを添加していない群と比較し，2 週間の培養後，添加群では細胞が大型になるなど明らかな形態変化が観察された．次に reverse transcriptase-polymerase chain reaction（RT-PCR）により肝細胞への分化誘導が起きているか否かの検討を行った（Fig. 1）．培養 2 週間，3 週間後にそれぞれ細胞を回収し肝細胞の初期分化マーカーである α-フェトプロテイン（AFP），成熟肝細胞のマーカーであるアルブミンの遺伝子発現を観察した．培養 2 週間後に誘導因子添加群で AFP の発現があり，3 週間後には AFP は消失し，添加群の細胞でアルブミン遺伝子の発現が確認された．

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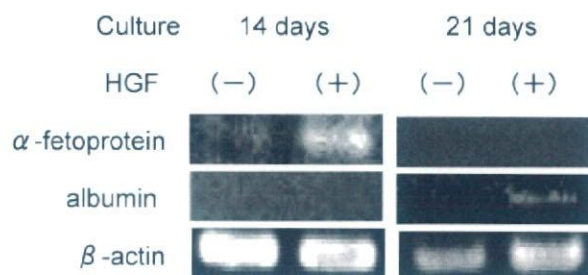
Fig. 1. RT-PCR of Hepatocyte Differentiation Marker Genes Expressed in Cultured BMSCs

Bone marrow cells were cultured in the presence or absence of HGF for 3 weeks. On the 14th and 21st days of culture, the cells were harvested and RT-PCR was performed using primers for AFP, albumin, and β-actin.
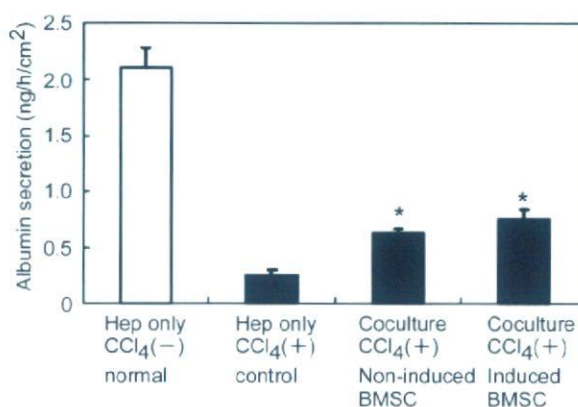

Fig. 2. Albumin Secretion from CCl$_4$-injured Hepatocytes Cocultured with BMSCs

BMSCs were cultured in the absence or presence of HGF for 2 weeks, and cocultured with hepatocytes treated or non-treated with CCl$_4$. The amount of secreted albumin was measured 24 h after the coculture. Open bar indicates albumin secretion from non-injured hepatocytes. Values are the means ± S.E. from three experiments. Asterisk indicates a significant difference from the values of injured hepatocyte monoculture (*$p < 0.05$).

またラットアルブミン抗体を用いた蛍光免疫染色の結果, アルブミンタンパクが合成されていることが明らかとなった. これらの結果より, HGF, デキサメタゾン, ITS 添加培地で BMSC を肝細胞様に方向付けできることが示された.

3-2. 傷害肝細胞に対する治癒効果 次にこの BMSC が傷害を受けた肝細胞に対して治癒効果をもたらすか否かを *in vitro* で検討を行った. コラゲナーゼ灌流法[21]を用いてラット肝細胞を単離し 6 穴のプレートに播種した. 培養 6 時間から 24 時間に 1 mM の四塩化炭素を添加して肝細胞に傷害を与えた. その傷害肝細胞と分化誘導処理をした MSC あるいは未処理の BMSC との共培養を 24 時間行い培地中に分泌されるアルブミン量を定量した (Fig. 2). 四塩化炭素処理により肝細胞のアルブミン分泌能は顕著に低下したが BMSC との共培養により有意な分泌能の回復が観察された. その効果は分化誘導の有無には関係なく BMSC が分泌する治癒因子によるものと考えられる. データには示さないが傷害肝細胞と BMSC を共培養することにより BMSC から HGF が分泌されることが示されており, 治癒効果をもたらしたと考えている. したがって *in vitro* の検討においては分化誘導を行わない BMSC においても肝再生, 肝保護に有用である可能性が示唆された.

3-3. 肝傷害ラットへの移植 4 週齢の Fisher344 系雄性ラットの腹腔内に四塩化炭素 (0.5 ml/kg body weight) を週 2 回, 4 週間投与し肝傷害を与えた. BMSC は分化誘導培地あるいは非誘導培地で培養後蛍光色素である PKH26 で染色し, 四塩化炭素初回投与 2 日後に尾静脈より 3×10^6 個の細胞を移植した. 移植 4 週間後に肝臓の凍結切片を作成し蛍光イメージアナライザーを用いて蛍光強度を測定した (Table 1). 培地のみを尾静脈から注入したコントロールも自家蛍光を発していたが, コントロール及び非誘導培地で培養した BMSC (−) に比べ誘導培地で培養した BMSC (＋) を移植した群では有意に高い蛍光強度が観察された. したがって機構は明らかではないが, 分化誘導処理により BMSC が傷害肝臓により生着し易くなることが示された.

肝傷害のマーカーである血清アスパラギン酸アミノトランスフェラーゼ (AST) とアラニンアミノトランスフェラーゼ (ALT) 値の正常値は 100 以下であるが四塩化炭素を与えたコントロールは非常に高い値であり, 肝傷害が惹起され炎症が起きていることが明らかとなった. 両酵素とも BMSC (−) 移植群ではコントロールに比べ, 顕著な差は得られなかったが, BMSC (＋) 移植群では有意に低下していた (Table 1). この結果は移植した BMSC (＋) が四塩化炭素の慢性毒性から肝細胞の壊死を抑制していることを示している. 血清アルブミン値を測定したところ障害を与えたコントロール群では正常値 3.9 g/dl から顕著に低下していた. BMSC (−) 移植群では効果がみられなかったが BMSC (＋) 移植群では正常値までアルブミン濃度が回復していた. この効果は移植した細胞が増殖してアルブミン

Table 1. Effect of BMSC Transplantation into Rats with Chronic Liver Injury

	Control not transplanted	Non-induced BMSC transplantation	Induced BMSC transplantation
Engraftment (arbitrary unit)	18250±1915	20643±1661	35624±1964*
AST (KU)	4089±605	3968±499	1973±478*
ALT (KU)	2418±342	1987±392	1459±369*
Albumin (g/dl)	3.27±0.23	3.44±0.27	3.98±0.11*
Fibrosis ratio (%)	0.916±0.063	0.802±0.292	0.649±0.053*

* $p < 0.05$ vs Control.

産生を維持したのか，あるいはレシピエントの肝細胞が保護されかつ再生が促進された結果なのか明らかではないが分化誘導の有用性が示された．

肝臓において持続的な炎症が続くと肝星細胞が活性化されコラーゲンなどのマトリックスが過剰産生され肝線維化が起こる．そして肝硬変から肝がんへ進行するケースが多いと言われている．そこで線維化の程度をアザン染色によって評価し BMSC の有効性を検討した．染色によって青く染まった領域を数値化し効果の判定を行った（Table 1）．その結果 BMSC（＋）の移植により，線維化が有意に抑制されることが示された．

本実験は骨髄細胞を分離し接着系の細胞を培養したものを使用している．したがって BMSC のみでなく造血系の細胞を維持する働きをする骨髄間質細胞と呼ばれているものも含まれており，BMSC 単独の効果であることを証明するためにはシングルセルクローニングを行い効果を判定する必要がある．

4. 親知らず由来間葉系幹細胞

4-1. MSC の単離 再生医療用の細胞源としてこれまで ES 細胞，骨髄細胞などが主に検討されてきたがわれわれは通常廃棄される組織から有用な幹細胞を単離することができれば倫理的な問題も回避でき有用であると考えた．歯科領域では歯髄からMSC が単離されたことが報告されており，[13,14] 抜歯され廃棄される歯に着目した．虫歯の場合，病原菌が含まれ再生医療に適用することは困難であるため，歯科矯正時に抜歯される第 3 大臼歯，通称"親知らず"を用いることとした．矯正時に抜歯されるものは埋伏した状態であり，未分化な歯胚組織が維持されている可能性が高く，分化が進むと象牙質，歯髄となる歯乳頭組織には有用な MSC が存在することが予想された．そこでインフォームドコンセントを得たのち，破棄された親知らずより歯乳頭組織

を採取し MSC のクローン単離を試みた（Fig. 3）．

歯乳頭組織をハサミで細かく切断し，コラゲナーゼにより細胞を分散後組織培養用ディッシュに播種し α-MEM を用いて培養を行った．接着性の細胞を回収し fluorescence activated cell sorting (FACS) を用いて 96 穴プレートの 1 ウェル当たり 1 つの細胞が入るように播種した．単一細胞からコロニー形成したものを継体しさらに増殖させ，2×10^4 cells を分化能の評価に使用し，残りの細胞を凍結保存した．カルセインを利用した骨分化能を指標として幹細胞としての特性を有するクローンの選択を行った結果，コロニー形成能を有するものの約 30% が骨分化能を発現した．その中から特に高い骨分化能を示したクローンを用いて以下の検討を行った．表面抗原の発現パターンをほかの MSC と比較するため，造血幹細胞マーカー CD34，血球系マーカーCD45，接着細胞マーカー CD29，CD44，MSC マーカー SH2, SH3, SH4 の発現について解析した．その結果 CD34, CD45 は陰性でほかはすべて陽性となった．これまで報告のある歯髄，脂肪組織，臍帯血，骨髄由来 MSC と発現パターンが一致したため本クローンは MSC であることが明らかとなった．以下本クローンを歯胚由来前駆細胞（tooth germ progenitor cell; TGPC）と略す．

4-2. 肝細胞への分化誘導 ES 細胞から肝細胞への分化誘導を試みた Hamazaki らの方法[22]に準じ，HGF，デキサメタゾン，ITS に加えて線維芽細胞増殖因子（FGF），オンコスタチン M（OSM）を用いた．FGF は肝発生のイニシエーションにおいて前腸に隣接する心臓中胚葉から分泌される必須因子である．[23] また OSM は造血細胞が産生し肝細胞の機能的成熟を促進するとの報告がある．[24] DMEM 培地に上記の因子，2% ウシ胎児血清を添加して培養を行うと誘導因子無添加のものに比べ著
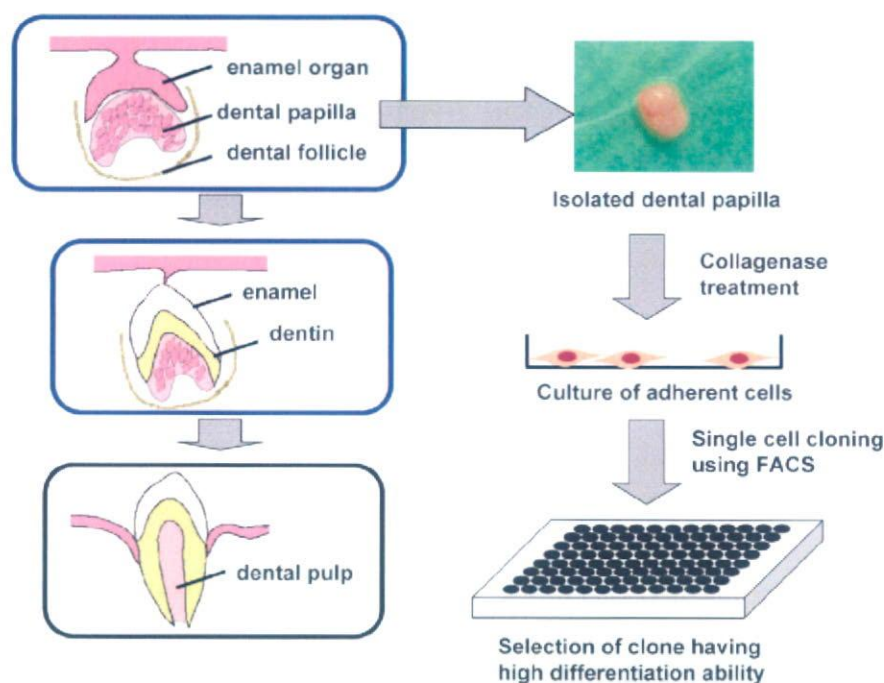
Fig. 3. Clonal Isolation of Mesenchymal Stem Cell Having High Differentiation Ability from Dental Papilla

しい形態変化が観察された（Fig. 4）．培養初期には細長い線維芽細胞用の形態であるが分化誘導を継続するにつれ，2週間後にはサイズの大きい多角の形態へと変化した．RT-PCR による解析の結果，分化誘導 10 日でアルブミン遺伝子の発現が観察され，逆に初期分化マーカーである AFP 遺伝子発現は減少する傾向にあった．肝芽細胞は肝細胞及び胆管上皮細胞へ分化するが胆管上皮細胞のマーカーである CK19 遺伝子発現も同時に減少していた．さらに蛍光免疫染色，ウエスタンブロッティングによる解析によりアルブミンタンパクの産生が確認され TGPC が肝細胞様に分化していることが示された．

4-3. TGPC 移植の効果 ヒト細胞を移植するため拒絶反応を起こさない免疫不全のヌードラットを使用した．9週齢のフィッシャー 344 系ヌードラットの腹腔内に四塩化炭素（1 ml/kg body weight）を週 2 回，4週間投与し肝傷害を与えた．TGPC は分化誘導培地あるいは非誘導培地で培養後蛍光色素である PKH26 で染色し，四塩化炭素初回投与 2 日後に門脈より 1×10⁷ 個の細胞を移植した．コントロールとしては四塩化炭素の替わりにオリーブオイルを腹腔内投与したもの，及び四塩化炭素を投与し細胞の替わりに生理食塩水を門脈から投与したもの（sham operation）を用意した．凍結肝臓切片を作
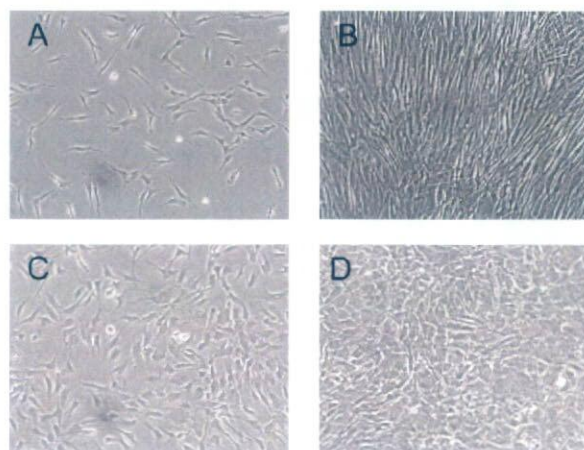

Fig. 4. Morphological Change of MSC Derived from Dental Papilla during the Induction Culture
MSCs were cultured in basal (A and B) and induction (C and D) medium. Pictures were taken at 7 (A and C) and 14 (B and D) days.

成し蛍光観察を行った．細胞移植群においては誘導培地，非誘導培地で培養した双方で生着が確認された．BMSC を移植した際には個々の蛍光が散在していたが TGPC 移植の場合はコロニー状の像が観察されたことから生着後に増殖したものと思われる．*In vitro* の培養において TGPC は BMSC に比べ旺盛な増殖能を有しており，生着後の増殖を可能にしたものと思われる．ヒト特異的な DNA の配列であ

る alu 配列の有無を PCR で確認したところ細胞移植群の肝臓でその存在が確認された．ほかの臓器，及び sham operation したラットの肝臓では PCR 産物は検出されなかった．これらの結果よりヒト歯胚由来の TGPC が確かに肝傷害を惹起したヌードラットの肝臓に生着したことが示された．

分化誘導した細胞の移植群では有意に血清 AST，ALT 値の低下，肝線維化の抑制が観察された．非誘導培地で培養した細胞を移植した群は肝臓内に生着していたにも係わらず有意な治癒効果は現われなかった．これらの結果より肝細胞への方向付けを行うことが重要であることが示された．このように破棄される組織から再生医療に有用な幹細胞が得られることは重要であり，自己の親知らずを抜歯した際，TGPC を細胞バンクに保存しておけば自身の細胞を肝疾患の治療に利用することが可能となる．またデータは示さないが TGPC は骨，肝臓以外に神経細胞への分化能も確認されており，多様な疾患に対して利用可能な能力を有している．

5. MSC に対する機能性付与

MSC は培養が可能であり造血幹細胞などと比較し遺伝子導入がし易い細胞である．われわれはこれまでアデノウイルスベクターを利用し肝細胞にチオレドキシン，C/EBP-β などの遺伝子を導入し酸化的ストレスやアポトーシスに対する抵抗性を付与することに成功している．[25,26] MSC に対しても細胞死に抵抗性を付与する遺伝子あるいは治癒を促進する因子の遺伝子を導入できれば最小限の移植細胞数で治癒効果を発現することが可能となる．そこで現在医薬基盤研究所の水口裕之博士と共同で MSC に対する最適な遺伝子導入法の検討を開始している．アデノウイルスベクターは受容体（coxsakievirus and adenovirus receptor; CAR）を発現する肝細胞などに非常に高い感染効率で遺伝子導入が可能であるが，MSC にはほとんど感染しない．Mizuguchi らはアデノウイルスのファイバー部分に RGD ペプチドやポリリジン配列を付加することにより CAR を発現しない細胞にも効率よく遺伝子導入が可能であることを報告している．[27-29] そこで改変型アデノウイルスベクターに LacZ 遺伝子を挿入し BMSC に感染させたところ良好な遺伝子発現が観察された．現在，種々の遺伝子を改変型アデノウィルスベクターに連結し，BMSC に対して機能性を付与す

ることを試みている．そして最終的には組織特異的なデリバリー機能を有した「細胞性医薬品」として育種することを目的として検討を続けている．

6. 結語

2006 年，京都大学の Yamanaka らにより ES 細胞の多能性維持に係わる因子が解析され Oct3/4，Sox2，c-Myc，Klf4 の遺伝子をレトロウイルスベクターでマウス皮膚細胞に導入することにより ES 細胞類似の induced pluripotent stem（iPS）細胞を樹立したことが報告された．[30] ES 細胞同様テラトーマを形成するなど解決すべき点も多いが自己の細胞，例えば皮膚の細胞を無限増殖能，多分化能を有する幹細胞に変身させる技術が確立できれば理想的であろう．本総説において成体に存在する BMSC と TGPC を利用した肝疾患治療の可能性を紹介したが今後，ES 細胞，iPS を含め様々な幹細胞が日常的に臨床使用される日が 1 日も早く訪れることを期待している．

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Fucoidan partly prevents CCl₄-induced liver fibrosis

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Abstract

Fucoidan, a sulfated polysaccharide extracted from brown algae, has a wide range of biological activities, including anti-inflammatory, anti-viral, and anti-tumor activities. In the present study, we investigated the effects of fucoidan on CCl₄-induced liver fibrosis. Administration of fucoidan reduced CCl₄-induced acute and chronic liver failure. Hepatic fibrosis induced by CCl₄ was also attenuated by injection of fucoidan. Damage to hepatocytes and activation of hepatic stellate cells are key events in liver fibrosis, and, interestingly, treatment of hepatocytes with fucoidan prevented CCl₄-induced cell death and inhibited the proliferation hepatic stellate cells. These results indicate that fucoidan might be a promising anti-fibrotic agent possessing dual functions, namely, protection of hepatocytes and inhibition of hepatic stellate cell proliferation.
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Keywords: Fucoidan; Liver fibrosis; Hepatocyte; Hepatic stellate cell

1. Introduction

Hepatic fibrosis results from chronic damage to the liver in conjunction with the progressive accumulation of fibrillar extracellular matrix proteins (Friedman, 1993; Gressner, 1995; Lieber, 1999). The main causes of liver fibrosis are infection with hepatitis B or C, alcohol abuse, and non-alcohol steato-hepatitis. There are over 100 million people with hepatic fibrosis in the world.

The liver consists of parenchymal (hepatocytes) and non-parenchymal cells (Kupffer, stellate, and endothelial cells). Hepatic fibrosis is triggered by specific intercellular interactions among some of these cells. Kupffer cells are activated by membrane components from damaged hepatocytes and infiltrating inflammatory cells. The activated Kupffer cells release pro-fibrotic factors, such as transforming growth factor-β, reactive oxygen species, and other factors (Wu et al., 1998). These pro-fibrotic factors act on hepatic stellate cells, which are key players in hepatic fibrosis (Gressner, 1995).

Hepatic stellate cells are normally quiescent and produce only small amounts of extracellular matrix components, such as laminin and collagen type IV, during the formation of basement membrane (Maher and Bissell, 1993). Exposure of hepatic stellate cells to the pro-fibrotic factors activates hepatic stellate cells, leading to a changed to a myofibroblast phenotype and an increase in the production of collagen (Friedman, 1999; Geerts, 2001). Preventing the activation of hepatic stellate cells is thus a promising anti-fibrotic strategy. Indeed, administration of antioxidants has been shown to suppress the activation of hepatic stellate cells, thereby preventing liver fibrosis, and inhibition of hepatocyte cell death can reduce liver fibrosis (Houglum et al., 1997; Maher et al., 1997; Horie et al., 2003; Canbay et al., 2002; Song et al., 2003).

Brown algae seaweeds contain both soluble dietary fiber polysaccharides, including alginates, fucans, and laminarans, and insoluble fibers, which are mostly composed of cellulose (Lahaye and Kaeffer, 1997; Kloareg and Quatrano, 1988). The fucans are cell wall polysaccharides that contain variable amounts of fucose, uronic acids, galactose, xylose, and sulfate. They are classified in three types according to their chemical composition: fucoidan, ascophyllan, and glucuronofuco-galactan sulfate (Lahaye and Kaeffer, 1997; Kloareg and Quatrano, 1988; Mabeau et al., 1990). Fucoidan is a complex sulfated polysaccharide derived from *Fucus vesiculosus*, and it has a variety of biological activities, including anti-inflammatory, anti-viral, anti-liver failure, and anti-tumor activities (Boisson-Vidal et al., 1995; Saito et al., 2006). Furthermore, fucoidan

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interacts with transforming growth factor-β and has antioxidative properties (Xue et al., 2001; McCaffrey et al., 1994). These findings indicate that fucoidan may have anti-fibrotic activity, but whether fucoidan can attenuate hepatic fibrosis is unknown. Therefore, in the present study, we evaluated the effects of fucoidan on hepatic injury and fibrosis. We found that fucoidan may be useful as a novel type of anti-fibrotic agent.

2. Materials and methods

2.1. Reagents

CCl₄ was purchased from Wako Pure Chemicals, Co. Ltd. (Osaka, Japan). Fucoidan was obtained from Sigma Chemical, Co. Ltd. (St. Louis, MO). CCl_4 and fucoidan were dissolved in olive oil and saline in animal experiment, respectively. In cellular experiments, fucoidan was dissolved in cultured medium for hepatocytes or hepatic stellate cells as described below. CCl_4 was dissolved in dimethylsulfoxide at 100 mM.

2.2. Animals and experimental protocols

All of the experimental protocols conformed to the ethics guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University. Male Sprague–Dawley rats (200–250 g) and male ddy mice (6 weeks old) were obtained from SLC (Shizuoka, Japan). The mice were housed in an environmentally controlled room (lights on from 8:00 to 20:00; temperature, 23 ± 1.5 °C). Animals had free access to water and commercial chow (Type MF, Oriental Yeast, Tokyo, Japan).

In the acute liver injury model, CCl_4 was intraperitoneally administrated to mice at 0.3 ml/kg body weight with or without intravenous injection of fucoidan (25 and 50 mg/kg body weight). After 24 h of fucoidan injection, the mice were anesthetized, and the blood was recovered. In the chronic liver injury model, CCl_4 (0.3 ml/kg body weight) and fucoidan (50 mg/kg body weight) were administered orally and intravenously, respectively, to mice twice a week for 8 weeks. The liver and blood were recovered from the mice under anesthesia. The serum was separated from the blood and stored at −20 °C before assay.

2.3. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) assays

Serum AST and ALT levels were measured using commercially available kits (Mitsubishi Kagaku Iatron Inc., Tokyo, Japan) according to the manufacturer's instructions.

2.4. Analysis of fibrosis

Liver specimens were fixed with 10% formaldehyde and embedded in paraffin. Tissue sections were mounted on slides, and Azan staining was performed to analyze the extent of fibrosis. After establishing a background for each micrograph, the number of pixels showing a blue color (stained collagen fibers) was determined with Scion Image (National Institutes of

Health, Bethesda, MD), and the percentage of fibrosis in the liver was calculated as the ratio of the blue-colored area to the total area of the liver.

2.5. Isolation of hepatocytes and assay of viability

Hepatocytes were isolated from rats by perfusion of the liver with collagenase (Seglen, 1976). The cells were cultured in William's medium E containing 1 nM insulin, 1 nM dexamethasone, and 10% fetal bovine serum. The cells were seeded onto a dish at 1×10^5 cells/cm² in the absence or presence of fucoidan at 0, 0.3, 0.5, and 1.0 mg/ml. After 6 h of treatment with fucoidan, CCl_4 was added to the cells at 1 mM. After 3 h, the viability of the cells was assayed by staining with Trypan blue.

2.6. Isolation of hepatic stellate cells and assay of viability

Hepatic stellate cells were isolated from rat livers by perfusion with collagenase and pronase, followed by centrifugation on a Nycodenz cushion as described previously (Kawada et al., 1993). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The cells were then seeded onto a dish at 5×10^5 cells/cm² and then treated with fucoidan at 0, 0.3, 0.5, and 1.0 mg/ml for 24 and 48 h. The viability of the cells was determined by mitochondrial conversion of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Mosmann, 1983). The viability of the cells was calculated compared to cells at 0 h (i.e., prior to treatment with fucoidan).

3. Results

3.1. Effect of fucoidan on acute liver injury

We first examined the effect of fucoidan on acute liver failure induced by single injection of CCl_4. Intraperitoneal injection of mice with CCl_4 increased biochemical markers of liver injury. Specifically, 24 h after injection of CCl_4, serum AST and ALT had increased from 125 to 707 and from 21 to 752 karumen unit/ml, respectively (Fig. 1). Intravenous administration of
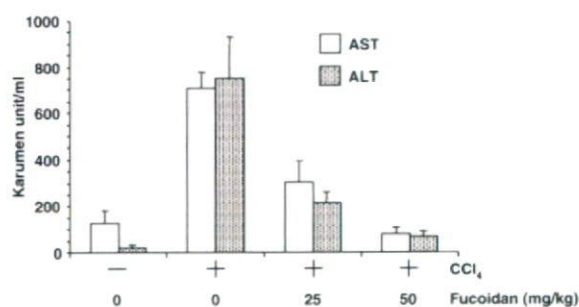

Fig. 1. Effect of fucoidan on CCl_4-induced hepatic injury. Mice received intraperitoneal injection of CCl_4 and intravenous injection of fucoidan. After 24 h, blood was recovered, and the serum AST (open column) and ALT (slashed column) levels were determined using commercially available kits. Results represent means ± S.D. ($n = 4$).

fucoidan attenuated this elevation of serum AST and ALT (304 and 214 karumen unit/ml, respectively, at 25 mg/kg fucoidan). Notably, injection of 50 mg/kg fucoidan restored serum AST and ALT to normal levels (77 and 67 karumen unit/ml, respectively). Together, these results indicate that fucoidan is a potent inhibitor of acute CCl_4-induced liver injury.

3.2. Effect of fucoidan on chronic liver injury

To evaluate the effect of fucoidan on chronic liver injury, we continuously administered CCl_4 to mice twice a week for 8 weeks. Serum AST and ALT levels increased from 149 to 433 and from 101 to 568 karumen unit/ml, respectively (Fig. 2A). Azan staining revealed that collagen accumulated in the liver, indicating the onset of liver fibrosis (Fig. 2B). Indeed, the fibrotic area in the liver increased from 1.9% to 14.8% of the
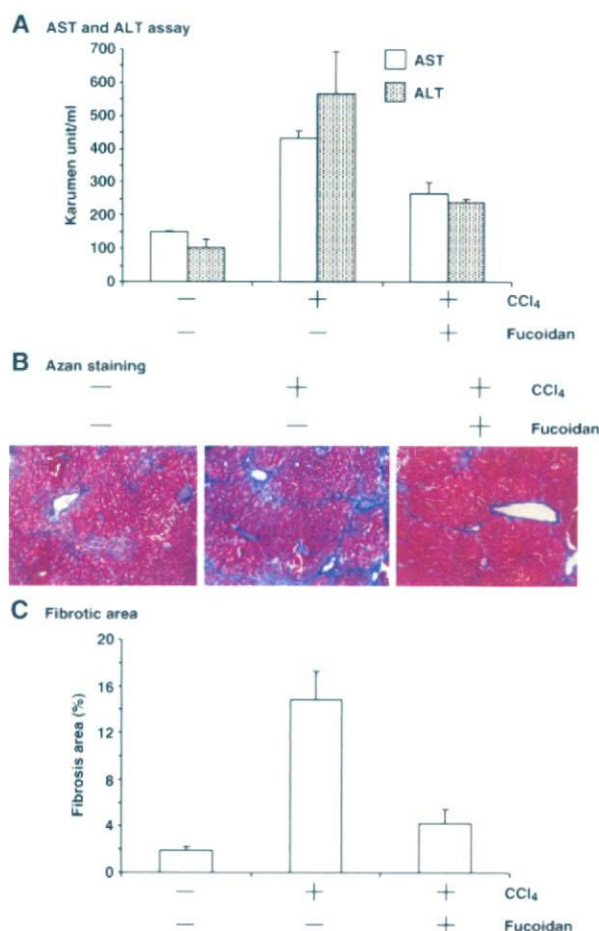

A   AST and ALT assay

B   Azan staining

C   Fibrotic area

Fig. 2. Effect of fucoidan on CCl_4-induced chronic hepatic injury. Mice were injected orally with CCl_4 (0.3 ml/kg body weight) and intravenously with fucoidan (50 mg/kg body weight) twice a week for 8 weeks. (A) Blood was recovered for determination of serum AST (open column) and ALT (slashed column). Results indicate are means ± S.D. (n = 4). (B) Livers were recovered, sectioned, and stained with Azan. Blue and red areas correspond to fibrotic and normal regions, respectively. (C) The ratio of the fibrotic blue area to the blue and red area of the liver was calculated from the sections using NIH Scion Image. The calculation was performed on five images. Results represent means ± S.D. (n = 4).
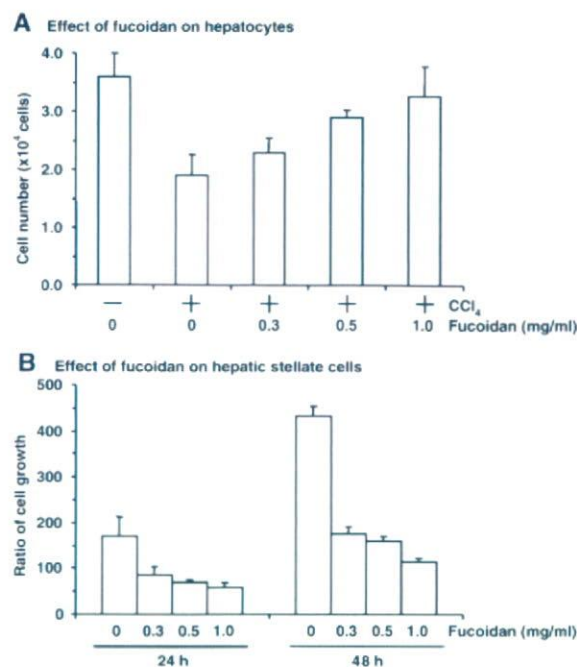

A   Effect of fucoidan on hepatocytes

B   Effect of fucoidan on hepatic stellate cells

Fig. 3. Effect of fucoidan on CCl_4-treated hepatocytes and hepatic stellate cells. (A) Effect of fucoidan on CCl_4-induced cell death in hepatocytes. Hepatocytes were treated with CCl_4 (1.0 mg/ml) in the absence or presence of fucoidan at the indicated concentration for 3 h. Viable cells were counted by Trypan blue staining. Results represent means ± S.D. (n = 4). (B) Effect of fucoidan on growth of hepatic stellate cells. Hepatic stellate cells were treated with fucoidan at the indicated concentration for 24 and 48 h. The viability of the cells was assayed by mitochondrial conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. The growth ratio was calculated as a percentage of the viability at 0 h. Results represent means ± S.D. (n = 4).

whole liver (Fig. 2C). The elevation of AST and ALT levels was significantly reduced by administration of fucoidan (from 433 to 265 and from 568 to 238 karumen unit/ml, respectively; Fig. 2A). The increase in fibrotic area induced by CCl_4 was also attenuated by fucoidan (from 14.8% to 4.3%; Fig. 2C). Together, these findings show that fucoidan may be useful for treatment of hepatic fibrosis in chronic liver injury.

3.3. Effect of fucoidan on hepatocytes and stellate cells

As described above, our preliminary results indicated that fucoidan may be useful for the treatment of hepatic fibrosis. During the initiation of hepatic fibrosis, hepatocytes release a paracrine factor that stimulates hepatic stellate cell growth, leading to the damage of hepatocyte membranes (Gressner, 1995; Gutierrez-Reyes et al., 2007). Therefore, to determine how fucoidan prevents liver injury, we evaluated its effects on hepatocytes and hepatic stellate cells. As shown in Fig. 3A, treatment of hepatocytes with CCl_4 (1 mM) reduced their viability to 63.3% of control (untreated) cells. In addition, fucoidan dose-dependently reduced CCl_4-induced cell death, with complete prevention of cell death at 1 mg/ml. Alone, fucoidan did not show any cytotoxicity at 1.0 mg/ml in hepatocytes (data not shown). CCl_4-induced cell death was not inhibited by pre-incubation of CCl_4 with fucoidan (data not

shown), indicating that the protective effects of fucoidan on CCl$_4$-induced cell death is not due to absorption of CCl$_4$ by fucoidan. In contrast, the viability of hepatic stellate cells was reduced to 26.4% of the control by a 48-h treatment with 1.0 mg/ml fucoidan (Fig. 3B). Thus, both the protective effects of fucoidan against CCl$_4$-induced cell death in hepatocytes and its cytotoxicity to hepatic stellate cells might contribute to its anti-fibrotic activity.

4. Discussion

Fucoidan, the sulfated polysaccharides of brown algae, contains L-fucose residues as the main sugar constituent along with sulfate esters. Although fucoidan is known to have many biological activities, including anti-coagulant, anti-thrombosis, anti-inflammatory, anti-liver failure, and anti-tumor activities (Boisson-Vidal et al., 1995; Berteau and Mulloy, 2003; Saito et al., 2006), this is the first investigation of its anti-fibrotic activity. Our results show for the first time that fucoidan can reduce hepatic fibrosis in an animal model.

Studies in animal models of hepatic fibrosis show that extracellular matrix components accumulate in interstitial regions of the liver around central veins or in the portal tracts. Normally, hepatic stellate cells exist in a quiescent state, but they become activated following liver injury. These activated hepatic stellate cells are primarily responsible for the excess production of extracellular matrix (Senoo et al., 1998). Thus, reduction of extracellular matrix production by activated hepatic stellate cells is crucial for the prevention of fibrogenesis. Damage to hepatocytes is the primary and continuing factor leading to hepatic stellate cell activation. Components released from the damaged cells, including lipid peroxides and reactive oxygen species, activate Kupffer cells in the liver, leading to their secretion of transforming growth factor-β (Tsukamoto, 1999). These pro-fibrogenic factors activate hepatic stellate cells, which results in liver fibrosis. Here, we showed that treatment of hepatocytes with fucoidan attenuates CCl$_4$-induced cell death.

Fucoidan has been reported to interact with transforming growth factor-β and to scavenge reactive oxygen species (Boisson-Vidal et al., 1995; McCaffrey et al., 1994; Xue et al., 2001). CCl$_4$ elevates serum transforming growth factor-β levels and acts as a hepatotoxin by inducing the production of reactive oxygen species (Weiler-Normann et al., 2007). In agreement with this, we have found that administration of fucoidan reduces CCl$_4$-induced lipid peroxidation (data not shown). Taken together, our results suggest that the anti-fibrogenic activity of fucoidan is due, at least in part, to attenuation of hepatic stellate cell activation by inhibition of transforming growth factor-β and/or by scavenging of reactive oxygen species, which can suppress the cascade of events that leads to hepatic stellate cell activation.

Hepatocyte injury is thought to promote fibrosis, leading to the release of activators from Kupffer cells. In contrast, hepatic stellate cell apoptosis is thought to be essential for the resolution phase of fibrosis (Canbay et al., 2002; Song et al., 2003; Iredale, 2001; Iredale et al., 1998; Issa et al., 2001). Thus,

a compound that can prevent hepatocyte injury and/or induce the death of hepatic stellate cells should be useful for the treatment of hepatic fibrosis. In the current studies we showed that fucoidan reduces the growth of hepatic stellate cells and that it can protect hepatocytes from injury. We also found that the fucoidan-treated hepatic stellate cells were stained with annexin V, a marker of apoptosis (data not shown). Taken together, these findings suggest that fucoidan may be useful for treating hepatic fibrosis.

Most studies on fucoidan have used a commercially available crude extract from F. vesiculosus, and we used the crude fucoidan in the current study. Fucoidan contains heteropolysaccharides of various kinds besides those consisting predominantly of sulfate and fucose (Nishino et al., 1994). Patel et al. found that crude commercial fucoidan was more active than the purified fucoidan at inhibiting the proliferation of vascular smooth muscle cells, and then they speculated that a specific structure in the crude fucoidan may mediate its biological activities (Patel et al., 2002). Indeed, the content of the sulfated groups in fucoidan determines its anti-proliferative and anti-coagulant activities in fibroblasts (Haroun-Bouhedja et al., 2000). The biological activities may differ among the various structures of pure fucoidan as well as the different components of crude fucoidan. Identification of the structures of fucoidan that protect hepatocytes from hepatotoxins and that inhibit hepatic stellate cell growth is needed for the development of fucoidan as an anti-fibrotic agent.

In summary, we found that fucoidan prevents hepatocyte cell death and induces the death of hepatic stellate cells in an animal model of hepatic fibrosis. Future studies will examine the molecular mechanisms of fucoidan in hepatocytes and hepatic stellate cells. This is the first report that fucoidan has anti-fibrotic activity and that it is a promising lead for the development of anti-fibrotic agents. Identification of the molecular target and the active structure of fucoidan may lead to the development of novel anti-fibrotic agents.

Acknowledgements

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