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〔ノ ー ト〕

ラクトース修飾絹フィブロイン基材上における初代培養ラット肝細胞のスフェロイド形成と維持

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1 緒 言

カイコが生産する繭糸の主成分が構造タンパク質の絹フィブロイン(SF)であり，繭糸を熱水処理(精練)することでSFが得られる．SFは古くから衣料素材として用いられてきたが，その一方で優れた強度と生体適合性を有することから外科用縫合糸としても用いられてきた[1]．近年SFを溶解したSF水溶液から，フィルム・ゲル・スポンジ・不織布などに加工する技術が開発されている[1),2)]．この成形性の良さと生体適合性に加え，昆虫生産物ゆえにBSEなどのヒト感染病原体の混入の危険性が極めて低いことから，SFを医療用材料特に組織再生用足場材料として利用する研究が進められている[1)~3)]．

肝臓は生体内においてアルコール・薬・毒物などの外来性異物の解毒，アンモニア処理のための尿素合成，アルブミンを初めとする血漿タンパク質の合成と分泌などさまざまな機能を有しており，これらの機能を担っているのが肝実質細胞(肝細胞)である．筆者らは肝細胞培養用足場基材としてのSFの新しい利用を目的に，肝細胞の認識糖鎖であるβ-ガラクトース残基を有するラクトースによるSFの化学修飾を行った[4)]．この新規に作り出したラクトース修飾SF(Lac-CY-SF)を足場基材に用いて初代ラット肝細胞の培養を行ったところ，対照区の未修飾SFでは肝細胞の接着がほとんど観察されなかったのに対し，Lac-CY-SFではコラーゲン基材に匹敵する高い細胞接着性を示した[4)]．そしてLac-CY-SF基材上に接着した初代ラット肝細胞の培養をさらに続けると，ラット肝細胞は軽度伸展形態で単層の島状集合体を形成した[4),5)]．しかし，Lac-CY-SF基材上においては，ホルモン因子であるインスリンとデキサメサゾンの培地への添加濃度が低い場合，培養3日目に基材から脱離した死細胞の増加が観察されている[5)]．

一般に単層状態の肝細胞では短期間に肝機能発現が低下し，細胞自身の生存率も低下することが知られている[6)~8)]．それに対し，三次元的な形状を有する多層の球状細胞凝集体(スフェロイド)となった肝細胞は，高い肝機能を発現したまま長期間生存し基材上に維持されることが報告されている[6)~10)]．なお，基材上における初代ラット肝細胞のスフェロイドの形成には，上皮細胞増殖因子(EGF)とインスリンを培地中に加えることが必要である[6)~10)]．これまでにプロテオグリカンでコートした基材上や親水性陽性荷電ポリスチレン基材上で，肝細胞スフェロイドが形成されることが報告されている[6),7)]．また，赤池らによって開発されたラクトース置換ポリスチレン誘導体(PVLA)のコート基材上でも肝スフェロイドが形成されることが知られている[8)~10)]．しかしそれ以外の平面基材を用いてのスフェロイド形成の報告は少ない．

本報では，β-ガラクトース残基を導入したLac-CY-SFの平面基材を用いてEGFとインスリンを添加した条件下でラット肝細胞を初代培養し，スフェロイドの形成を検討するとともに，基材上におけるスフェロイドの維持についても調べたので，その結果について報告する．

2 実 験

2.1 試薬

試薬は，市販品をそのまま使用した．Lac-CY-SFの作製に使用したラクトース一水和物と塩化シアヌル(CY)は，和光純薬工業(株)から購入したものを用いた．肝細胞培養用のウィリアムスのE培地は，ICN Biomedicals Inc. 製の市販品から調製した．アプロチニンはタカラバイオ(株)から購入したものをそのまま用いた．EGFは東洋紡績(株)から購入し，インスリンはSigma Aldrich Co. から購入した．肝細胞培養実験に必要なそのほかの試薬については，田中らの報告[11)]に従って入手した市販品を使用した．

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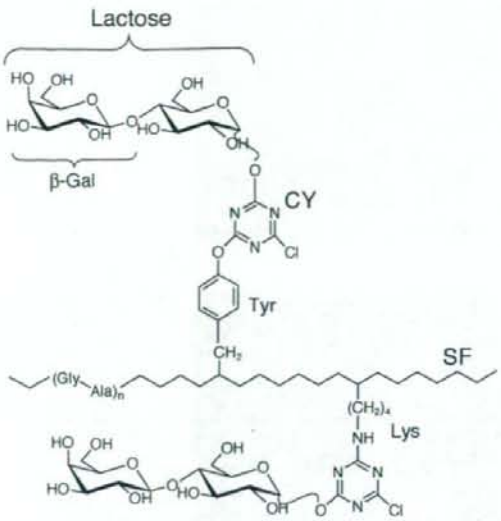

Scheme 1. Chemical structure of lactose-silk fibroin conjugates (Lac-CY-SF) prepared using cyanuric chloride (CY) as a coupling reagent.

2.2 Lac-CY-SF 培養基材の作製

原料の SF 水溶液は，家蚕の繭を精練して得た SF の繊維を，9 M 臭化リチウム水溶液に溶解し透析による脱塩を行って調製した[4]．Lac-CY-SF の作製は，既報の方法に従って CY をカップリング試薬に用いて行った[4),5]．アルカリ条件下ジオキサン-水の溶媒系でラクトースと CY を 4°C で反応させて，修飾剤となる CY 活性化ラクトースを作製した．この修飾剤溶液に SF 水溶液を加えて，修飾剤と SF のアミノ酸残基(チロシン・リジン残基)との反応を 37°C で行うことにより，目的の Lac-CY-SF を作製した(Scheme 1)[4),5]．反応液の中和による反応停止後，蒸留水を用いた透析と限外ろ過で Lac-CY-SF 水溶液の精製と濃縮を行った．得られた Lac-CY-SF におけるラクトース含有量は 17 wt%である[4),5]．

0.1%(w/v)濃度に調製した Lac-CY-SF 水溶液をシリンジフィルター(日本ミリポア(株)製，孔径 0.45 μm)を用いてろ過滅菌した．ろ液各 0.5 mL を細胞培養用のポリスチレンディッシュ(イワキ硝子(株)製，6 ウエル平底プレート，直径 35 mm)の各ウエルに注入し 1 時間静置することで，ディッシュ表面への試料の吸着コートを行った．ディッシュから試料水溶液を除いた後，Dulbecco's PBS(−)緩衝液にて洗浄を行い，試料コートディッシュを使用直前まで D-PBS(−)緩衝液で満たして保存した．

2.3 肝細胞培養実験

ラット肝細胞は，体重 180〜200 g のウィスター系ラットから Seglen のコラゲナーゼ灌流法[12]により調製した．この肝細胞を 1 nM インスリンと 1 μg/mL アプロチニンを添加したウィリアムスの E 培地に懸濁し，播種細胞懸濁液とした．なお，トリパンブルーによる染色で 85%以上の生存率を有する肝細胞を培養に供した．Lac-CY-SF コートディッシュ上に 2.5×10^5 cells/cm^2 の細胞密度になるように肝細胞懸濁液 2 mL を加え，培養を行った．培養は 5%CO_2-30% O_2-65%空気を気相として，CO_2 インキュベーター中で行った．2.5 時間培養後基材に接着しなかった細胞を回収し，培地を 20 ng/mL EGF，100 nM インスリン，1 μg/mL アプロチニンを含むウィリアムスの E 基本培地と交換してさらに 10 日間培養を行った．2 日ごとに培地交換を行い，その直後細胞の形態を位相差顕微鏡((株)ニコン製 DIAPHOT TMD)で観察した．

3 結果と考察

Lac-CY-SF 基材上における接着時のラット肝細胞の形態と，接着後 EGF とインスリンを添加した培地条件下でさらに培養した肝細胞の形態変化を Figure 1 に示す．培養 2.5 時間後基材に接着した細胞は，これまでの接着実験で示した形態と同様の小さな丸い形態を示した(Figure 1(a))[4)]．EGF とインスリンを含む培地に交換して接着細胞の培養を続けたところ，細胞は伸展を始め，培養 2 日目には軽度伸展形態で単層を形成した(Figure 1(b))．培養 3 日目から細胞の移動と集合が始まり，培養 4 日目にはスフェロイドが形成された(Figure 1(c))．形成されたスフェロイドは基材接着部の下層細胞において伸展形態が観察され，直径 100 μm 前後の比較的小さいスフェロイドの他に，全長 200〜300 μm の細長く大きなスフェロイドも観察された．培養 4 日目以降はスフェロイドの大きさの増加は見られず，6 日目においても 4 日目とほぼ同じ大きさと形状で基材上に維持されていた(Figure 1(d))．しかし，8 日目からは死細胞の基材からの脱離とスフェロイドの縮小が観察され始め(Figure 1(e))，10 日目にはスフェロイドの縮小が顕著になった(Figure 1(f))．

これまでに赤池らは PVLA を足場材料として用いた場合，ラット肝細胞は基材接着後 EGF とインスリンの添加条件下における培養 2 日目(48 時間)で直径 80〜100 μm のスフェロイドを形成することを確認し，PVLA 基材上で 3 週間以上にわたるスフェロイドの生存を報告している[9),10)]．一方，Lac-CY-SF 基材ではスフェロイドの形成に時間がかかり，形成されたスフェロイドも短期間しか基材上に維持されない．

スフェロイドの形成には細胞と基材の間の相互作用の強さが深く関係し，細胞と基材が非常に強く接着しているコラーゲンのような基材表面では，細胞どうしが動き

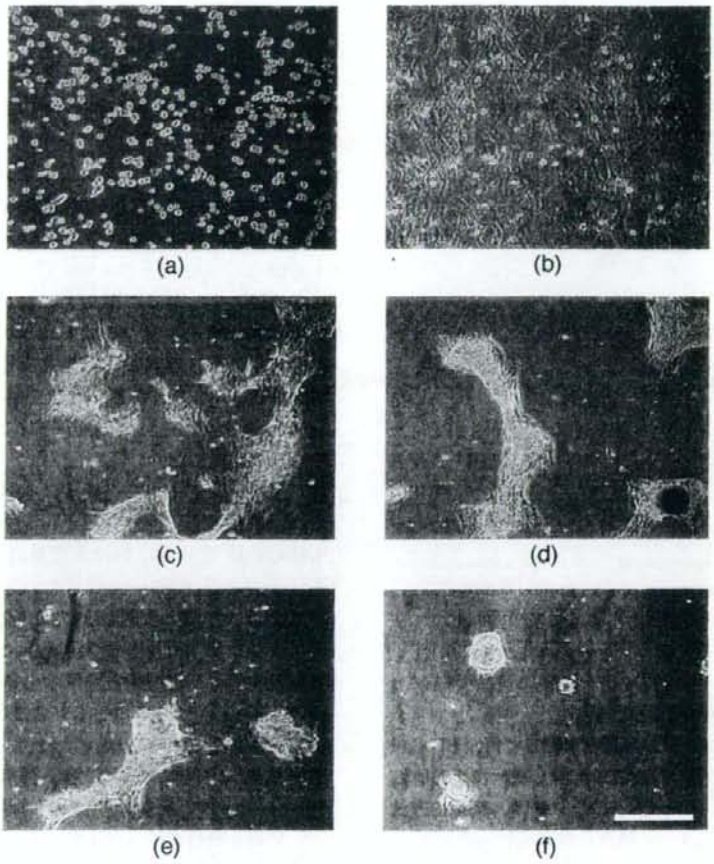

Figure 1. Phase-contrast micrographs of rat hepatocytes cultured on dishes coated with Lac-CY-SF. The cells were cultured in Williams' E medium containing 1 nM insulin for 2.5 h (a) and subsequently in the medium containing 20 ng/mL EGF and 100 nM insulin for 2 days (b), 4 days (c), 6 days (d), 8 days (e), and 10 days (f). Scale bar indicates 100 μm.

あって接触・接着することができず細胞の集合化が困難であることが知られている[8),9)]．したがってLac-CY-SF基材上でのスフェロイド形成に時間を要するのは，肝細胞とLac-CY-SFとの間の相互作用がPVLAとの間の相互作用に比べ強いためと推定される．またLac-CY-SF基材上のスフェロイドがきれいな球状の形態を示さず，スフェロイドの基材接着部の下層細胞が伸展形態を示すのも，細胞とLac-CY-SFとの間の相互作用の強さによると考えられる．

Lac-CY-SFにおいて肝細胞のアシアロ糖タンパク質レセプターが認識するβ-ガラクトース残基を有するラクトース分子は，CY由来のトリアジン環を介して，SFのチロシン残基(含有量5 mol%)のフェノール性ヒドロキシル基およびリジン残基(含有量0.3 mol%)のε-アミノ基と結合している(Scheme 1)[4),5)]．そのためLac-CY-SFではPVLAに比べラクトース糖鎖密度は低いが，ポリスチレンディッシュ表面に吸着していると考えられるSFのポリペプチド主鎖からラクトースは離れて水層側に存在し，末端のガラクトース残基の立体障害は少ないと推定される．そして肝細胞のレセプターは立体障害の少ないガラクトース残基に容易に接近し強く結合できるので，肝細胞はLac-CY-SFと強く相互作用するものと推定される．

この肝細胞とLac-CY-SFとの強い相互作用による肝細胞の移動速度の低下が，スフェロイド形成に時間を要する原因になっていると考えられるが，それに加えてスフェロイド巨大化の原因にもなっているかもしれない．すなわち基材上の細胞の移動速度が遅ければスフェロイ

ド形成に必要な細胞どうしの接触までに時間を要するが，その反面いったん細胞間の接触が起こると，移動速度の遅さゆえに接触時間が長くなると考えられる．そして接触時間が長くなれば，集合化が容易になるとともに，細胞どうしが強く結合した安定な集合体が形成されるので，スフェロイドが大きくなると思われる．

一方，基材のスフェロイド維持能の違いの一因として，形成されるスフェロイドの大きさの違いが影響している可能性がある．一般にスフェロイドの直径が大きくなるにつれ，スフェロイドの中心部への酸素や栄養分の供給不足となりネクローシス(壊死)が起こることがよく知られている[13),14)]．Török らはスフェロイドの直径が200 μm より小さいとネクローシスは見られないが，200 μm 以上になるとネクローシスが発生することを報告している[14)]．また Glicklis らは，スフェロイドの直径が100 μm より大きくなるとスフェロイド中心部は酸素濃度が低い状態になり，細胞の生存率が低下することを示している[15)]．Lac-CY-SF 基材においては最初から200 μm 以上の大きなスフェロイドが数多く形成されることから，スフェロイド形成から比較的短期間にネクローシスが発生している可能性がある．

以上の結果から，Lac-CY-SF コートディッシュを基材に用いたラット肝細胞の初代培養において，EGF とインスリンの添加によってスフェロイドが形成されることを確認した．しかしながら，形成されたスフェロイドを Lac-CY-SF 基材上に5日以上維持するのは困難であることが示された．

今後 Lac-CY-SF 基材上で長期間維持される良好なスフェロイドを形成させるためには，誘導因子である EGF とインスリンの添加条件の検討が必要と考えられるが，一方で足場基材の形状を変えることはより有効な手段と考えられる．近年の組織工学において，足場材料を基板全面にコートしたこれまでの平面基材に加え，微細加工技術を用いて細胞非接着性の基板表面に数十～数百ミクロン単位の大きさの細胞接着空間を規則的に配したマイクロパターン基材の作製が行われている[16)~18)]．この細胞接着領域が制限されたマイクロパターン基材を用いることにより，肝細胞では形成されるスフェロイドの大きさや機能の制御が可能になっている[16),17)]．繊維タンパク質由来の Lac-CY-SF は耐熱性・耐溶媒性に優れ，フォトリソグラフィー技術を用いる微細加工工程にも耐えられる材料である．それゆえ今後 Lac-CY-SF を細胞接着領域としたマイクロパターン基材を作製することができれば，細胞接着領域の制限により基材と肝細胞との相互作用が弱められて短期間でのスフェロイド形成が期待されるとともに，形成されるスフェロイドの大きさも制御され，ネクローシスの発生の抑制と基材上での長期間細胞維持が期待される．

また最近の肝細胞を対象とした組織工学研究では，マイクロパターン基材だけでなく，生体内の細胞外マトリックスに類似した三次元構造を持つポリウレタンフォームやアルギン酸スポンジなどの多孔質体を用いて肝スフェロイドを形成させ，長期間高い肝機能を発現した状態で基材に細胞を維持させる試みが行われている[15),19)~22)]．こうした多孔質体ではスフェロイドは空孔内に形成され，酸素や栄養分の供給，老廃物の除去に有利であるとともに，試料コート平面基材を用いた場合に比べて細胞の基材接着は抑えられている[15),22)]．それゆえ，マイクロパターン基材と同様に多孔質体も，細胞接着の制限で基材と細胞との相互作用が弱められ，細胞どうしの相互作用によってスフェロイド形成が促進される足場基材と考えられる．SF 水溶液と同様に Lac-CY-SF 水溶液から，凍結乾燥法を用いてスポンジの作製が可能である．したがって，足場となる Lac-CY-SF を三次元構造体のスポンジに成形することでも，肝スフェロイド形成と維持がより効果的に行われると期待される．

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[Notes]

Formation and Maintenance of Rat Hepatocyte Spheroids on Lactose-Silk Fibroin Conjugates in Primary Cultures

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We examined the formation and maintenance of spherical multicellular aggregates (spheroids) of rat primary hepatocytes on dishes coated with lactose-silk fibroin conjugates bearing galactose residues (Lac-CY-SF) as substrate materials. Rat hepatocytes that had attached onto the conjugate-coated dishes were subsequently cultured in a medium supplemented with epidermal growth factor (EGF) and insulin. After the rat hepatocytes extended flat on the conjugate-coated dishes in 2 days of culture, these hepatocytes formed spheroids about 100 to 300 μm in diameter at day 4. After 6 days of culture the spheroids were maintained without any obvious change in size on the conjugate-coated dishes. However, the detachment of the spheroids from the conjugate-coated dishes began from day 8, and the spheroids clearly shrank at day 10. These results suggested that hepatocyte spheroid formation was induced on the Lac-CY-SF conjugate-coated dishes in the presence of EGF and insulin, but the conjugate-coated dishes were not capable of keeping the spheroids for more than 5 days.

KEY WORDS Lactose-Silk Fibroin Conjugates / Rat Hepatocytes / Epidermal Growth Factor / Insulin / Spheroids /
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Annexin A3 Expression Increases in Hepatocytes and is Regulated by Hepatocyte Growth Factor in Rat Liver Regeneration

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Annexin (Anx) A3 increases and plays important roles in the signalling cascade in hepatocyte growth in cultured hepatocytes. However, no information is available on its expression and role in rat liver regeneration. In the present study, AnxA3 expression was investigated to determine whether it also plays a role in the signalling cascade in rat liver regeneration. AnxA3 protein and mRNA level both increase in liver after administration of carbon tetrachloride (CCl_4) or 70% partial hepatectomy. AnxA3 protein level increases in isolated parenchymal hepatocytes, but not in non-parenchymal liver cells, in these rat liver regeneration models. AnxA3 mRNA increases in hepatocytes after CCl_4 administration. Anti-hepatocyte growth factor antibody suppresses this increase in AnxA3 mRNA level. These results demonstrate that AnxA3 expression increases in hepatocytes through a hepatocyte growth factor-mediated pathway in rat liver regeneration models, suggesting that AnxA3 plays an important role in the signalling cascade in rat liver regeneration.

Key words: annexin A3, carbon tetrachloride, hepatocyte growth factor, parenchymal hepatocytes, partial hepatectomy.

Abbreviations: Anx, Annexin; CCl_4, carbon tetrachloride; HGF, hepatocyte growth factor.

Annexin (Anx) A3 is a member of the Anx family, which binds to phospholipids and membranes in a Ca^{2+}-dependent manner (*1–4*). AnxA3 has been shown to have anti-coagulant and anti-phospholipase A_2 properties *in vitro* (*5*, *6*), plus to promote Ca^{2+}-dependent aggregation of isolated specific granules from human neutrophils (*5*, *6*). Some reports describe its regulation and role in cultured cells (*7–11*); however, there are no reports describing these characteristics *in vivo*.

We recently reported that AnxA3 is expressed in cultured rat hepatocytes, but not in isolated hepatocytes and that inhibition of AnxA3 expression by RNA interference results in a significant inhibition of hepatocyte growth (*10*, *12*, *13*). These findings indicate that AnxA3 plays an important role in the signalling cascade in hepatocyte growth in cultured hepatocytes, although the mechanism remains to be elucidated. The significance of AnxA3 in hepatocyte growth is also supported by the finding that known stimulatory or inhibitory actions of various factors to hepatocyte growth correlated well with the increase or decrease in AnxA3 expression (*14*).

These findings indicate that AnxA3 increases and is likely to play an important role in the signalling cascade in rat liver regeneration. AnxA1 increases in rat and mouse liver regeneration models, *e.g.* after administration of carbon tetracholoride (CCl_4) and 70% partial hepatectomy (*15*, *16*). Suppression of AnxA1 expression using anti-sense technology inhibits proliferation in a mouse hepatocyte cell line (*15*). Therefore, AnxA1 is also likely to play an important role in the signalling cascade in rat liver regeneration.

In the present study, AnxA3 expression in rat liver regeneration models was investigated to explore the possibility that AnxA3 plays important roles in the signalling cascade in rat liver regeneration.

MATERIALS AND METHODS

Animals and Experimental Conditions—Adult male Wistar rats (180–200 g) were purchased from Japan SLC Co., Ltd. (Shizuoka, Japan) and used for all studies. They were maintained in a 12 h light/dark cycle, allowed food and water *ad libitium*. All animal care and procedures were approved by the institutional care committee and carried out in accordance with the guidelines established by the National Institute of Health.

For studies of liver regeneration after toxic injury, rats received CCl_4 intraperitoneally (2 ml/kg body weight of 50% solution of CCl_4 in olive oil). Control rats received olive oil intraperitoneally (1 ml/kg body weight of olive oil). Animals given CCl_4 or olive oil were sacrificed at 3–24 h after administration.

A 70% partial hepatectomy was performed according to Higgins and Anderson (*17*). In the sham operation, livers were exposed and manipulated but not removed. These procedures were performed under anaesthesia with Nembutal (Abbot, Chicago, IL, USA). Animals subjected to

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partial hepatectomy or sham operation were sacrificed at 2.5–20 h after the operation.

For infusion of anti-human hepatocyte growth factor (HGF) antibody, rats were intravenously injected with 0.2 ml goat anti-human HGF IgG (Sigma–Aldrich, St Louis, MO, USA) (1.25 mg/kg body weight) diluted in phosphate-buffered saline (PBS) through the tail vein, then received CCl_4 intraperitoneally, as described earlier. Control rats were injected with the same volume and amount of control goat IgG, and then received CCl_4 intraperitoneally in a similar manner. Parenchymal hepatocytes were prepared from the rats after 6 h, as described subsequently.

Preparation of Liver Lysate—The procedures were performed at low temperature, unless described otherwise. Liver was *in situ* perfused with PBS *via* the portal vein, then removed from the body. Liver was homogenized with a Potter-Elvehjem homogenizer in 4× (v/w) buffer A [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA and 2.5% (v/v) Triton-X 100] containing 1 mM benzylsulphonyl fluoride, 0.3 mM leupeptin and 0.5 mM aprotinin. The homogenate was shaken for 15 min at room temperature, then sonicated four times for 15 s each time. After centrifugation at 100,000*g*, the cytosolic fraction was stored at −70°C until use.

Cell Isolation—Parenchymal hepatocytes were isolated from rats by *in situ* perfusion of the liver with collagenase (*18*). Non-parenchymal liver cells were isolated from the supernatant of parenchymal cells by differential centrifugation, as described by Shimaoka *et al.* (*19*). In this article, hepatocytes are also referred to as parenchymal hepatocytes to distinguish between hepatocytes and non-parenchymal liver cells.

Preparation of Cell Lysate—Cell lysates were prepared by a modification of the reported by Römisch *et al.* (*20*). Procedures were performed at low temperature, unless described otherwise. Cells were resuspended in three volumes of buffer A containing 1/100 (v/v) protease inhibitor cocktail (Sigma–Aldrich, St Louis, MO, USA). They were then shaken for 15 min at room temperature and sonicated four times for 15 s each time. After centrifugation at 100,000*g*, the cytosolic fraction was stored at −70°C until use.

Western Blot Analysis—An equal amount of cytosolic protein from each experiment was subjected to SDS–PAGE on a 10% gel and electroblotted to PVDF membrane (GVHP; Millipore, Bedford, MA, USA). After blocking the membrane with 5% skimmed milk, a western blot analysis was performed using rabbit anti-human AnxA3 antibody serum (1: 5,250) (a gift from Drs F. Russo-Marie and C. Ragueness-Nicol), mouse anti-human GAPDH monoclonal antibody (1: 5,000) (Abcam, Cambridge, UK), or rabbit anti-beta-actin polyclonal antibody (1: 500) (BioLegend, San Diego, CA, USA). Detection was performed using the ECL detection system (GE Health care Bioscience, Buckinghamshire, UK). Housekeeping protein, GAPDH and beta-actin, were selected based on results of preliminary studies. Intensity of each band was measured over a proportional range. A computer-assisted analyser was used to quantitatively analyse intensity, with intensity of the AnxA3 band normalized to the intensity of the appropriate housekeeping protein. Protein amount from liver and cell lysate was measured using a previously described method (*21*), with bovine serum albumin used as a standard.

Total RNA Extraction and Real-Time Quantitative PCR—Total RNA was extracted from liver by a modification of guanidine thiocyanate–phenol–chloroform extraction method (*22*, *23*). Total RNA was extracted from cells using Trizol® reagent (Invitrogen, Cergy Pontoise, France) in accordance with the manufacture's protocol. Equal amounts of RNA (~1 μg) from each experiments were reverse-transcribed using a THERMOSCRIPT™ RT–PCR System (Invitrogen, Cergy Pontoise, France) and oligo$(dT)_{20}$ in a final volume of 40 μl, in accordance with the manufacturer's protocol. Subsequently, 2 μl of cDNA was used as templates for real-time PCR analysis using a LightCycler system (Rosche Diagnostics, Tokyo, Japan) according to the manufacture's instructions. For AnxA3 and 28S rRNA, the PCR programme consisted of 40 cycles of 10 s at 94°C, 10 s at 60°C and 12 s at 72°C. Primer sequences for AnxA3 were 5′ -CAA ATT CAC CGA GAT CCT GT-3′ and 5′ -TGC TGG AGT GCT GTA CGA AA-3′ (*14*) and for 28S rRNA 5′ -CCA GAG CGA AAG CAT TTG CCA-3′ and 5′ -GGC ATC ACA GAC CTG TTA TTG CTC-3′ (*14*). AnxA3 levels were normalized to the levels of 28S rRNA.

Statistical Analysis—Data were analysed using Student's *t*-test, and *P*-values <0.05 were considered to be statistically significant.

Immunohistochemical Examination—Serial liver sections cut at 3 μm thick from the paraformaldehyde-fixed and paraffin-embedded blocks. De-paraffinated and re-hydrated sections were heated for 5 min at 100°C in 10 mM citrate buffer (pH 6.0) followed by the treatment with 10 μg/ml Proteinase K (TAKARA BIO Inc., Shiga, Japan) for 5 min at room temperature. These activated sections were then subjected to blocking with 10% bovine serum albumin for 1 h at room temperature. After washing with PBS, sections were simultaneously incubated for 2 h with antibodies, *e.g.* anti-rat hepatic sinusoidal endothelial cells mouse IgG (SE-1, Immuno-Biological Laboratories Co., Ltd. Gunma, Japan) 1:20 and rabbit anti-human AnxA3 antibody serum 1:200. The fluorescence-labelled secondary antibodies were AMCA-labelled sheep anti-mouse IgG (Jackson Immuno Research Laboratories, Inc., PA, USA) 1:200 and FITC-labelled sheep anti-rabbit IgG (MP Biomedicals Inc., Ohio, USA) 1:200. The liver sections were thus mounted on a cover glass with a mounting medium, Vectashield (Vector Laboratories, CA, USA), and subjected to microscopic observation.

RESULTS

AnxA3 Expression in Liver Following CCl_4 Treatment—AnxA3 protein level increased ~3-fold at 6 h after administration of CCl_4 and this increased level was maintained to 24 h (Fig. 1). AnxA3 mRNA level started to increase at 3 h after administration, reaching an ~17-fold increase at 24 h (Fig. 2).

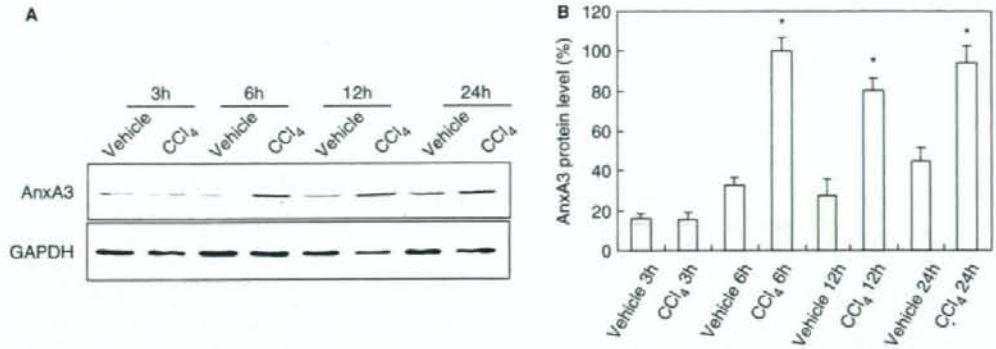

Fig. 1. **AnxA3 protein level in liver following treatment with CCl_4.** (A) Data shown are representative of western blot analysis results. Approximately 35 and 1.5 μg of protein were used for detection of AnxA3 and GAPDH, respectively. (B) Results are presented relative to the value produced by liver in rats at 6 h after CCl_4 administration. AnxA3 protein levels were normalized to the housekeeping protein, GAPDH. Data are expressed as mean ± S.D. ($n = 4$ at each time point) $^*P < 0.01$, compared to the value produced by liver in rats after olive oil administration.

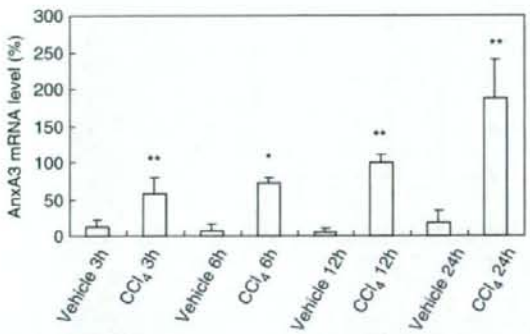

Fig. 2. **AnxA3 mRNA level in liver following treatment with CCl_4.** Results are presented relative to the value produced by liver in rats at 6 h after CCl_4 administration ($n = 4$ at each time point). AnxA3 mRNA levels were normalized to housekeeping gene, 28S rRNA. Data are expressed as the mean ± SD ($n = 4$ at each time point) $^*P < 0.01$, $^{**}P < 0.05$, compared to the value produced by liver in rats after olive oil administration.

AnxA3 Expression in Parenchymal Hepatocytes and Non-parenchymal Liver Cells Following CCl_4 Treatment—Parenchymal hepatocytes and/or non-parenchymal liver cells are involved in the increase of AnxA3 expression in liver following CCl_4 treatment. AnxA3 protein level increased ~5-fold in parenchymal hepatocytes at 6 h after CCl_4 treatment, but did not change in non-parenchymal liver cells (Fig. 3). AnxA3 mRNA level increased ~5-fold in parenchymal hepatocytes at 6 h after CCl_4 treatment; however, it did not change in non-parenchymal liver cells (Fig. 4).

AnxA3 Expression in Liver after Partial Hepatectomy—AnxA3 protein level started to increase at 5 h after partial hepatectomy, reaching a 1.6-fold increase at 20 h (Fig. 5). AnxA3 mRNA level increased to ~2,800-fold at 2.5 h, then began decreasing at 5 h, falling back to basal level at 20 h (Fig. 6).

AnxA3 Expression in Parenchymal Hepatocytes and Non-parenchymal Liver Cells After Partial Hepatectomy—AnxA3 protein level increased ~1.5-fold in isolated parenchymal hepatocytes at 6 h after partial hepatectomy, but did not change in non-parenchymal liver cells (Fig. 7). AnxA3 mRNA level decreased to ~80% in hepatocytes at 6 h after partial hepatectomy; however, AnxA3 mRNA did not change in non-parenchymal liver cells (Fig. 8).

AnxA3 Expression in Hepatic Sinusoidal Endothelial Cells—Non-parenchymal liver cells expressing AnxA3 were investigated by immunohistochemical staining. Hepatic sinusoidal endothelial cells were chosen as a candidate, as human umbilical vein endothelial cells express AnxA3 (*20*). AnxA3- and SE-1-positive cells were observed in normal rat liver section (Fig. 9 panel A and B, respectively), with localization of AnxA3-positive cells corresponding to SE-1-positive cells (Fig. 9, panel C).

Effect of Anti-HGF Antibody on AnxA3 mRNA Level in Hepatocytes Following CCl_4 Treatment—To investigate whether HGF is involved in the increase in AnxA3 mRNA level in hepatocytes following CCl_4 treatment, effect of anti-HGF antibody on mRNA level was investigated. Anti-HGF antibody decreased AnxA3 mRNA level to ~60% compared to control IgG (Fig. 10).

DISCUSSION

In the present study, we demonstrate that expression of AnxA3 increases in two rat liver regeneration models and in parenchymal hepatocytes, but not non-parenchymal liver cells. AnxA3 protein levels in the liver increased at 5 h and 6 h in partially hepatectomized rats and rats treated with CCl_4, respectively. DNA synthesis begins to change at ~16 and 24 h in partially hepatectomized rats and rats treated with CCl_4, respectively (*24*). AnxA3 plays an important role in the signalling cascade in hepatocyte growth for cultured rat hepatocytes (*10*), therefore is also likely to have the same role in rat liver regeneration.

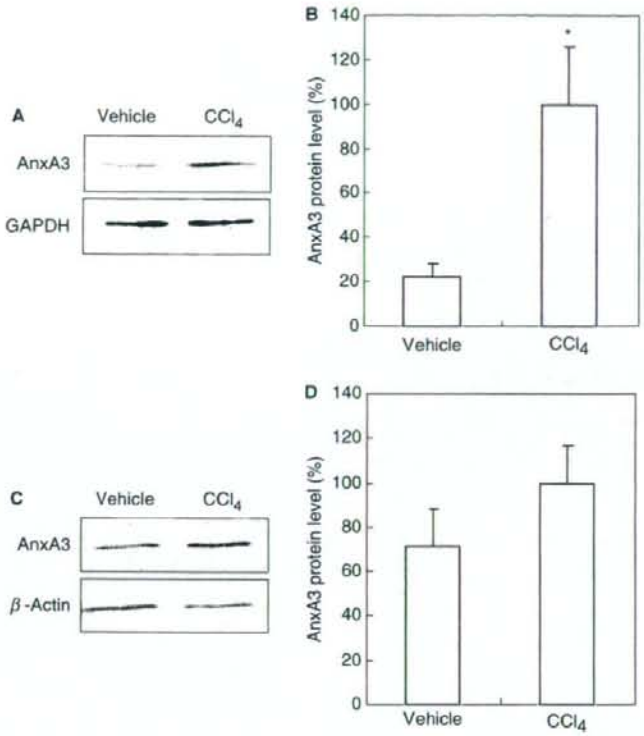

Fig. 3. **AnxA3 protein level in parenchymal hepatocytes and non-parenchymal cells isolated from liver in rats following treatment with CCl_4.** (A) Parenchymal hepatocytes and (C) non-parenchymal cells were isolated from liver in rats at 6 h after either CCl_4 or olive oil treatment. Data shown are representative western blot analysis results for parenchymal hepatocytes and non-parenchymal cells, respectively. Approximately 90 and 0.94 μg of protein was used for the detection of AnxA3 and GAPDH in parenchymal hepatocytes, respectively. Approximately 2.8 μg of protein was used for detection of AnxA3 and beta-actin in non-parenchymal cells. Results for parenchymal hepatocytes (B) and non-parenchymal cell (D) are presented relative to parenchymal hepatocytes and non-parenchymal liver cells from rats at 6 h after CCl_4 administration, respectively. AnxA3 protein levels in parenchymal hepatocytes and non-parenchymal liver cells were normalized to housekeeping protein, GAPDH and beta-actin, respectively. Data are expressed as mean ± SD ($n = 4$) $^{*}P < 0.01$, compared to the value for parenchymal hepatocytes or non-parenchymal liver cells from rats at 6 h after olive oil treatment.

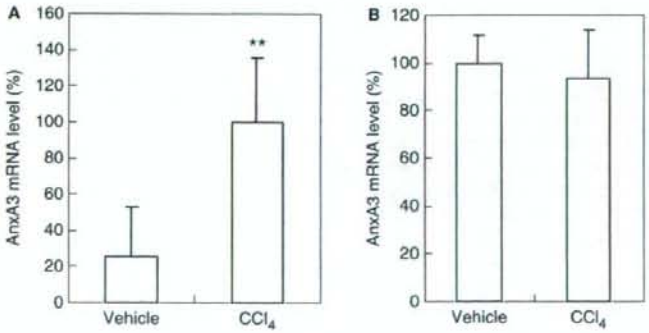

Fig. 4. **AnxA3 mRNA level in parenchymal hepatocytes and non-parenchymal cells isolated from livers in rats following treatment with CCl_4.** (A) Parenchymal hepatocytes and (B) non-parenchymal liver cells were isolated from liver in rats at 6 h after either CCl_4 or olive oil treatment. AnxA3 levels were normalized to the housekeeping gene, 28S rRNA. Results for parenchymal hepatocytes and non-parenchymal liver cells are presented relative to hepatocytes and non-parenchymal cells from rats at 6 h after CCl_4 treatment, respectively. Data are expressed as the mean ± SD ($n = 4$) $^{**}P < 0.05$, compared to parenchymal hepatocytes and non-parenchymal liver cells from liver in rats at 6 h after olive oil treatment.

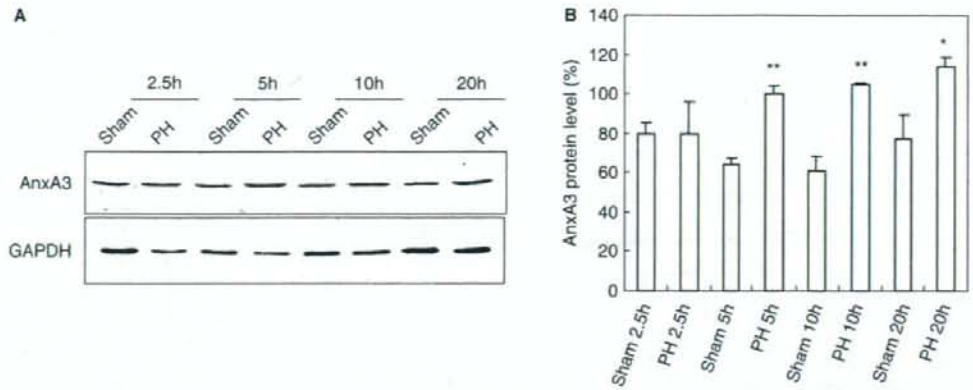

Fig. 5. **AnxA3 protein level in liver after partial hepatectomy.** (A) Data shown are representative of western blot analysis results. Approximately 35 and 1.5 μg of protein were used for detection of AnxA3 and GAPDH, respectively. (B) Results are presented relative to the values for liver in rats at 5 h after partial hepatectomy. AnxA3 protein levels were normalized to levels of housekeeping protein, GAPDH. Data are expressed as mean ± SD ($n = 4$ at each time point) $^{*}P < 0.01$, $^{**}P < 0.05$, compared to the value produced by liver in rats after sham operation.

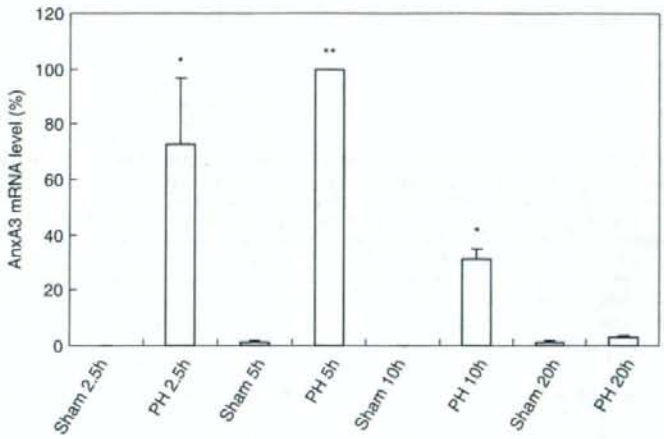

Fig. 6. **AnxA3 mRNA level in liver after partial hepatectomy.** Results are presented relative to the value produced by liver in rats at 5 h after partial hepatectomy. AnxA3 mRNA levels were normalized to housekeeping gene, 28S rRNA. Data are expressed as mean ± SD ($n = 4$ at each time point) $^{*}P < 0.01$, $^{**}P < 0.05$, compared to after sham operation.

Extent of increase in AnxA3 protein level was lower than in AnxA3 mRNA level in rat liver regeneration models, suggesting that AnxA3 protein, for which synthesis is enhanced, degrades rapidly in these conditions. Several proteases are induced or activated in rat liver regeneration (*25–31*). Therefore, AnxA3 may be rapidly degraded by some of these proteases, resulting in the relatively low level of increase in AnxA3 protein expression compared to mRNA expression.

AnxA3 in the liver from rats at 24 h after CCl_4 treatment was investigated using immunohistochemical analysis, to determine whether proliferating cells are AnxA3-positive parenchymal cells. AnxA3 was not detected in parenchymal hepatocytes, but was detected in non-parenchymal liver cells (data not shown). This failure of detection in parenchymal hepatocytes may be because expression of AnxA3 in these cells is too low to detect compared to non-parenchymal liver cells.

AnxA3 protein level increased in hepatocytes after partial hepatectomy; however, AnxA3 mRNA level after sham operation was even higher than after partial hepatectomy, inconsistent with the results for AnxA3 protein level. AnxA3 protein levels did, however, correlate with AnxA3 mRNA levels in cultured rat hepatocytes (*14*). AnxA3 mRNA was undetectable in hepatocytes from normal rats that were not sham operated (*10*, *12*). Therefore, sham operation may induce some signal that leads to an increase in AnxA3

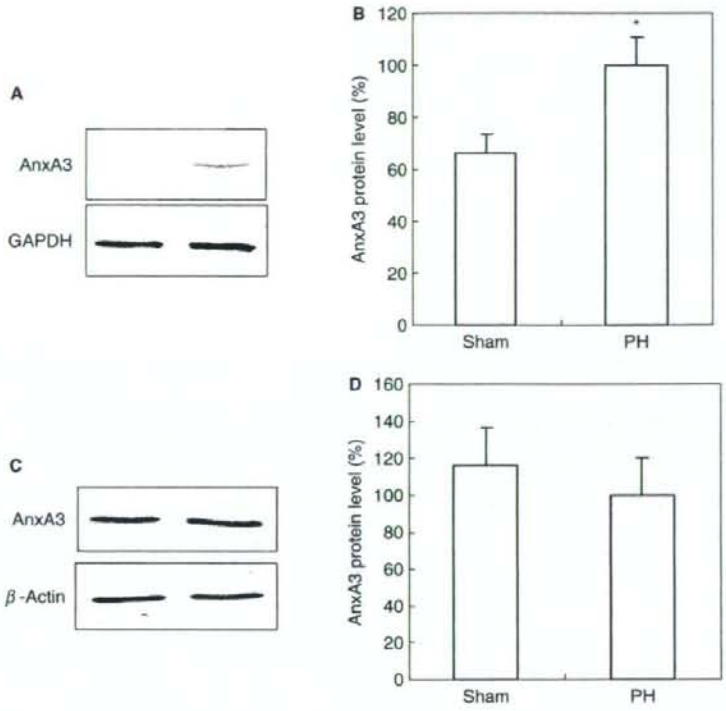

Fig. 7. **AnxA3 protein level in parenchymal hepatocytes and non-parenchymal liver cells after hepatectomy.** (A) Parenchymal hepatocytes and (C) non-parenchymal liver cells were isolated at 5 h after partial hepatectomy or sham operation. Data shown are representative of western blot analysis results for parenchymal hepatocytes and non-parenchymal liver cells, respectively. Approximately 90 and 2.8 μg of protein were used for detection of AnxA3 and GAPDH in parenchymal hepatocytes, respectively. Approximately 2.8 μg of protein was used for detection of AnxA3 and beta-actin in non-parenchymal liver cells. AnxA3 protein levels in parenchymal hepatocytes and non-parenchymal liver cells were normalized to housekeeping proteins GAPDH and beta-actin, respectively. Results for parenchymal hepatocytes (B) and non-parenchymal liver cells (D) are presented relative to the value produced by parenchymal hepatocytes and non-parenchymal liver cells from rats at 5 h after partial hepatectomy, respectively. Data are expressed as mean ± SD ($n = 4$) $^{*}P < 0.01$, compared to parenchymal hepatocytes and non-parenchymal liver cell from rats at 5 h after sham operation.

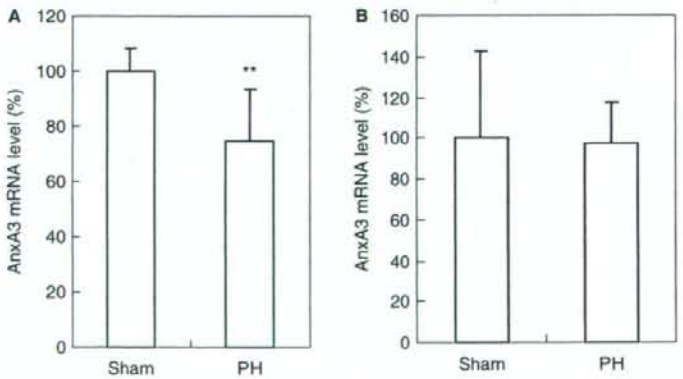

Fig. 8. **AnxA3 mRNA level in parenchymal hepatocytes and non-parenchymal liver cells after partial hepatectomy.** (A) Parenchymal hepatocytes and (B) non-parenchymal liver cells were isolated from liver in rats at 5 h after either partial hepatectomy or sham operation. AnxA3 mRNA levels were normalized to housekeeping gene, 28S rRNA. Results for parenchymal hepatocytes and non-parenchymal liver cells are presented relative to parenchymal hepatocytes and non-parenchymal liver cells from rats at 5 h after partial hepatectomy, respectively. Data are expressed as mean ± SD ($n = 4$) $^{**}P < 0.05$, compared to parenchymal hepatocytes and non-parenchymal liver cells from rats at 5 h after sham operation.

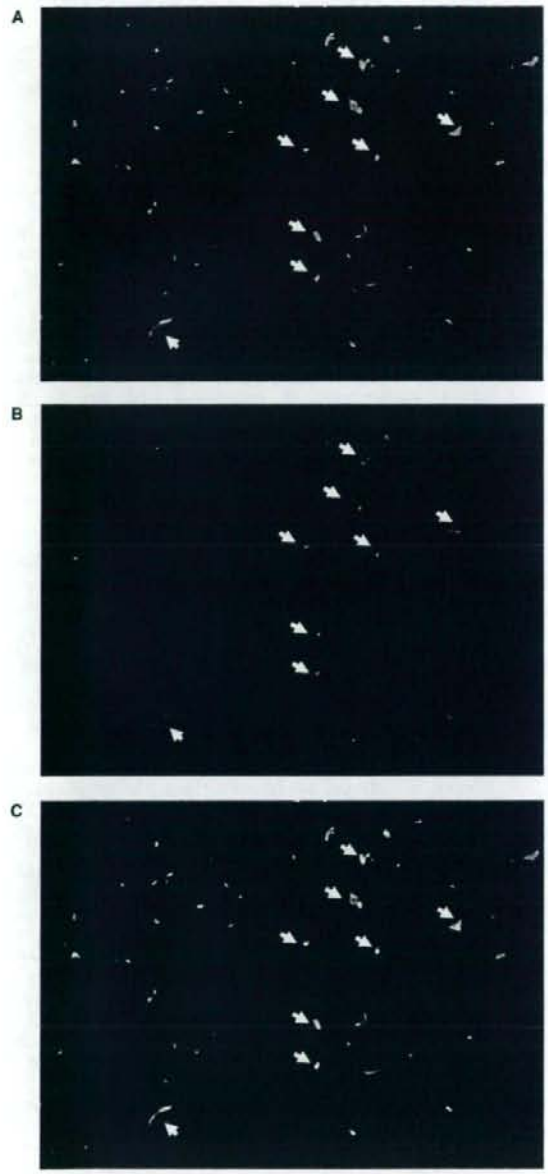

Fig. 9. **AnxA3 expression in hepatic sinusoidal endothelial cells in normal rat liver.** (A) AnxA3-positive cells; (B) SE-1-positive cells; (C) Merged image of AnxA3- and SE-1-positive cells. In (A–C), arrows show examples of positive immunoreactive cells.

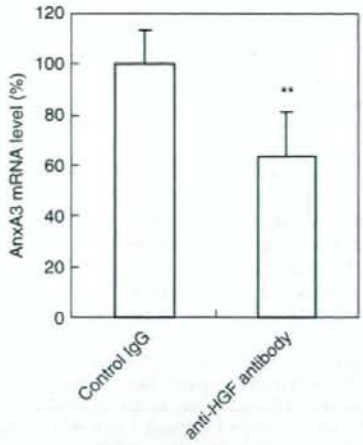

Fig. 10. **Effect of anti-HGF antibody on AnxA3 mRNA level in parenchymal hepatocytes following treatment with CCl_4.** Hepatocytes were isolated from liver in rats at 6 h following treatment with either anti-HGF IgG or control IgG, then CCl_4. AnxA3 levels were normalized to housekeeping gene, 28S rRNA. Results are presented relative to the value produced by hepatocyte isolated from liver in rats at 6 h following treatment with control IgG, then CCl_4. Data are expressed as mean ± SD ($n = 4$) $^{**}P < 0.05$, compared to hepatocytes from rats at 6 h following treatment with control IgG, then CCl_4.

mRNA level only in hepatocyte isolation procedures, including perfusion with collagenase at 37°C. This possibility may be supported by the finding that AnxA3 mRNA level is greatly enhanced in the liver from rats after partial hepatectomy, compared to after sham operation in analysis using total RNA directly extracted from liver perfused with cold PBS.

Increase in AnxA3 mRNA level was inhibited by anti-HGF antibody in hepatocytes from rats at 6 h after CCl_4 administration, indicating that HGF is involved in increasing AnxA3 mRNA expression in hepatocytes. Consistent with this finding, HGF increased AnxA3 mRNA level in hepatocytes cultured on Matrigel (*14*), on which hepatocytes maintain functions similar to those within a normal animal (*32*). HGF protein needs to increase in blood within 6 h at the latest after CCl_4 administration for HGF to increase AnxA3 mRNA level. This was indicated by the finding that HGF protein dramatically rises in the plasma at 2 h after partial hepatectomy and CCl_4 administration (*33*).

Effect of anti-HGF antibody on AnxA3 protein level was investigated; however, reproducible results were not obtained for AnxA3 and GAPDH protein levels in the experiments using control IgG and anti-HGF IgG antibodies. Also, there was a decreased recovery of total protein compared to the parenchymal hepatocytes isolated from liver in rats without these treatments. As administration of IgG was performed only *via* tail vein in this experiment, this procedure may be a factor in this variation. It is likely that the increases in fluid pressure to liver cause liver injury followed by enhancement of protein degradation by some proteases. This is supported by the finding that alanine transaminase transiently elevates in serum from rats after administration of PBS *via* the tail vein (*34*). However, strict control of fluid pressure is difficult in practice. Therefore, variation in

these sequential cascades may result in no reproducible results.

AnxA3 was demonstrated to be expressed in non-parenchymal liver cells, although proteins levels do not change in the liver regeneration models. Further immunohistochemical analysis showed co-localization of AnxA3-positive and SE-1-positive cells indicating that AnxA3 is expressed in hepatic sinusoidal endothelial cells.

In conclusion, the results of this study demonstrate that AnxA3 expression increases in hepatocytes through an HGF-mediated pathway in rat liver regeneration models, suggesting that AnxA3 plays an important role in the signalling cascade in rat liver regeneration.

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Research Article

A comparison of the tube forming potentials of early and late endothelial progenitor cells

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ABSTRACT

The identification of circulating endothelial progenitor cells (EPCs) has revolutionized approaches to cell-based therapy for injured and ischemic tissues. However, the mechanisms by which EPCs promote the formation of new vessels remain unclear. In this study, we obtained early EPCs from human peripheral blood and late EPCs from umbilical cord blood. Human umbilical vascular endothelial cells (HUVECs) were also used. Cells were evaluated for their tube-forming potential using our novel *in vitro* assay system. Cells were seeded linearly along a 60 μm wide path generated by photolithographic methods. After cells had established a linear pattern on the substrate, they were transferred onto Matrigel. Late EPCs formed tubular structures similar to those of HUVECs, whereas early EPCs randomly migrated and failed to form tubular structures. Moreover, late EPCs participate in tubule formation with HUVECs. Interestingly, late EPCs in Matrigel migrated toward pre-existing tubular structures constructed by HUVECs, after which they were incorporated into the tubules. In contrast, early EPCs promote sprouting of HUVECs from tubular structures. The phenomena were also observed in the *in vivo* model. These observations suggest that early EPCs cause the disorganization of pre-existing vessels, whereas late EPCs constitute and orchestrate vascular tube formation.

Introduction

In healthy individuals, endothelial cells are rarely found in the blood. However, mature endothelial cells can be found in the circulation following detachment from injured vessels. For example, circulating endothelial cells substantially increase in a wide variety of pathological conditions associated with profound vascular insult. Therefore, circulating endothelial cells are a useful marker of vascular damage [1,2]. Endothelial progenitor cells (EPCs), derived from bone marrow, are also found in the circulation and are involved in tumor vasculogenesis and wound healing

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[3,4]. The discovery of EPCs in human blood has led to a new paradigm in which vasculogenesis and angiogenesis occur in adult tissues [5]. These results have suggested a potential new approach to the treatment of cardiovascular and ischemic diseases. Preclinical studies have shown that transplantation of human EPCs to nude mice with hind limb ischemia improved blood flow recovery and capillary density resulting in a significant reduction in the rate of limb loss [5–10]. Clinical trials using autologous EPC transplantation have already been performed and significant improvements have been observed in myocardial function and ischemic diseases [11–15]. The strict roles of EPCs in neovascularization are not well understood because various populations of cells with varying differentiation potentials were transplanted.

EPCs consist of two different subpopulations, termed early and late EPCs [16–18]. Although both EPCs are derived from mononuclear cells (MNC) and express endothelial cell markers, they have different morphologies and growth patterns [18,19]. Early EPCs exhibit a spindle-like morphology and the majority of them are derived from CD14(+) subpopulations [16,19–21]. Late EPCs, named after their late outgrowth potential, exhibit a cobblestone morphology and are derived from CD14(−) fractions [17,19]. These two populations have been characterized for production of vascular endothelial growth factor (VEGF), VEGF receptor expression, cytokine secretion, and tube forming activity *in vitro* and *in vivo* [19].

Using photo-catalytic lithography, we have developed a new cell culturing technology for capillary engineering [22]. In this approach, endothelial cells are patterned on a substrate and then transferred to Matrigel. The endothelial cells change their morphology and form tubular vessels as confirmed by electron microscopy and dye microinjection. In the present study, using this novel technology, we focused on the tube-forming activity of early and late EPCs. In addition, we asked whether EPCs were incorporated into preexisting tubular structures.

Materials and methods

Human samples

Human peripheral blood was provided by 7 healthy human volunteers. Human umbilical cords and cord bloods were obtained from 12 healthy newborns. Informed consent was obtained from all donors, and samples were handled according to the tenets of the Declaration of Helsinki, with the approval of university review boards.

Cell isolation

Blood was diluted 1:1 with PBS containing 2 mM EDTA, and overlayed on Lymphoprep (AXIS-SHIELD, Oslo, Norway). Cells were centrifuged at 1100×*g* for 10 min. The resulting mononuclear cells (MNC) were collected and washed three times in PBS. Human umbilical vein endothelial cells (HUVECs) were isolated enzymatically.

Cell culture

Isolated MNC were resuspended in endothelial basal medium-2 (EBM-2) (Clonetics, San Diego, CA) supplemented with the EGM-2 bullet kit (Clonetics), plated on culture dishes precoated with human fibronectin (Sigma, Saint Louis, MO), and maintained in the medium. To obtain early EPCs from peripheral blood MNC and cord blood MNC, medium was changed every 2 days, and after 5 to 7 days of culture, early EPCs were isolated. Late EPCs were obtained from cord blood as previously described by Gulati et al. [17]. Medium was then changed daily for 7 days and on alternate days thereafter. The characteristic colonies of late EPCs were observed under a phase-contrast microscopy (IMT-2; Olympus Optical, Tokyo, Japan) and these cells were cloned by colony isolation. HUVECs were used at passages 1–3.

Cell characterization

Early and late EPCs were seeded onto a chamber slide glass (Nalge Nunc, Naperville, IL) coated with fibronectin (Sigma) and fixed in 4% paraform aldehyde (PFA) next day. Immunocytochemistry was performed using PE-conjugated anti-human CD31 antibody (555446; BD Pharmingen, San Diego, CA; diluted 1:50) and primary antibodies against human VEGFR2, (sc-6251; Santa Cruz Biotechnology, Santa Cruz, CA; 2 μg/ml) or von Willebrand factor (vWF) (M0616; Dako, Glostrup, Denmark; 1.2 μg/ml), followed with Alexa 488-conjugated anti-mouse IgG (A-11029; Molecular Probe, Leiden, Netherlands; 2 μg/ml). Non-immune mouse IgG_1 (sc-3877; Santa Cruz; diluted 1:100) was used as a control. Cell nuclei were stained with 1 μg/ml of To-Pro3 (T3605; Molecular Probe). Samples were observed and photographed under a laser confocal microscope (LSM510META; Carl-Zeiss, Jena, Germany).

Flow cytometric analysis of CD14, CD31, CD45 and VEGFR2 expression in early EPCs and late EPCs

Cells were collected non-enzymatically using Cell Dissociation Buffer (Invitrogen Corp, San Diego, CA) and labeled with FITC-conjugated anti-CD45 monoclonal antibody (Beckman Coulter, Marseilles, France), FITC-conjugated anti-CD31 monoclonal antibody (Pharmingen) and anti-VEGFR2 monoclonal antibody (Santa Cruz Biotechnology) at 4 °C for 30 min. Cells incubated with anti-VEGFR2 monoclonal antibody were subsequently stained with PE-conjugated anti-mouse IgG antibody at 4 °C for 30 min. After washing with PBS containing 1% BSA, flow cytometric analysis was performed with a FACS Calibur (Becton-Dickenson, NJ).

Photo-mask preparation

STK-03 titanium dioxide (TiO_2) photo-catalyst aqueous dispersion (Ishihara Sangyo; Osaka, Japan) was diluted to 33 wt.% with isopropyl alcohol, stirred for 1 h and filtered using a 3.0 μm pore size polytetrafluoroethylene filter (Advantec, Tokyo, Japan). Cr-Quartz photo-masks with slit width 60 μm and slit interval 300 μm were cleaned using a VUM-3184 UV-ozone washing machine (Oak Manufacturing, Tokyo, Japan) to

decompose low molecular weight adsorbents on the mask surface. The mask was coated with the TiO_2 dispersion by spin-coating at ~700 rpm for 15 s and then baking at 150 °C for 10 min.

Substrate preparation

NA35 polished glass substrates (NH Techno Glass, Yokohama, Japan) were cleaned using the VUM-3184 for 420 s. Next, 1.5 g of heptadecafluorodecyltrimethoxysilane solution (TSL-8233; GE Toshiba Silicone, Tokyo, Japan), 5.0 g of tetramethoxysilane solution (TSL-8114; GE Toshiba Silicone), and 2.4 g of 0.005 N HCl were mixed and stirred for 24 h at room temperature to make fluoro-alkyl-silane (FAS) mixture solution. The mixture was diluted with 1 wt.% isopropyl alcohol, stirred for 15 min, filtered by Chromato-Disc (filter type 0.45 μm; Kurabo) and then coated onto the glass substrate by spin-coating at ~1000 rpm for 15 s. The FAS-coated substrate was then baked at 150 °C for 10 min.

Surface modification and patterning

Both TiO_2-coated photo-masks and FAS-coated substrates were immersed and sonicated in deionized water for more than 5 min, and then baked at 120 °C for 5 min. The TiO_2-coated side of the photo-mask was irradiated with UV for 15 min at irradiation energy of ~30 J/cm^2 to rejuvenate the photo-catalytic activity of TiO_2. The FAS-coated side of the glass substrate was placed facing and in contact with the TiO_2 layer of the photo-mask and UV-irradiated for several minutes through the mask to form hydrophilic regions on the FAS layer.

Cell patterning

Prior to cell seeding, glass substrates were placed on cell culture dishes. The cells were trypsinized and suspended, and labeled with PKH26 Red (Sigma) according to the manufacturer's instructions. Cells were counted, seeded on the substrates, and incubated for 18 h at 37 °C. A total of 2×10^5 cells were seeded per substrate. During incubation, cells in hydrophobic areas moved to hydrophilic areas.

Transplant to matrigel

The patterned cells on the substrate were turned over onto Matrigel (Becton Dickinson, Bedford, TX) and incubated with the culture medium containing 0.2% FBS for 24 h. When the substrate was removed, the cells were transferred from the substrate to the Matrigel.

Observation of the tube formation

Firstly, the pattern of cells on Matrigel was observed by a phase-contrast microscopy (IMT-2; Olympus Optical). Next, live cells which formed tubular structures were labeled with 500 ng/ml Calcein-AM (Molecular Probes) for 30 min in 37 °C, and three-dimensional structures of capillaries were analyzed by a laser confocal microscope. Furthermore, the engineered capillaries on Matrigel were fixed with 4% PFA and stained with 4 μg/ml of anti-human VE-cadherin rabbit polyclonal antibody (210-232-c100; ALEXIS, San Diego, CA), followed by 4 μg/ml of Alexa 488-conjugated anti-rabbit IgG (Molecular Probe). Capillaries were observed by a laser confocal microscope.

EPC incorporation into tube-like structure

Early and late EPCs were pre-labeled with PKH26 Red, mixed with unlabeled HUVECs, and seeded on the substrate. Twenty-four hours after transplant to Matrigel, the cells were labeled with Calcein-AM, the fluorescence of EPCs and HUVECs were analyzed by a laser confocal microscope.

To quantify EPC incorporation into tube-like structure, early and late EPCs were pre-labeled with PKH26 Green. These cells were mixed with HUVECs which were pre-labeled with PKH26 Red, and cultured on the substrate. The fluorescence of EPC and HUVECs was analyzed by a fluorescence microscopy (BZ-8000; Keyence, Osaka, Japan). The incorporation ratio of EPCs into the tube structure was calculated by the cellular area of EPC (μm^2)/mm of tube structure.

In vivo angiogenesis assay

We developed an ear vessel occlusion model for an *in vitro* angiogenesis assay. After occlusion of the murine auricular vessel, cells were introduced into the subcutaneous pocket below the occlusion point, and newly formed vessels were analyzed. Twelve hours before the cell injection, the mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) and auricular vessels were occluded. Collateral circulation was observed under a stereomicroscope (LEICA Mz9, Leica Microsystems, Wetzlar, Germany). Early or late EPCs were labeled with PKH26 and suspended in growth media, and 5×10^4 of each EPCs were subcutaneously injected into the occluded pinna within 12 h of vessel occlusion. Forty-eight hours after the cell injection, the mice were given 250 μg of *Banderiaea simplicifolia* lectin 1 (BS1-lectin) (Vector Laboratories, Burlingame, CA) intravenously and sacrificed 45 min later. Sections (4-μm thickness) of cell-transplanted pinna were made for histological observation. This experiment was performed using 7-week nude mice and all procedures were carried out with the full approval of the ethical committee of Tokyo Medical and Dental University.

Calculations and statistical analysis

The statistical significance of differences in the data was evaluated by use of analysis of Welch's t-test or Student t-test. A value of $P<0.05$ was accepted as statistically significant.

Results

Characterization of two types of EPCs

MNC were harvested from human peripheral blood of healthy individuals or from cord blood and seeded on fibronectin-coated dishes. Following 5 to 7 days of cultivation, adherent cells were observed in clusters. The cells exhibited a spindle-

like shape (Fig. 1A, left) and displayed several endothelial cell markers including CD31, vWF and VEGFR2 (Fig. 1B, left). When MNC were isolated from human cord blood, early EPCs were also observed after 3 days of cultivation. Their morphology and expression of endothelial markers used here were almost the same as those of early EPCs derived from peripheral blood (data not shown). They proliferated and thereafter gradually disappeared over the next 1 week.

After early EPCs had disappeared, colonies with different morphology emerged over 12–21 days of cultivation. They exhibited a cobblestone morphology, spindle-like shape (Fig. 1A, right) and displayed several endothelial cell markers such as CD31, vWF and VEGFR2. Also, eNOS expression was observed in both early and late EPCs (data not shown). Thus, these cells were late EPCs as reported by Hur et al. [18]. To characterize two types of EPCs, we carried out a flow

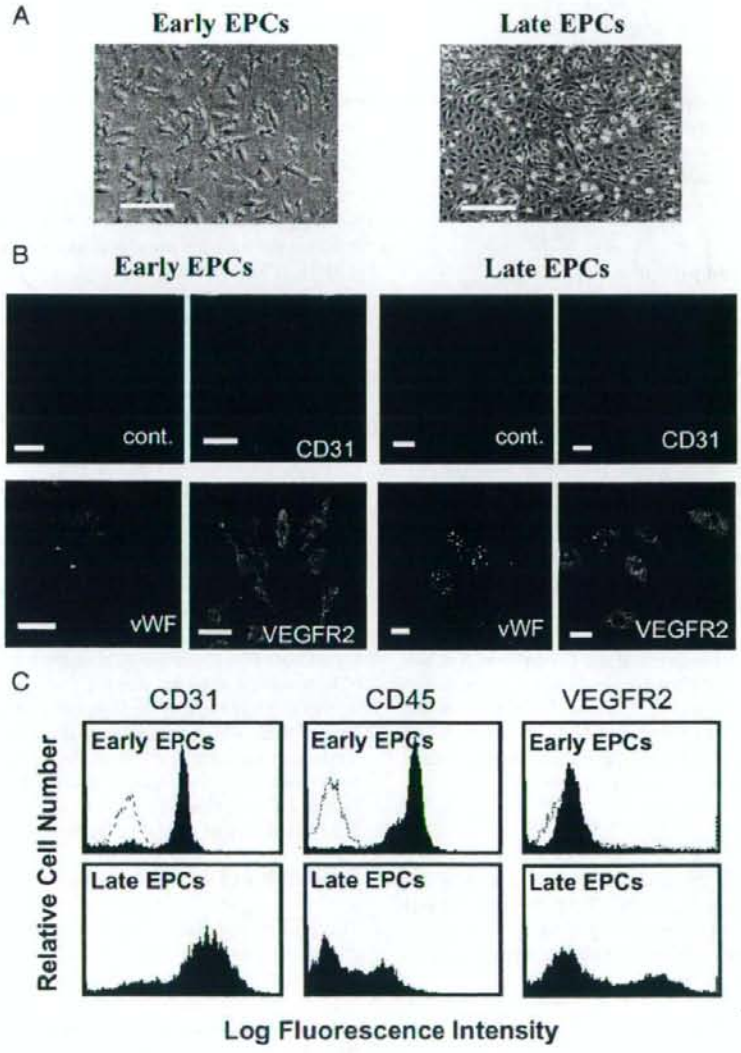

Fig. 1 – Morphological and immunophenotypical characterization of early and late EPCs. (A) Early EPCs cultured for 7 days and late EPCs cultured for 14 days. Scale bar=100 μm. (B) Immunocytochemistry of CD31, vWF and VEGFR-2 was demonstrated. Scale bar=20 μm. Shown are representative data from 7 independent experiments using early EPCs isolated from different peripheral blood and 7 independent experiments using late EPCs isolated from different cord blood with similar results. (C) Flow cytometric analysis of CD31, CD45 and VEGFR2 expression in early EPCs and late EPCs. Shown are representative data from 5 independent experiments using early EPCs and late EPCs isolated from different cord blood with similar results. Isotype controls are overlaid in a dot line on each histogram for each surface antigen tested.