Ⅲ依存性の血小板・内皮細胞接着があることのみならず、それ以外の蛋白質フィブリノゲン、von Willebrand因子、フィブロネクチン（FN）などによる接着があることは明らかである[17]。これらの蛋白質は、血小板のα顆粒にも存在し、活性型FXⅢの基質になることも共通点である。

血小板の細胞質内の蛋白質も、細胞外に放出された後に活性型FXⅢの基質として働くことがある。例えば、thymosin β4（Tβ4）は43アミノ酸からなるペプチドで、ほとんどの細胞における主要な細胞内蛋白質であり、G-actinに結合してF-actinへの多量体化を抑制するが、細胞外ではマトリックス成分上で内皮細胞の接着、伸展反応を促進し、遊走を刺激することが知られている。このTβ4は、トロンビン刺激により血小板から放出され、恐らく活性型FXⅢによってフィブリンに架橋接合される（図2）[18]。こうして、Tβ4は組織障害と血栓の部位に局在化され、創傷治癒、血管新生、炎症反応に貢献する。

血管新生因子であるVEGFも大量に血小板のα顆粒に存在し、活性化によって放出されて血栓に取り込まれるので、血管新生や血管透過性に関与する[19]。FXⅢ-Aとの関連については後述する。

b. FXⅢと単球/マクロファージの相互作用

1．FXⅢ-Aは単球/マクロファージの細胞表面に発現する

単球/マクロファージがFXⅢ-Aを持つことも古くからよく知られている[20][21]。McDonaghらは、FXⅢ-AがU937の細胞表面に存在し、lipopolysaccharide（LPS）、phorbolmyrisacetate（PMA）、interferon-γ（IFN-γ）などの刺激によって増加すると報告している[21]（図1）。IFN-γ添加後にFXⅢ-A蛋白質の量の変化はないと述べているので、後述する最新のデータとは相反している。彼らは、U937や肺胞マクロファージをトロンビン処理してフィブリノゲンとカルシウムを加えるとγ-dimerが検出されると述べている。Greenbergらも、ヒト末梢血単球と腹腔マクロファージの表面にFXⅢ-Aが存在し、前者はFXⅢ欠損症患者の血漿との混合後にフィブリン架橋結合産物が検出されているので、末梢血単球表面のFXⅢ活性は凝固、炎症反応において生理的な役割を果たしている可能性があると結論している[22]。比較的最近の報告でも、ヒト単球性白血病細胞株THP-1の細胞表面にFXⅢ-Aが検出されている[23]。このTHP-1細胞表面のFXⅢは、FNを塗付したディッシュでTPA＝PMAを用いて分化させると約1/4に減少する。

こうしてみると、単球/マクロファージでは分化の刺激の種類、程度によっては、細胞内で生合成されたFXⅢ-Aが未知のメカニズムによって細胞表面に移送されて、これが活性化されれば細胞周辺の基質を架橋結合すると考えられる（図1）。血漿中のFXⅢ-A量に比べればごく微量ではあるが、炎症局所にはマクロファージが浸潤、集簇するので、細胞表面のFXⅢ-A活性も局所的には高濃度となりうる。

ただし、Belkinらは、THP-1細胞表面のFXⅢ-Aは、FN上では細胞の接着/伸展にも、遊走にも関与しないと結論しているので[23]、*in vivo*機能との関連では、他の種類の細胞、あるいは他の接着性蛋白質との相互作用の存在を想定するべきであろう。

2．FXⅢ-Aの産生量は単球/マクロファージの分化によって変化する

ヒト末梢血単球を培養すると、最初はFXⅢ-A蛋白質が細胞内に存在するが、マクロファージに成熟すると検出されなくなる[24]。また、採取したての成熟した肺胞マクロファージにはFXⅢ-Aは存在しない。前述したヒトTHP-1細胞でもFXⅢ-A産生は、PMA処理で著しく減少する[23]。

ところが、ヒト末梢血単球を培養すると、3日目にFXⅢ-A mRNA量がピークに達してFXⅢ-A蛋白質の合成が確認されるという報告もあるので[25]、培養条件や活性化の違いなどが異なる結果の原因であると推定される。事実、培養ヒト単球をINF-γかBCGを用いて古典的経路で活性化すると（Th1 cytokine）FXⅢ-Aの発現は抑制され、interleukin(IL)-4を用いて第二経路で活性化すると（Th2 cytokine）FXⅢ-Aの発現は著しく亢進することが報告されている（図1）[26]。喉頭腫瘍周囲のマクロファージにはFXⅢ-Aが検出され、リンパ節の結核性肉芽腫にあるマクロファージには検出されないので、前者は第二経路で、後者は古典的経路で活性化されていると推論されている。

ヒト末梢血単球をTh2 cytokineであるIL-4存在下で3日間培養すると、FXⅢ-A発現量は40倍増加するという報告もある[27]。同じTh2 cytokineであるIL-13存在下では4.7倍である。同様に著しく増加する15-lipoxygenase-1（15-LOX；290倍）、FN（185倍）、monoamine oxidase-A（MAO-A；55倍）などが炎症反応の鎮静化に働くので、FXⅢ-Aを「抗」炎症因子と位置付けられている。ちなみに「向」炎症因子であるtumor necrosis factor α（TNFα）、MCP-1、IL-8、-18、COX-1、-2などは、IL-4存在下での培養によって軒並み発現が低下している。また、Chaitidisらは、活性型FXⅢは基質からammoniaを放出するので、酸性化した炎症部位の細胞外pHを中和して抗炎症的に働くかもしれないと述べている[27]。

他の因子に比べてFXIII-Aの発現は遅く誘導されるので、15-LOXやMAO-Aなどの早期誘導遺伝子産物が後期誘導遺伝子であるFXIII-A発現のシグナル伝達カスケードに関与している可能性がある点が、筆者にとってはとても興味深い。炎症反応の後期の「修復相」において、先にFNのような細胞外マトリックス蛋白質を合成、分泌、蓄積させておいて、その後FXIII-Aが出現して基質であるFNを架橋結合するというストーリーである。

実際、ヒトの各種の組織を免疫学的手法で染色すると、FXIII-A陽性と陰性の単球/マクロファージがさまざまな割合で、さまざまな染色強度（＝発現の程度）で検出されるのは、活性化刺激の種類と強度が多彩で不均一であるためであろう。

3．FXIII発現はLPS（により発現誘導されたサイトカイン）刺激により低下する

単球の活性化は細菌感染に対する自然免疫の鍵となるステップである。ラットにLPSを持続的に注入すると、3時間後に血漿FXIII活性は50%に低下するという[28]。また、ヒト単球を16時間LPS存在下で培養すると、IL-1β、IL-6、IL-8、IL-10、TNFα、granulocyte/macrophage colony stimulating factor（GM-CSF）など向炎症性cytokineの発現が著しく増加し、逆にFXIIIの発現は低下する[29]。培養ヒト単球をINF-γ（Th1 cytokine）かBCGを用いて古典的経路で活性化するとFXIII-Aの発現は抑制されるので[26]、FXIII-Aは炎症反応の早期の「破壊相」では働かず、後期の「修復相」に貢献しているものと思われる。

*in vivo*では事態はより複雑である。ラットの心筋梗塞モデルでは、ミネラルコルチコイド受容体阻害薬投与3日後には、「向」炎症性cytokineであるMCP-1、TNFα、IL-1β、IL-6、IL-10とともに抗炎症性cytokineであるIL-4の発現が梗塞巣で増加し、FXIII-A発現も増加している[30]。この効果は、clodronateをリポソームに入れて投与して単球/マクロファージの梗塞巣への浸潤を阻害すると消失し、FXIII-A発現増加もキャンセルされる。

いずれにせよ、*in vivo*では梗塞巣に浸潤したマクロファージの多くがFXIII-Aを発現している。

c. 好中球や補体系との相互作用

1．FXIIIは顆粒球エラスターゼによって活性化/分解される

炎症巣には、走化因子によって大量の白血球が集族してくるが、刺激を受けた好中球は強力なlysosome酵素を顆粒から放出する（図1）。そのうち、顆粒球エラスターゼは各種の蛋白質を分解することが知られており、FXIIIも切断されて不活性化される[31]。最近、Muszbekらは、顆粒球エラスターゼがトロンビンとは異なる部位を切断してFXIIIを活性化することを分子モデルで示している[32]。また、顆粒球エラスターゼは活性型FXIIIを緩徐に分解して不活性化することも再確認されている[33]。

2．FXIIIは補体系プロテアーゼによって活性化される

補体系は、古典的経路、第二経路、レクチン経路で活性化され、最終的には侵入した微生物や障害された宿主細胞を溶解するシステムである。凝固系と同様カスケード反応であり、感染部位に限局して働くように調節されている。最近、FXIIIがヒトmannan-binding lectin（MBL）-associated serine protease-1（MASP1）によって活性化されることが報告された（**図1**）[34]。さらに、fiscolinと結合したMASP複合体（MASP1、2、3からなる）をヒト血清から精製し、FXIII活性化とフィブリン架橋結合を起こすことが確認されている[35]。トロンビンに比べると活性化速度は著しく遅いが、フィブリンの架橋結合は生成できるので、反応の限局化に貢献しうる。

d. 炎症反応におけるFXIII-Aの機能

1．マクロファージの貪食能を促進する

FXIII-Aが単球/マクロファージの細胞質内に存在することもよく知られているが、その機能は確定されていない。Adanyらは、ヒト単球で、感作ヒツジ赤血球を用いたFcγ受容体を介する貪食と、酵母を用いた補体受容体を介する貪食がFXIII活性阻害薬であるmonodansylcadaverine（MDC）によって著明に阻害されることを示した[25]。また、Fcγ受容体を介する貪食と補体受容体を介する貪食は、FXIII欠損症患者の単球でも著しく減少していたので、FXIIIは単球/マクロファージの貪食作用に役割を果たしていると考えている。MDCは感作赤血球や酵母の単球への結合を阻害しないので、FXIIIはそれらの粒子のendocytosisの段階に関与していることになる。

ところが、FXIII欠損症患者において細菌の貪食障害に基づく易感染性についての臨床報告は皆無である。多くの重要な生体作用には冗長性があるので、*in vitro*と*in vivo*の所見は必ずしも一致しないという例の1つであろう。種の違いが原因である可能性もある。

一方、マウスの心筋梗塞モデルでは、マクロファージと好中球の梗塞巣への浸潤はFXIII欠損マウスで野生型マウスに比べて低下しているが、細胞当りの貪食活性を比較するとFXIII欠損マウスの好中球では低下しているのに対してマクロファージの方は変化がみられない[36]。これは、

Adanyらの*in vitro*の実験結果と異なるが[26]、上述したようにマクロファージは心筋梗塞モデルの生体内では炎症性サイトカインなどによる多彩で異なる刺激を受けているためであるかもしれない。

2．好中球・マクロファージの遊走能を修飾する

ラットにLPSを持続的に注入して腸管の粘膜下集合静脈と毛細管後静脈で白血球のローリング量を計測した実験では、LPSにより著増した内皮への白血球粘着はFXIII投与で若干減少した[28]。また、ラットの上腸間膜動脈閉塞による虚血再還流後の肺と回腸への好中球の浸潤は組み換えFXIII投与で有意に低下する[37]。

心筋梗塞後の炎症性反応は組織修復に強く影響する。Nahrendorfらはマクロファージと好中球の梗塞巣への浸潤を蛍光標識した分子を用いてマウスの生体内で観察した[36]。野生型マウスに比べ、FXIII欠損マウスでは、マクロファージと好中球の梗塞巣への浸潤が有意に低下しているので、FXIIIは白血球の粘着や遊走を促進していると考えられる。なお、彼らの以前の報告でも、梗塞巣の境界域への好中球の遊走はFXIII欠損マウスで有意に低下しているが、FXIIIの投与によって部分的に回復しているので、好中球の走化性メカニズムにもFXIIIが関与していると思われる[38]。

心筋梗塞とは病態が異なるが、熊本大学の山本らは、ヒト関節リウマチ組織のアポトーシス細胞から2量体化したS19リボゾーム蛋白質（RP-S19）を検出し、活性型FXIIIの架橋結合反応によって形成されうることを示した[39]。2量体化RP-S19は走化活性を獲得し、単球のC5a受容体に結合して組織への遊走を促進してアポトーシス細胞の除去に働くが、好中球に対しては逆に遊走を抑制してアポトーシスを促進するので、炎症組織の過剰な破壊を抑制するという。この点でも、FXIIIは炎症の沈静化の方向、すなわち抗炎症性に働いているといえる。FXIIIが新たに補体受容体のリガンドを生成して細胞機能を制御する点が、とても興味深い。彼らは、動脈硬化巣に2量体化RP-S19様活性を検出しており、さらに血漿中のRP-S19様前駆体が、トロンビンによって活性化された血小板を足場にして活性型FXIIIによって2量体化されるという。したがって、血栓の分解処理をするためにマクロファージを呼び寄せる役割を果たしていると思われる。

一方、三次元フィブリンゲルマトリックスの中でのマクロファージの遊走能は、カバーガラスやボイデンチェンバーの多孔性フィルターとは異なる。活性型FXIIIはフィブリン同士を架橋結合するため、モルモットの腹腔マクロファージの遊走を抑制する[40]。また、その中にFNを加えるとさらにマクロファージ遊走能は低下する。これは、単球/マクロファージがFNに結合するからであり、RGDSペプチドを添加して結合を阻害すると遊走能は有意に増加する。

このように、FXIIIは、病態、器官、組織、細胞の種類の違いによって、遊走能を抑制したり、促進したりする。これは、FXIIIと他の蛋白質との相互作用によって、どちらにスイッチが入るのか決定されるためであろう。

3．血管透過性を抑制する

FXIIIが、血管透過性に関与しているということはかなり古くから知られている。例えば、内皮細胞に対する抗体をモルモットの背部の皮膚内に注射すると、FXIIIの投与量依存的に血管透過性が抑制される[41]。また、抗内皮細胞抗体の注射部位の皮膚は、FXIIIの投与量依存的に浮腫が抑制されるので、FXIIIは血管透過性を抑制することによって抗炎症性に働くといってよい。Nollらは、単層のブタ大動脈の培養内皮細胞を用いてトロンビン活性化FXIIIが酵素活性依存的に透過性を低下させること、FXIIIは隣接する内皮細胞の接触面に沈着することを報告している[42]。また、彼らは、エネルギー枯渇によって惹き起こした内皮細胞の透過性亢進を活性化FXIIIが阻害すること、さらにラットの心臓の虚血再還流による心筋含水量の増加を活性化FXIIIが阻害することも示した。FXIIIの内皮バリアー機能は、単層内皮細胞の細胞間隙の通路を酵素作用により修飾することを介しており、細胞間隙に存在するマトリックス蛋白質を架橋結合させるためであると推測している。最近、ラットの上腸間膜動脈閉塞による虚血再還流後の気管支肺胞への血管透過性を測定し、組み換えFXIII-Aが透過性亢進を抑制することが示された[37]。FXIIIは、LPSによるラット腸管の機能的毛細血管密度の減少を有意に緩和して、粘膜毛細血管の循環を保護することも報告されている[28]。

なお、内皮透過性の亢進には白血球の内皮細胞層への粘着増加も関与しているので、その影響を除外する必要がある。白血球非依存性のendotoxemiaを惹起するためにL-selectinインヒビターであるfucoidinを用いて白血球のローリングと粘着を阻害した実験では、FXIIIがLPSを持続的に注入して惹起された微小血管の透過性を有意に低下させることが確認されている[43]。

このように、FXIIIは内皮透過性を制御することによっても炎症反応を修飾する。

4．血管新生を促進する

創傷治癒は、各種の段階に多数の細胞や、多数の酵素や構造蛋白質、多数の成長因子、サイトカインなどが関与す

る、極めて複雑な生体現象である。その一部にはFXIIIがかかわっているため、その先天性欠損症の患者の約1/4には創傷治癒異常が認められる。これは、FXIII-AのKOマウスでも確かめられている[44](小関と一瀬、未発表データ)。FXIII-Aが内皮細胞に結合することから[8)15)]、Farrellらは、ヒト皮膚の微小血管から樹立された内皮細胞株HMEC-1を用いて内皮細胞がマイクロプレートに塗付されたFXIII-Aに酵素活性非依存性に結合すること、フィブリンゲルの中では脈管形成を阻害することを報告した[14]。ところが、細胞外マトリックス成分により近いMatrigelでは、Inbalらはヒト臍帯静脈内皮細胞(HUVEC)を用いた実験で、活性型FXIIIは内皮細胞成分を増加させて脈管の厚さを増し、脈管形成を促進すると報告しており[45]、前述したように内皮細胞の由来やマトリックスの性状の違いが、相反する結果に繋がったと思われる。

Inbalらは、比較的大量の活性型FXIIIを培養HUVECに投与すると、細胞遊走、DNA合成、増殖が促進され、アポトーシスが抑制されること、この作用の基礎は活性型FXIIIが内皮細胞の増殖を阻害するThrombospondin-1(TSP-1)の発現を抑制することにあることを明らかにした[45]。さらに、*in vivo*でもウサギの眼角膜に活性型FXIIIを注射すると、血管新生を促進することも示している。同様に、ラットの脛骨に欠損を作製してhydroxyapatiteを充填/移植すると、血漿由来のFXIIIを補充した場合には血管の形成が促進されることが報告されている[46]。

Inbalらは、FXIIIの血管新生作用の分子メカニズムをHUVECにおいて詳細に検討し、FXIIIは酵素活性依存性にintegrin $\alpha v\beta 3$とVEGF受容体を架橋結合し、両者の非共有結合的な相互作用を促進すること(図2)、VEGF受容体のリン酸化と活性化を促進すること、c-JunとEgr-1の発現を亢進させること、WT-1のTSP-1遺伝子のプロモーターへの結合を促進させることなどを示した[47]。また、新生仔マウスの心臓を同系統のマウスの耳介に移植して、その周囲に活性型FXIIIを注射すると移植心周囲の血管形成が促進されている[48]。この移植心におけるc-Junの発現は1.4倍に増加し、TSP-1発現は活性型FXIIIにより著しく減少していた。Matrigelをマウスの鼠径部に注入した*in vivo*の実験でも、FXIII-A KOマウスでは新生血管数が野生型マウスの1/2であり、あらかじめ活性型FXIIIをMatrigelに添加しておくと、新生血管数は野生型マウスとほぼ同等に増加する[48]。

いずれにせよ、活性型FXIIIは、少なくともマウスでは血管新生に重要な役割を果たしているといってよい。ヒトでも先天性FXIII欠損症の患者の約1/4に創傷治癒異常が認められるので、他の因子との関連で症状出現の有無が決まるものと思われる。

e. 中休み;炎症性疾患におけるFXIIIの変動と役割

最早、約束の紙面が尽きたし、原稿の〆切も過ぎてリミットが来たので、ここで筆を置く。本稿は基礎編だけになってしまったが、編集主幹の許可を得たので、いずれ続編、すなわち臨床編を書きたい。そこでは、炎症性疾患におけるFXIIIの変動と役割について、具体的には、慢性炎症性腸疾患、アレルギー性血管炎(Henoch Schonlein紫斑病)、関節リウマチ、DIC、敗血症、肝炎など、動脈硬化症、先天性心疾患手術、後天的血友病XIIIなどにおけるFXIIIの動きと働きを概説する。

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Degradation of cross-linked fibrin by leukocyte elastase as alternative pathway for plasmin-mediated fibrinolysis in sepsis-induced disseminated intravascular coagulation

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ABSTRACT

An alternative pathway for fibrinolysis that comprises leukocyte elastase and its interaction with the plasminogen activator-plasmin system has been suggested. Plasma levels of cross-linked fibrin degradation product by leukocyte elastase (e-XDP) were significantly increased in patients with sepsis induced disseminated intravascular coagulation (DIC) compared with healthy subjects (18.6 ± 19.9 vs 0.58 ± 0.47 U/mL, $p<0.001$). Twenty seven unique spots were identified from e-XDP dominant patients by immune-purification and two-dimensional difference gel electrophoresis, and they contained fibrinogen Bβ-chain derived fragments Bβ Asp-164, Ser-200, Gln-301, Ala-354, Ile-484 and γ-chain derivatives γ Val-274 at their amino-termini by acquired and processed tandem mass spectrometer. The Sequential Organ Failure Assessment Scores in patients with e-XDPs levels 3-10 U/mL were significantly lower than those with e-XDPs levels -3 U/mL, 10-30 U/mL, and 30- U/mL. The adjusted odds for 28-day mortality rate in patients with e-XDP levels less than 3 U/mL (hazard ratio, 4.432; 95% CI, 1.557-12.615 [$p=0.005$]) were significantly higher than those in patients with e-XDP levels of 3–10 U/mL. These data suggest that leukocyte elastase might contribute to the degradation of cross-linked fibrin in sepsis-induced DIC.

Introduction

DIC is an acquired syndrome characterized by persistent activation of blood coagulation in the microvasculature, and is currently accepted to be a pathologic state that occurs over the course of a severe underlying disease [1–3]. Sepsis is a distressing disorder with systemic activation of the inflammatory and coagulation cascades in response to microbial infection, and may be the most common pathogenic state that leads to the development of DIC [4]. In spite of the apparent association between sepsis-induced DIC and the risk of death [5], it remains uncertain to what extent intravascular fibrin or coagulation proteases are critical factors in determining the clinical course.

The degradation of fibrin is usually performed by the serine protease plasmin, which is generated from plasminogen by plasminogen activators. However, suppression of the plasminogen activator-plasmin system is mediated by increased plasma plasminogen activator inhibitor-1 (PAI-1) levels in patients exhibiting systemic inflammatory response syndrome as well as sepsis-induced DIC [6,7]. Alternative systems for fibrinolysis that comprise proteases other than plasmin and their interactions with the plasminogen activator-plasmin system have been thought to play important roles in the digestion of fibrin [8,9]. Leukocytes are known to release intrinsic proteolytic enzymes, including leukocyte elastase as well as cathepsin G, in a variety of clinical conditions [10,11]. Although exposure to inflammatory mediators and interaction with leukocytes cause endothelial activation and damage, leukocyte elastase has been found to digest factor XIIIa mediated cross-linked fibrin and to yield different molecular species of cross-liked fibrin digests from those generated by plasmin [12,13].

Here, we demonstrate that the alternative pathway for fibrinolysis by leukocyte elastase is activated, and contribute to the degradation of cross-linked fibrin in sepsis-induced DIC.

Abbreviations: DIC, disseminated intravascular coagulation; FDP, fibrinogen and fibrin degradation product; XDP, cross-linked fibrin degradation product; e-XDP, cross-linked fibrin degradation product by leukocyte elastase; p-XDP, cross-linked fibrin degradation product by plasmin; TAT, thrombin-antithrombin complex; PIC, plasmin-α_2-plasmin inhibitor complex; SFMC, soluble fibrin monomer complex; PAI-1, plasminogen activator inhibitor-1; SOFA score, Sequential Organ Failure Assessment score.

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Materials and methods

Study population

All samples were obtained with informed consent from patients or their family members according to the Declaration of Helsinki. Blood was drawn from 117 patients with sepsis-induced DIC (Table 1). Samples from aged and sex matched healthy volunteers with consent (23 males and 23 females, 44.6 ± 10.5 years old) were also analyzed as normal controls. Sepsis was defined as infection plus systemic manifestations of infection based on the diagnostic criteria by 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference [14]. DIC was diagnosed according to the diagnostic criteria established by the Japanese Ministry of Health and Welfare (JMHW DIC criteria [15,16]). In brief, the presence of basic diseases, the clinical conditions (namely, bleeding symptoms and organ dysfunction), and the results of the examination (platelet counts, prothrombin time, fibrinogen, and fibrinogen and fibrin degradation products [FDP]) were quantified on a score basis (maximum = 13; minimum = 0). If the number was ≥ 7, DIC was established. In individuals with a DIC score of six points, two or more positive findings on supplementary tests (namely, soluble fibrin monomer complex [SFMC], D-Dimer, thrombin-antithrombin complex [TAT], and plasmin- α_2-plasmin inhibitor complex [PIC]) were needed to make a diagnosis of DIC. 75 patients (64.1%) diagnosed by JMHW DIC criteria were also diagnosed with overt DIC by the ISTH criteria [2], which coincided with the previous study by Wada H, et al [17].

Quantification of molecular markers of coagulation and fibrinolysis

The prothrombin time and the activated partial thromboplastin time were measured with coagulation-based activity assays. Plasma fibrinogen levels were measured by clotting methods using Fibrinogen Test Sankyo (Sankyo, Tokyo, Japan). FDP levels in sera were determined by the latex agglutination assay using LPIA-FDP (Mitsubishi Chemical Medience, Tokyo, Japan). Plasma PIC levels were measured by enzyme-linked immunosorbent assay (ELISA) (Kokusai-Shiyaku, Kobe, Japan). Plasma levels of TAT were also quantified by ELISA (Sysmex, Kobe, Japan). SFMC levels were determined by the latex agglutination assay and using monoclonal antibody IF-43 [18]. For the PAI-1 assay, we used a latex photometric immunoassay (LPIA-200; Mitsubishi Chemical Medience), as previously described [19]. The levels of XDPs by plasmin (p-XDPs) were measured by latex-agglutination assays utilizing JIF-23 [20]. Plasma levels of e-XDPs were also measured by the automated latex photometric immunoassay using IF-123 monoclonal antibody, which is specific for the fibrin fragment D species generated by granulocyte-elastase digestion as previously described [13,19]. The variance (CV) of e-XDP assay was 0.00 - 3.78% within-run, 0.00 - 3.35% between runs.

Table 1
Baseline demographics and disease characteristics.

	Septic DIC, n=117
Age (years)	61.7±15.4
Male / Female	72 / 45
Basic disease	
Respiratory infection	70 (59.8)
GI tract or biliary tract infection	18 (15.4)
Urinary tract infection	10 (8.6)
Other infection	19 (16.2)
Positive blood culture	44 (37.8)
Results of Gram's staining of bacterial pathogen	
Purely Gram-positive	43 (36.5)
Purely Gram-negative	33 (28.5)
Mixed	11 (9.5)
Culture negative or not obtained	30 (25.3)
Type of organism	
Gram-positive	
Staphylococcus aureus	39 (44.6)
Other *staphylococcus* species	3 (3.5)
Streptococcus pneumonia	5 (5.4)
Other Gram-positive	2 (1.7)
Gram-negative	
Escherichia coli	6 (7.1)
Klebsiella species	6 (7.1)
Pseudomonas species	14 (16.1)
Other Gram-negative	9 (10.5)
Fungus	3 (3.5)

The values are given in (%).

Two-dimensional difference gel electrophoresis (2D-DIGE) and image analysis

The fibrinogen and fibrin degradation products were isolated from septic DIC patients' samples using anti-fibrinogen polyclonal antibody (Dako, Carpinteria, California, USA) coupled Sepharose. 2D-DIGE was performed as described elsewhere [21,22]. Briefly, each sample was loaded onto a pH 3-10NL IPG Strip (BIO-RAD Laboratories, Hercules, California, USA) for 16 h at 25 °C. Isoelectric focusing was performed using the PROTEAN IEF cell (BIO-RAD). The second dimension of electrophoresis was then performed in 10% SDS-PAGE slab gels. After electrophoresis, the gels were stained with SYPRO Ruby Protein Stain, and the resulting protein spot patterns were analyzed using Molecular Imager FX Pro and PD Quest software (BIO-RAD).

In-gel digestion and nano liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

Each protein spot was excised from 2D-DIGE gels with a spot cutter (ProteomeWorks, BIO-RAD). Each gel piece was washed with 50% v/v acetonitrile, 0.1% formic acid to extract the resulting peptides. Nano LC-MS/MS analysis was performed in positive ion mode on a Micromass Q-Tof Ultima Global mass spectrometer controlled by the software Mass LynxTM 4.0 (Waters-Micromass, Manchester, UK) coupled with an UltiMate Nano LC system (Dionex-LC Packings; GenTech, New York, NY, USA) [23,24]. Spectra were recorded under the condition of a source temperature 80 °C and cone voltage of 80 V. Acquired and processed MS/MS data were searched against the NCBInr database using the Mascot search program (MS/MS Ion Search; Matrix Science, Boston, MA, USA).

Statistical Analysis

The SPSS statistical software package (SPSS, Chicago, IL, USA) was used for all statistical analyses of data. Normally distributed variables are presented as the means ± SEM and were compared by means of the Student's t test. Variables not normally distributed were analyzed with the two-sided Mann-Whitney U test. A difference with $p<0.05$ was considered statistically significant. Multivariate logistic regression analyses were used to identify independent predictors of mortality in septic DIC cases. Kaplan-Meier product limits were computed for the freedom from endpoint, and the log-rank test was used to screen univariate group results regarding the outcomes. Multivariate Cox regression models were used to investigate the association of plasma e-XDPs levels with the 28-day mortality rate after DIC diagnosis.

Results

XDPs levels and their correlation with molecular markers of coagulation and fibrinolysis in sepsis-induced DIC patients

Baseline characteristics of sepsis-induced DIC patients are summarized in Table 1. Plasma concentrations of e-XDPs of patients with septic DIC were significantly higher than those of healthy subjects

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(18.6 ± 19.9 vs 0.58 ± 0.47 U/mL, p<0.001, Fig. 1). We could not find any correlation between e-XDP levels and peripheral white blood cell counts (Fig. 2, A). However, there was a negative correlation between e-XDP levels and percentage of immature neutrophil ($rs = 0.046$, $p = 0.033$, Fig. 2, B). The e-XDPs levels showed a mild correlation with FDPs levels ($rs = 0.528$, $p<0.001$) and with p-XDPs levels ($rs = 0.547$, $p<0.001$) (Table 2). By contrast, there was not any correlation between e-XDPs levels and platelet counts, prothrombin time, levels of fibrinogen, TAT, SFMC, PAI-1, or PIC.

2D-DIGE and mass spectrometry analysis of XDPs isolated from patients with sepsis-induced DIC

We recovered XDPs from patients who showed marked elevation of e-XDPs levels with low levels of p-XDPs (patients e1 to e5, Fig. 3 A) and those who exhibited low levels of e-XDPs with remarkably increased levels of p-XDPs (patients p1 to p5, Fig. 3 A). Each of the isolated XDPs was analyzed by 2-D DIGE with fluorescent SYPRO Ruby staining. As shown in Fig. 3 B, twenty-seven spots were unique to the e-XDPs dominant group (red squares) and 19 spots were unique to the p-XDPs high group (green squares). Although the limited material available precludes identification of proteins corresponding to less intense spots given the detection limits, we could analyze only three spots (#5901, #5902 and #7601 in Fig. 3 C) unique to the e-XDP dominant group. Comparing acquired and processed MS/MS data against the NCBInr database using the Mascot search program showed that spot #5901 contained Bβ-chain-derived fragments possessing fibrinogen Bβ Asp-164, Ser-200, Gln-301, Ala-354 and Ile-484, and that spot #5902 had Bβ Asp-164 and Ile-484 at their amino-termini, respectively (Fig. 3 D). In addition, the spot #7601 was found to contain a fibrinogen γ-chain fragment corresponding to γ Val-274 at its amino-terminal.

Relationship between plasma e-XDPs levels, multiple organ failure and prognosis in sepsis-induced DIC

We found that Sequential Organ Failure Assessment (SOFA) scores were significantly higher in the patients with p-XDP levels of 3-10 μg/mL,10–30 μg/mL or greater than 30 μg/mL compared with those in the group with p-XDP levels less than 3 μg/mL (Fig. 4). By contrast, the SOFA scores in patients with e-XDPs levels 3–10 U/mL were significantly lower than those with e-XDPs levels -3 U/mL, 10-30 U/mL, and 30- U/mL. In addition, the survival rate to 28 days after DIC diagnosis in the group with e-XDP levels less than 3 U/mL was significantly lower than those in the groups with e-XDP levels of 3–10 U/mL, 10–30 U/mL and 30- U/mL (Fig. 5). Cox regression analyses of time-to-event data among patients according to e-XDPs levels at the time of DIC diagnosis revealed that the adjusted odds for the 28-day mortality rate in patients with e-XDPs levels less than 3 U/mL (hazard ratio, 4.432; 95% confidence interval, 1.557-12.615 [$p = 0.005$]; Table 3) were significantly higher than those in patients with e-XDPs levels 3–10 U/mL. Interestingly, plasma e-XDPs levels might be an independent factor predicting survival as revealed

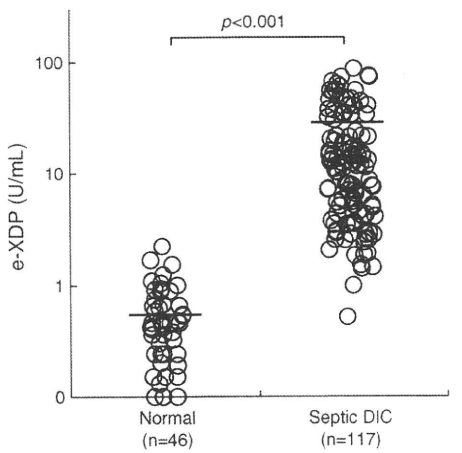

Fig. 1. Plasma e-XDPs levels in sepsis-induced DIC and healthy subjects. Plasma levels of e-XDPs of sepsis-induced DIC patients (n = 117) and normal healthy subjects (n = 46) are shown.

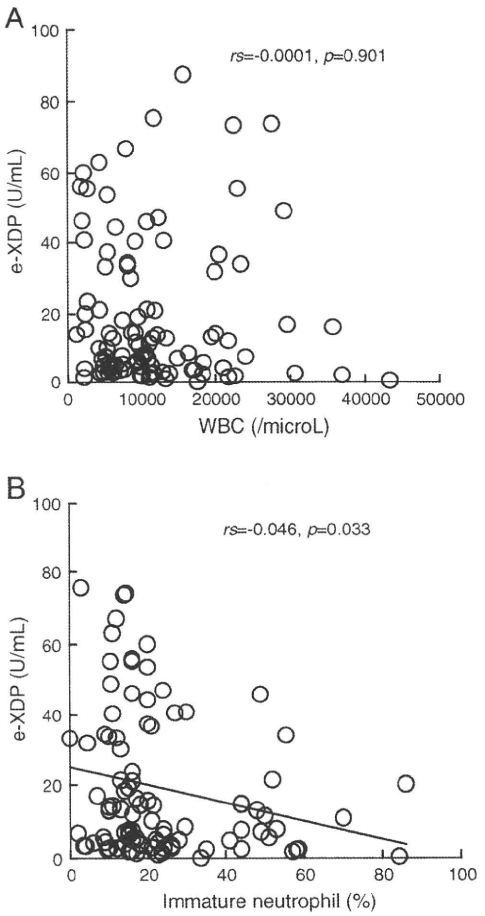

Fig. 2. Correlation between leukocyte and e-XDPs in patient with sepsis-induced DIC Correlations between peripheral white blood cell counts and plasma e-XDPs levels (A) and between immature neutrophil percentages and plasma e-XDPs levels (B) in patient with sepsis-induced DIC are shown. Values of rs are determined by Spearman rank correlation test.

Table 2
Correlation between XDP levels and molecular makers of DIC in patients with sepsis-induced DIC (n = 117).

Molecular markers	p-XDP		e-XDP	
	rs	*p*	*rs*	*p*
Platelet	-0.215	0.031	-0.056	0.575
PT	-0.133	0.183	-0.126	0.205
Fibrinogen	-0.222	0.025	-0.003	0.974
TAT	0.020	0.839	0.023	0.818
SFMC	0.119	0.233	0.147	0.140
PAI-1	0.045	0.654	0.043	0.666
PIC	0.348	<0.001	0.185	0.063
FDP	0.812	<0.001	0.528	<0.001
p-XDP	-	-	0.547	<0.001
e-XDP	0.547	<0.001	-	-

*Values of rs are determined by Spearman rank correlation test.

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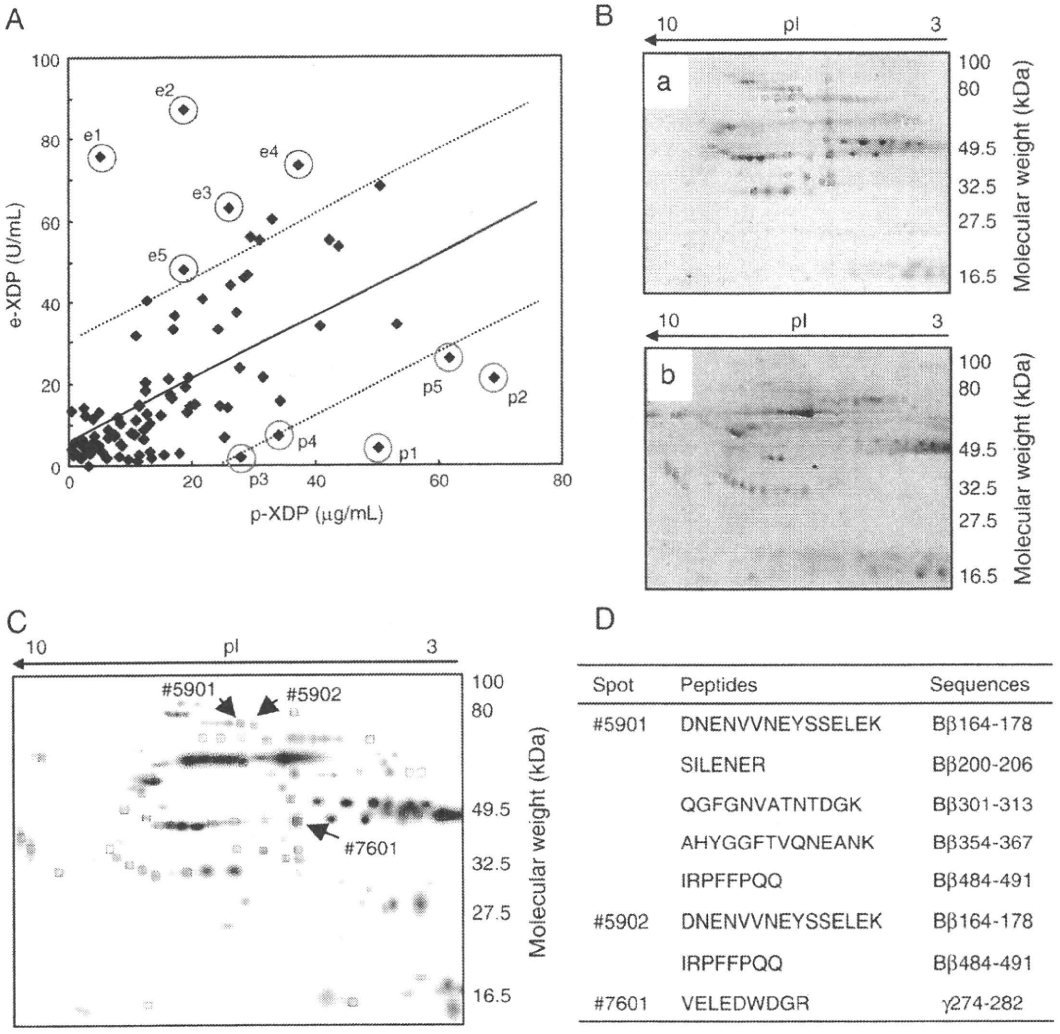

Spot	Peptides	Sequences
#5901	DNENVVNEYSSELEK	Bβ164-178
	SILENER	Bβ200-206
	QGFGNVATNTDGK	Bβ301-313
	AHYGGFTVQNEANK	Bβ354-367
	IRPFFPQQ	Bβ484-491
#5902	DNENVVNEYSSELEK	Bβ164-178
	IRPFFPQQ	Bβ484-491
#7601	VELEDWDGR	γ274-282

Fig. 3. 2D-DIGE analysis of fibrinogen and fibrin degradation products (FDPs) in sepsis-induced DIC patients. A. XDPs were recovered using anti-fibrinogen polyclonal antibody-coupled Sepharose from e-XDPs dominant (patients e1-e5) and p-XDPs dominant (patients p1-p5) patients with sepsis-induced DIC. B. Representative difference map comparison of five sets of e-XDPs dominant (a, red squqares) and p-XDPs dominant (b, green squares) subjects. Each sample was separated by isoelectric focusing (nonlinear gradient of pI 3 to 10) and SDS-PAGE (nominal range 16.5 kDa to 100 kDa) followed by Sypro Ruby-staining. C. Three spots (#5901, #5902 and #7601) were identified with MALDI-TOF, comparing acquired and processed MS/MS data by searching against NCBRInr databease using the Mascot search program. D. Sequences recovered from three spots.

by multivariate logistic regression analyses of molecular markers in sepsis-induced DIC patients (hazard ratio, 0.957; 95% confidence interval, 0.917-0.999 [$p = 0.045$]; Table 4), although platelet, PAI-1, PIC and FDP levels could also affect mortality.

Discussion

Leukocyte elastase may degrade major tissue constituent proteins, such as elastin and a variety of proteoglycans, as well as plasma proteins including fibrinogen and fibrin [8,25]. Gando and co-workers have demonstrated that leukocyte elastase mediated-fibrinolysis is activated to varying degrees depending on the amount of systemic inflammation such as major surgical procedure and sepsis [26–29]. The plasma levels of e-XDPs of sepsis-induced DIC patients were significantly higher than those of healthy controls (Fig. 1), and they showed no correlation with those of TAT or SFMC (Table 2). However, we did not measure anti-thrombin, protein C, or inflammatory cytokines levels in this study, we could not define any relationship between the fibrin formation and the leukocyte elastase-mediated fibrinolysis. We found that there was a negative correlation between e-XDP levels and percentage of immature neutrophil (Fig. 2). Previous reports showed that immature neutrophils are not fully developed as they have deficient phagocytic capacity, impaired bacterial killing, decreased chemotaxis and release of leukocyte elastase [30,31]. Thus, immature neutrophil could not effectively release leukocyte elastase to degrade cross-linked fibrin that might result in generation of e-XDP in sepsis-induced DIC patients.

The subsequent activity of leukocyte elastase is balanced by endogenous inhibitors, the predominant one being α_1-protease inhibitor [32]. In the clinical situation, leukocyte elastase level has been measured on the basis of the level of a leukocyte elastase and α_1-protease inhibitor complex [33]. Although the increase in the level of this complex in the plasma may be a marker of leukocyte elastase secretion from activated neutrophils, it may not be representative of actual leukocyte elastase-mediated proteolytic activity [34,35]. We could not find any correlation between e-XDP levels and the levels of elastase- α_1-protease inhibitor complexes ($rs = 0.095$, $p = 0.615$), even though they showed mild correlations with FDPs and p-XDPs levels (Table 2). Thus, monitoring the levels of degradation products

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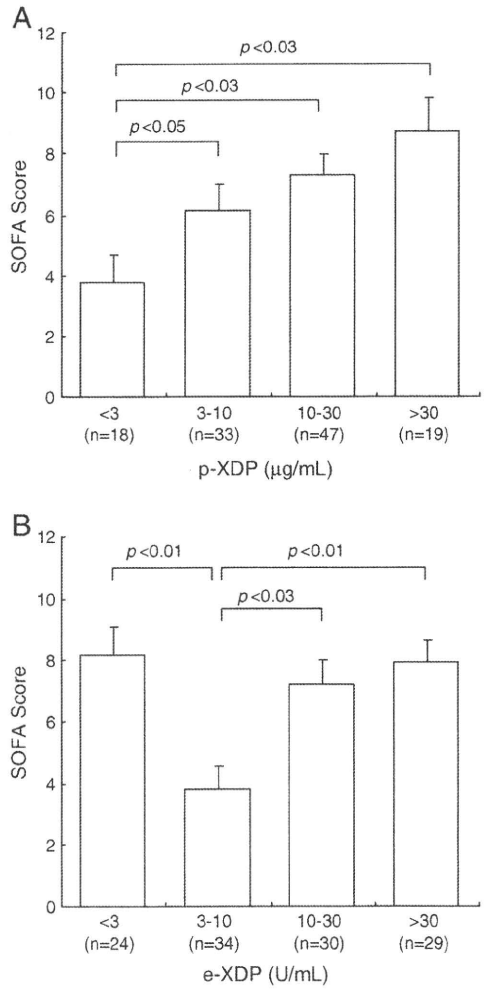

Fig. 4. Relationships between the SOFA score and XDPs levels in sepsis-induced DIC patients. A. Patients with sepsis-induced DIC were classified into four groups with p-XDPs levels (<3 μg/mL, 3-10 μg/mL, 10-30 μg/mL, >30 μg/mL) at the time of DIC diagnosed, and the groups were compared with respect to SOFA scores. B. Sepsis-induced DIC patients were classified into four groups according to e-XDPs levels (<3 U/mL, 3-10 U/mL, 10-30 U/mL, >30 U/mL) at the time of DIC diagnosis, and the groups were compared with respect to SOFA scores. Data are presented as means ± SEM.

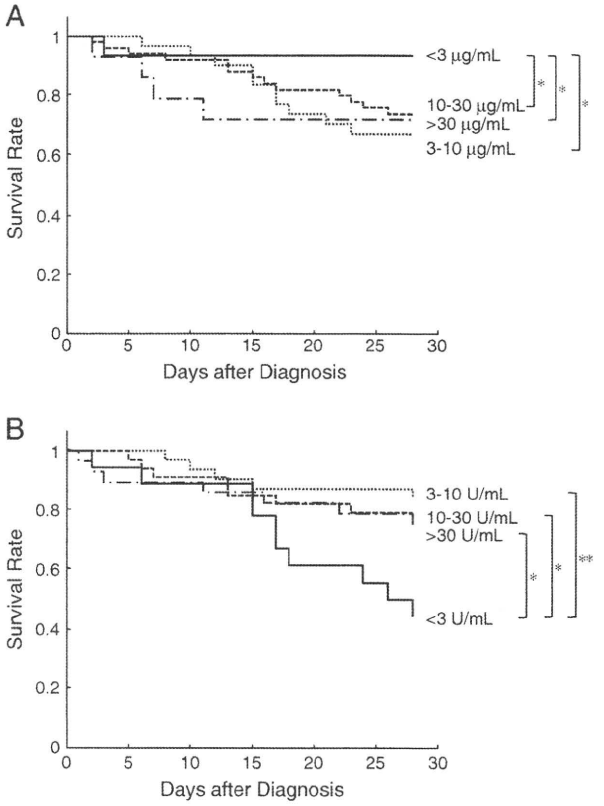

Fig. 5. The XDPs levels correlate with the mortality rate by 28 days after diagnosis in sepsis-induced DIC patients. A. Kaplan-Meier plots with respect to p-XDPs levels (<3 μg/mL [n = 18], 3-10 μg/mL [n = 33], 10-30 μg/mL [n = 47], >30 μg/mL [n = 19]) at the time of DIC diagnosis for sepsis-induced DIC patients. B. Kaplan-Meier plots with respect to e-XDPs levels (<3 U/mL [n = 24], 3-10 U/mL [n = 34], 10-30 U/mL [n = 30], >30 U/mL [n = 29]) at the time of DIC diagnosed for sepsis-induced DIC cases. *$p<0.05$; **$p<0.03$.

of cross-liked fibrin produced by leukocyte elastase might be important to evaluate the status of local fibrinolysis by leukocyte elastase as well as by plasmin.

The discrimination of e-XDPs from p-XDPs is necessary for a better understanding of sepsis-induced DIC, because a variety of molecular species collectively termed fibrin degradation products are released into the circulation [36,37]. We demonstrated that cross-linked fibrin degradation products subjected to 2-D DIGE analysis showed major differences between e-XDPs dominant and p-XDPs dominant patients (Fig. 3). Three spots unique to the e-XDPs dominant patients with mass spectrometry were fibrinogen Bβ-chain-derived fragments and fibrinogen γ-chain derivatives, which might possess plasmin-cleavage P1' sites (Bβ Asp-164, Ser-200, and Ala-354) and leukocyte elastase-cutting ones (Bβ Gln-301, Ile-484, and γ Val-274) [38]. In addition, the epitope for IF-123 is located in the carboxyl-terminal region (residues 196–204) of the α-chain remnant of fragment D residing at both ends of e-XDPs [13]. Thus, the combination of elastase and plasmin could digest cross-linked fibrin molecules in patients manifesting extensively increased fibrinolysis and generate the XDPs species seen in sepsis-induced DIC patients.

The SOFA scores were shown to be useful for evaluating organ failure in multicenter studies, and to be a valuable scoring system for predicting the outcome of DIC [39,40]. Several researchers have shown that plasma levels of e-XDPs are correlated with the level of sepsis-related organ dysfunction [28]. Interestingly, our study revealed that the SOFA scores in the group with e-XDPs levels of 3–10 U/mL were significantly lower than those with e-XDPs levels -3 U/mL, 10-30 U/mL, and 30- U/mL (Fig. 4). Gando et al. have demonstrated that leukocyte elastase-mediated fibrinolysis is activated in varying degrees depending on systemic inflammation [26]. Leukocyte elastase shows profibrinolyic effects, degrading fibrinogen and fibrin and inactivating PAI-1 [41]. In contrast, leukocyte elastase has antifibrinolytic potential to cleave fibrinolytic enzymes [42,43]. Recent study showed that neutrophil elastase can degrade tissue factor pathway inhibitor, which results in increasing blood clot formation [44]. Thus, the balance of all these interactions among the pro- and anti-fibrinolytic effects as well as

Table 3
Cox's regression analyses of time-to-event among patients according to e-XDP levels at the time of diagnosis of sepsis-induced DIC (n=117).

e-XDP (U/mL)	28-day mortality after DIC diagnosis	
	HR (95% CI)	p
<3	4.432 (1.557-12.615)	0.005
3-10	1.000	-
10-30	1.560 (0.510-4.770)	0.435
30<	1.450 (0.539-1.450)	0.539

HR: hazard ratio, CI: confidence interval.

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Table 4
Logistic regression analyses of molecular markers for 28-day mortality after diagnosis of sepsis-induced DIC (n = 117).

Molecular markers	28-Day Mortality after DIC Diagnosis		
	Hazard Ratio	95% CI	p
Platelet, $X10^3/\mu L$	0.872	0.778-0.978	0.019
Prothrombin time, s	1.136	0.974-1.325	0.105
Fibrinogen, mg/dL	0.999	0.995-1.003	0.648
TAT, ng/mL	0.985	0.960-1.010	0.229
SFMC, μg/mL	1.032	1.004-1.060	0.023
PAI-1, ng/mL	1.012	1.003-1.021	0.011
PIC, μg/mL	0.737	0.547-0.993	0.045
FDP, μg/mL	1.046	1.003-1.091	0.035
p-XDP, μg/mL	1.013	0.942-1.088	0.731
e-XDP, U/mL	0.957	0.917-0.999	0.045

procoagulant action of neutrophil elastase may allow lysis to progress or not progress, or even enhancement of coagulation in clinical situation [26]. However, coagulation-related and inflammation-associated data are required to elucidate the clear mechanisms which cause the insufficient or sufficient activation of leukocyte elastase-mediated fibrinolysis in sepsis-induced DIC patients.

The group with e-XDP levels less than 3 U/mL showed significantly lower survival rates to 28 days after DIC diagnosis than patients with e-XDPs levels of 3–10 U/mL or 10–30 U/mL by Kaplan-Meier analyses (Fig. 5), and the adjusted odds for the mortality rate of this group were 4.432 (95% CI, 1.557-12.615, $p = 0.005$, Table 3). Multivariate logistic regression analyses showed that plasma e-XDP levels at DIC diagnosis might be an independent factor for 28-day mortality in sepsis-induced DIC patients (Table 4). However, it might not biologically be very relevant, as the hazard ratio was modest (0.957, 95% CI, 0.917-0.999). Collectively, the degree of the local activation of leukocyte elastase might contribute to organ damage as well as the actual prognosis in sepsis-induced DIC patients. Poor activation of leukocyte elastase (e-XDPs levels, <3 U/mL) might result in massive fibrin deposition when the plasminogen activator-plasmin system is suppressed, balanced activation (e-XDPs levels, 3–10 U/mL) could effectively degrade fibrin thrombi to protect against ischemic organ damage, and excessive activation (e-XDPs levels, >10 U/mL) evading local inhibitors might result in organ injury with proteolytic cleavage of tissue and plasma components [10,19,45].

In conclusion, we have demonstrated that leukocyte elastase could contribute to the degradation of cross-linked fibrin, and that e-XDPs levels at the time of diagnosis for DIC might predict the prognosis of patients with sepsis-induced DIC. The evaluation of leukocyte elastase-mediated fibrinolysis and control of its activity by specific inhibitors such as sivelestat could improve the poor outcome of septic DIC [46,47].

Authorship contribution

S. Madoiwa designed and performed the research, analyzed data, and wrote the paper. H. Tanaka, Y. Nagahama, M. Dokai, Y Kashiwakura, A. Ishiwata, A. Sakata, and A. Yasumoto performed experiments; S. Madoiwa, T. Ohmori, J. Mimuro, and Y. Sakata analyzed data and revised the paper.

Conflict of interest statement

The authors state that they have no conflict of interest.

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Impact of acute cellular rejection on coagulation and fibrinolysis biomarkers within the immediate post-operative period in pediatric liver transplantation

Mimuro J, Mizuta K, Kawano Y, Hishikawa S, Hamano A, Kashiwakura Y, Ishiwata A, Ohmori T, Madoiwa S, Kawarasaki H, Sakata Y. Impact of acute cellular rejection on coagulation and fibrinolysis biomarkers within the immediate post-operative period in pediatric liver transplantation.
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Abstract: We studied restoration of the coagulation and fibrinolysis system in pediatric patients following liver transplantation and biomarkers of blood coagulation and fibrinolysis for suspecting the occurrence of acute cellular rejection. Coagulation activity recovered rapidly within two days following transplantation, but it took approximately 21–28 days for full recovery of the coagulation and fibrinolysis factors synthesized in the liver. PAI-1 levels were significantly higher in patients at the time of acute cellular rejection compared with levels after control of AR, and levels on days 14 and 28 in patients without AR. Plasma protein C and plasminogen levels at the time of rejection were significantly lower than those on day 14 in patients without AR. Statistical analysis suggested that an increase in plasma PAI-1 at a single time point in the post-operative period is a reliable marker among the coagulation and fibrinolysis factors for suspecting the occurrence of acute cellular rejection. These data suggested that appropriate anticoagulation may be required for 14 days after liver transplantation in order to avoid vascular complications and measurement of plasma PAI-1 levels may be useful for suspecting the occurrence of acute cellular rejection in pediatric patients following liver transplantation.

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Vascular thrombosis and immunological rejection of the transplanted liver in patients undergoing liver transplantation are frequent and serious post-operative complications (1). The majority of coagulation factors, factors regulating coagulation, and fibrinolysis factors are synthesized in the liver, and plasma coagulation factor levels may therefore fall for a short period after transplantation, but may return to normal levels upon regeneration of the grafted liver. Anastomosis of the vascular system of the grafted liver and the recipient vessels is carried out during liver transplantation, and anticoagulants are commonly administered for a period of time following surgery. However, restoration of the coagulation and fibrinolysis system following liver transplantation in pediatric patients has not been well studied (2, 3). In addition, the

Abbreviations: ADAMTS13, a disintegrin-like and metalloprotease with thrombospondin type 1 motif 13; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AR, acute rejection; AST, aspartate aminotransferase; ELISA, enzyme linked immunosorbent assay; LDH, lactate dehydrogenase; NAR, no acute rejection; PAI-1, plasminogen activator inhibitor 1; PELD, pediatric model for end-stage liver disease; PT-INR, prothrombin time-international normalized ratio; sES, soluble E-selectin; TM, thrombomodulin; vWF, von Willebrand factor; γ-GTP, γ-glutamyl transpeptidase.

thrombogenic state after liver transplantation is not well understood. We performed a single center study to investigate the coagulation and fibrinolysis system and the relationship between coagulation markers and acute cellular rejection following liver transplantation from living-related donors.

Materials and methods

Patients and study protocol

Sixty-three pediatric patients with liver failure due to biliary atresia (n = 59), ornithine transcarbamidase deficiency (n = 2), or Wilson's disease (n = 2) underwent living-related liver transplantation from April 2001 to March 2006 and were enrolled in this study. Most of the patients with biliary atresia had previously undergone hepatic portajejunostomies.

Description of patients

The patients were classified into two patient groups: one with acute cellular rejection (group AR, n = 24) and one with no acute cellular rejection (group NAR, n = 39). The diagnosis of acute cellular rejection was made by liver biopsy. There were no significant differences between group AR and group NAR in terms of age, gender, basal diseases, or the use of calcineurin inhibitors (data not shown). The PELD scores (AR, 13.0 ± 7.8; NAR, 15.1 ± 9.5), the amount of blood loss (AR, 85.0 ± 127.8 mL/kg; NAR, 125.8 ± 176.0 mL/kg), the amount of total blood transfusion (AR, 162.1 ± 109.1 mL/kg; NAR, 161.8 ± 170.8 mL/kg), the amount of plasma transfusion (AR, 64.1 ± 49.1 mL/kg; NAR, 94.2 ± 89.1 mL/kg), the cold ischemic time of graft liver (AR, 149.75 ± 126.4 min; NAR, 121.1 ± 69.1 min), and the warm ischemic time of graft liver (AR, 64.9 ± 18.1 min; NAR, 65.1 ± 13.3 min) upon operation were not significantly different between group AR and group NAR. Patients with severe infections or major bleeding episodes at the time of blood sampling for analysis were excluded from the analysis.

Immunosuppression and anticoagulation protocols

The standard protocol for immunosuppression was as follows. Both methylprednisolone and a calcineurin inhibitor (tacrolimus or cyclosporine) were used for immunosuppression. Intravenous administration of methylprednisolone (20 mg/kg) was started during the operation and the dosage was tapered to 3 mg/kg on day 1 and to 0.5 mg/kg on day 7 after liver transplantation. A calcineurin inhibitor was infused intravenously after transplantation and the blood concentration of tacrolimus or cyclosporine was adjusted to 18–20 ng/mL or 200–300 ng/mL till day 7 after liver transplantation, respectively. Intravenous injection of calcineurin inhibitor and methylprednisolone were converted to oral administration of these regimens after patient's oral intake had been fully confirmed and the blood concentration of tacrolimus or cyclosporine was adjusted 10–15 ng/mL or 100–150 ng/mL, respectively. The methylprednisolone dose was tapered to 0.06 mg/kg on day 30. Postoperative anticoagulation was performed with intravenous administration of dalteparin (low molecular weight heparin) at the dose of 2 U/kg/h, nafamostat mesilate (serine protease inhibitor with anticoagulant activity) at the dose of 0.1 mg/kg/h, and prostaglandin E1 at the dose of 0.01 μg/kg/min till day 7 after transplantation. Anticoagulation was continued with intravenous administration of heparin (unfractionated heparin) at the dose of 8 U/kg/h from day 8 to day 21 after liver transplantation.

Blood sample collection and analysis

All samples were obtained from patients with informed consent, according to the Declaration of Helsinki. Routine laboratory tests including complete blood counts, coagulation tests, blood chemistry analysis, and urinalysis were performed, and biomarkers of blood coagulation and fibrinolysis, i.e., PAI-1, TM, ADAMTS13, and sES were measured before and after liver transplantation on days 1, 3, 7, 10, 14, 21, and 28. Blood sampling was performed on days 35 and 49 in some patients. These were quantified using commercially available ELISAs (Mitsubishi Chemical Medience Co., Tokyo, Japan; Diaclone, Tepnel Research Products & Services, Cedex, France) (4, 5). The plasma activity levels of plasminogen and protein C were quantified using laboratory test kits (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA). Rationale for measurements of these biomarkers are as follows. PT-INR is currently used worldwide as a coagulation test to monitor the effects of anticoagulants such as coumarin in patients at risk of thrombosis. Protein C is a vitamin K-dependent protein synthesized in the liver that functions as an important regulatory factor for coagulation (6). Plasminogen is the zymogen of plasmin, a key enzyme in fibrinolysis, and is also synthesized in the liver (7). Therefore, plasma protein C and plasminogen levels were thought to be good markers for the restoration of the coagulation and fibrinolysis system following liver transplantation. Levels of these markers might correlate with protein synthesis in the liver, thereby reflecting regeneration of the graft liver. Additionally, measurement of these factors may also be important for patient management, because deficiency of protein C and type II plasminogen deficiency are thought to increase the risk of thrombosis (7, 8). The fibrin degradation product level, determined by the monoclonal antibody specific for degradation products of cross-linked fibrin, is a biomarker for the presence of a thrombus and is used to diagnose venous thrombosis and disseminated intravascular coagulation, however, the fibrin degradation product level may be affected by the presence of blood clots in the extravascular spaces (e.g., the peritoneal cavity), and may therefore not accurately reflect the thrombogenic state in the post-operative period. Thus, the soluble fibrin level was used to assess the thrombogenic state during the post-operative period following liver transplantation. PAI-1 is a primary regulator of fibrinolysis that is synthesized mainly in endothelial cells. Plasma PAI-1 levels change significantly in various pathological conditions (4). ADAMTS13 is the vWF cleaving protease that plays an important role in vWF multimer processing (9). It is synthesized in liver stellate cells and the liver is thought to be the primary source of ADAMTS13 in the circulation (9–11). In addition to the liver stellate cells, vascular endothelial cells in other organs may also be able to synthesize ADAMTS13 (12), and ADAMTS13 mRNA has been detected in the liver, kidneys and lungs in mice (13). ADAMTS13 deficiency results in platelet thrombus formation in the circulation, resulting in the development of a typical thrombotic microangiopathy (9). It is possible that ADAMTS13 deficiency might occur after liver transplantation, and plasma ADAMTS13 levels in patients were

therefore quantified following transplantation. TM, an important regulator of blood coagulation, is synthesized in vascular endothelial cells and is used as a marker of vascular injury (6). The sES level has been used as a marker for endothelial cell dysfunction (14). For example, the sES level is increased in systemic infections such as sepsis.

Diagnosis of acute cellular rejection

The diagnosis of acute cellular rejection was made by liver biopsy and was evaluated using the rejection activity index (3) scores (1, 15, 16). Patients suspected of suffering from acute cellular rejection because of deterioration of liver function (increased serum levels of bilirubin, AST, ALT, ALP, LDH, and γ-GTP compared with previous levels) were subjected to ultrasonography-guided liver biopsy. The liver biopsy specimens were examined for the presence of acute cellular rejection. Patients diagnosed with acute cellular rejection were subjected to intensive immunosuppressive therapy with intravenous methylprednisolone. Mycophenolate mofetil and/or OKT3 were also administered in some patients. Plasma samples obtained before starting administration of the intensive immunosuppressive regimens were evaluated in the following studies.

Statistical analysis

Statistical analyses were performed using SPSS software (SPSS Inc., Tokyo, Japan). Student *t*-tests were used to compare the mean values between groups. Multiple logistic regression analysis was used to investigate the association between biomarkers of blood coagulation and fibrinolysis and the occurrence of acute cellular rejection. p-values <0.05 were considered statistically significant.

Results

Analysis of the coagulation and fibrinolysis system following liver transplantation

Changes in mean values of coagulation tests in patients without acute cellular rejection, vascular complications, or severe infections are shown in Fig. 1. The coagulation activity after liver transplantation was assessed by measuring prothrombin time (PT-INR). The mean PT-INR value rose to approximately 1.8 on day 1, but quickly fell again to <1.5 on day 2, and then normalized gradually. These data suggest that the coagulation activity rapidly recovered after transplantation, once the graft liver started to function.

The mean protein C level of patients before liver transplantation decreased to 57.5% of the normal level. This may have been due to the decreased synthesis of protein C in the liver because most patients had liver failure. The mean protein C level fell to approximately 50% of the normal level on day 1 post-transplantation, and then increased gradually, reaching ≥80% of the normal level by day 14. The mean plasminogen level changed in a similar manner to protein C. By day 28, both protein C and plasminogen levels had returned to almost 90–100% of the normal levels. The nadir values of protein C and plasminogen on day 1 post-transplantation might

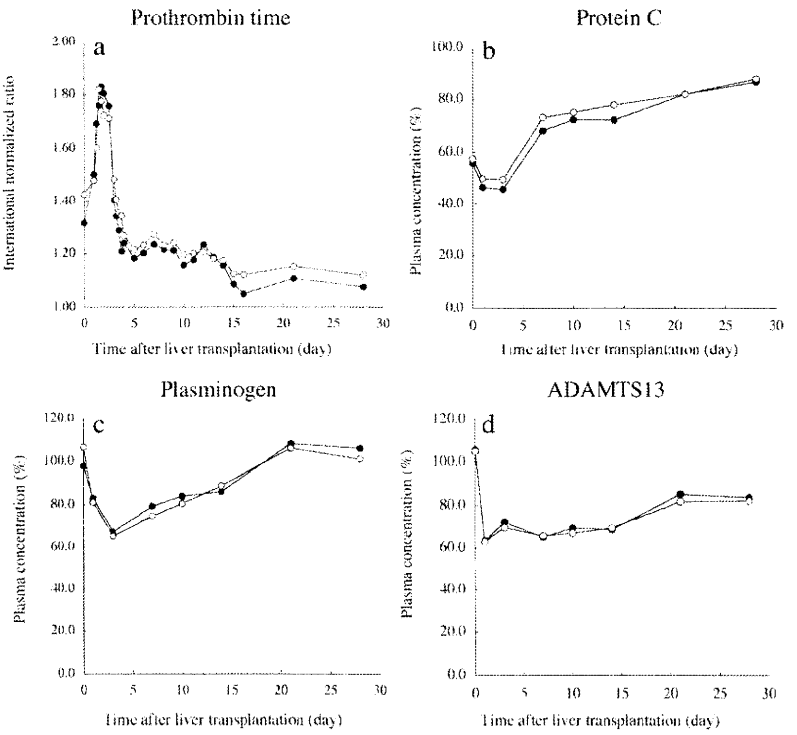

Fig. 1. Restoration of the coagulation and fibrinolysis system following liver transplantation. (a) The mean values of coagulation activity assessed by PT-INR and (b) plasma levels of protein C (normal range 67.1–129.0%), (c) plasminogen (normal range 85.0–120.0%), and (d) ADAMTS13 (normal range 100 ± 15%) of patients without complications (open circle) and of patients with acute cellular rejection (closed circle) are shown.

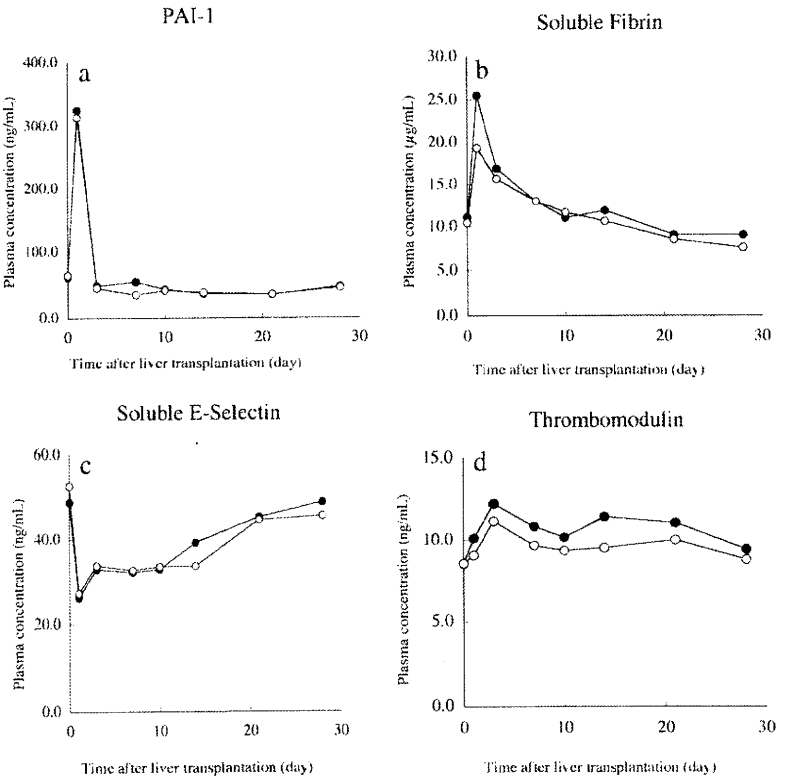

Fig. 2. Analysis of biomarkers of coagulation and fibrinolysis following liver transplantation. The mean plasma levels of PAI-1 (normal range 20–30 ng/mL), soluble fibrin (normal range: < 7.5 μg/mL), sES (normal range: < 37.5 ng/mL), and TM (normal range 4.46 ± 1.36 ng/mL) of patients without complications (open circle) and of patients with acute cellular rejection (closed circle) are shown.

be affected by plasma transfusion during and after surgery. These data suggest that the synthesis of coagulation factors in the graft liver may start on day 1, resulting in rapid recovery of coagulation activity, but it may take up to 14 days for recovery of the coagulation and fibrinolysis system to near normal levels, and 21–28 days for full restoration of the system after liver transplantation. These data also suggest that graft livers may regenerate to the appropriate size within four wk, though graft livers may vary in size depending on their recipients and donors. The average levels of the coagulation and fibrinolysis factors in patients with acute cellular rejection was not significantly different from those in patients without acute cellular rejection in the post-operative periods, but rate of restoration of the protein C and plasminogen levels on day 14 in group AR was slow.

Soluble fibrin levels in patients with no complications increased significantly on day 1 and then gradually decreased, normalizing by day 14 as shown in Fig. 2. These data suggest that the thrombotic state may continue for 14 days after liver transplantation, and that appropriate antithrombotic therapy may therefore be required during this period.

The mean plasma PAI-1 level was increased approximately 10-fold on post-operative day 1, compared with the normal level, but returned quickly to the normal level on day 3 after transplantation (Fig. 2). These data, together with the changes in the plasminogen level during the post-operative period (Fig. 1), suggest that fibrinolysis activity was suppressed on day 1 after liver transplantation.

The average plasma ADAMTS13 level decreased significantly on day 1 post-transplantation (Fig. 2), but the decrease was not as severe as that of protein C or plasminogen (Fig. 1). However, low levels of ADAMTS13 were maintained for 14 days after liver transplantation. These changes in plasma ADAMTS13 levels after liver transplantation did not parallel those of protein C or plasminogen (Fig. 1), reflecting the extrahepatic synthesis of ADAMTS13 and the possibility that ADAMS13 is synthesized not in hepatocytes, but in stellate cells in the liver. The plasma ADAMTS13 level fell to 28.4% of the normal level in one patient, but she showed no typical signs of thrombotic microangiopathy.

The TM level was increased on day 3 post-transplantation and remained at the upper limit of the normal range after day 7 (Fig. 2). The sES level was significantly increased in patients before liver transplantation (Fig. 2), which may be explained by the fact that many patients enrolled

in the study had undergone hepatic porta-jejunostomies and therefore had biliary tract infections before transplantation. The sES level was reduced post-transplantation, and remained almost within the normal range until day 14, but then was significantly increased on days 21 and 28 (Fig. 2). This increase in the sES level was not associated with the presence of infection or other disease states. The average changes of biomarkers of the coagulation and fibrinolysis system in patients with acute cellular rejection was not significantly different from those in patients without acute cellular rejection in the post-operative periods.

Three patients in this study suffered from hepatic artery thrombosis after liver transplantation, and an increased PT-INR (prolongation of prothrombin time) was detected in all three patients. Increase of plasma PAI-1 in the following samples of two patients was observed. Decrease of plasma protein C and plasma ADAMTS 13 in the following samples were observed in two patients. Other biomarkers did not change significantly. However, due to the small sample size, the predictive value of this test for the development of vascular complication was inconclusive.

Relationship between coagulation and fibrinolysis markers and acute cellular rejection

Patients were divided into two groups, group AR and group NAR, based upon the presence of acute cellular rejection as described above. The mean onset time of acute cellular rejection in group AR was on day 15 ± 8.7 after liver transplantation, while the mean time for data collection was on day 14 ± 7.9. Laboratory data and coagulation markers for each group at two time points were subjected to statistical analysis. Measurements taken immediately before the diagnosis of acute cellular rejection in group AR were compared with those taken after the cessation of rejection by intensive treatment with methylprednisolone in group AR, and those taken on days 14 and 28 in group NAR.

Statistical analysis of the mean levels of coagulation and fibrinolysis markers (Table 1) revealed that the PAI-1 level at the time of acute cellular rejection in group AR was significantly higher than that after cessation of rejection in group AR, and those on days 14 and 28 in group NAR (data for day 28 of group NR are not shown in Table 1).

The plasma protein C and plasminogen levels at the time of AR diagnosis in group AR were significantly lower than those on day 14 in group NAR.

Table 1. Coagulation and fibrinolysis biomarkers following liver transplantation

	Group AR (n = 24)		Group NAR (n = 39)
	Before*	After†	Day 14
PAI-1 (ng/mL)	79.3 ± 103.9‡	23.0 ± 10.7	38.5 ± 30.4
Plasminogen (%)	85.2 ± 22.8‡	99.4 ± 29.0	97.68 ± 13.8
Protein C (%)	65.7 ± 23.0‡	89.3 ± 37.9	87.2 ± 25.5
ADAMTS13 (%)	67.5 ± 24.1	77.8 ± 23.6	72.5 ± 17.4
ATIII (%)	96.3 ± 17.3	111.5 ± 57.4	99.3 ± 14.9
PT-INR	1.17 ± 0.21	1.08 ± 0.13	1.13 ± 0.13
Fibrinogen (mg/mL)	295.3 ± 116.4	296.3 ± 106.7	280.6 ± 74.0
Thrombomodulin (U/mL)	10.2 ± 3.8	10.8 ± 4.8	8.7 ± 5.2
Soluble E-selectin (μg/mL)	43.8 ± 16.7	46.4 ± 19.0	33.5 ± 17.2
Soluble fibrin (μg/mL)	13.57 ± 17.3	8.64 ± 14.9	10.2 ± 13.9

*Values at the time immediately before acute cellular rejection.
†Values after cessation of acute cellular rejection.
‡Values taken from the time point proximate to acute cellular rejection (before) are significantly different from those of group AR after cessation of acute cellular rejection (after) and those on day 14 in group NAR ($p < 0.01$).
Values are mean ± s.d.

The ADAMTS13 level at the diagnosis of AR in group AR appeared to be lower than that after cessation of rejection in group AR, and those on day 14 in group NR, though the differences were not statistically significant.

There were no significant differences between the levels of other coagulation and fibrinolysis markers in patients at the time of rejection diagnosis and after cessation of acute cellular rejection in group AR, or the levels on days 14 and 28 in group NAR.

The changes of the coagulation and fibrinolysis factors and biomarkers before the diagnosis of acute cellular rejection by liver biopsy were studied. These biomarkers levels of samples obtained from the patients proximate to the diagnosis of acute cellular rejection (AR-proximate sample in Fig. 3) were compared with those obtained before the AR-proximate sample (earlier sample in Fig. 3). The PAI-1 level in the AR-proximate samples were significantly higher than that in the earlier samples. The mean values of protein C, plasminogen, and ADAMTS13 in the AR-proximate samples was expected to be higher than those in the earlier samples, but they were lower than the earlier samples though the differences were not statistically significant. The mean values of other biomarkers in the two time points were not significantly different.

Multiple logistic regression analysis was performed to identify the coagulation and fibrinolysis markers for suspecting the occurrence of acute cellular rejection. Absolute values of coagulation and fibrinolysis factors (protein C, plasminogen, ADAMTS13) synthesized in the liver

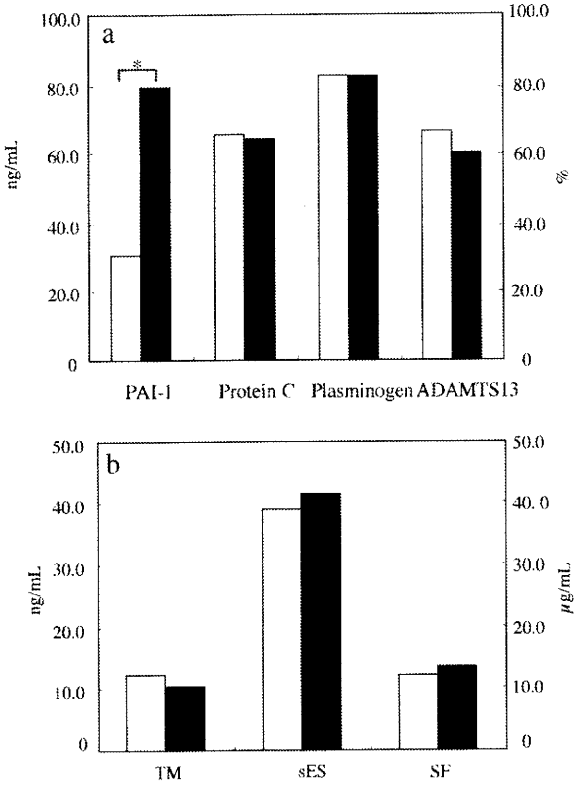

Fig. 3. Changes of coagulation and fibrinolysis factors and biomarkers in patients with acute cellular rejection. The mean plasma levels of coagulation and fibrinolysis factors and biomarkers obtained from patients with acute cellular rejection at two time points were shown. The AR-proximate samples (closed square) were obtained from the patients proximate to the diagnosis of acute cellular rejection. The earlier samples (open square) were obtained before the AR-proximate samples.

were difficult to ascertain using this method, and the changes in levels between time points were therefore analyzed. PAI-1 levels are independent of regeneration of the liver and an increase in plasma PAI-1 levels of >40 ng/mL at a single time point was therefore taken into account. Increases in soluble fibrin, TM, or sES since the previous time point, and above the normal range, were also taken into account. A summary of the multiple logistic regression analysis of coagulation and fibrinolysis markers is shown in Table 2. These data suggest that an increase in PAI-1 levels, and a decrease in protein C, plasminogen, or ADAMTS13 levels, were independently related to the occurrence of acute cellular rejection. Other markers were not related to the occurrence of acute cellular rejection (Table 2). Among these markers, an increase in plasma PAI-1 levels was observed in almost 80% of the patients in group AR.

Table 2. Multiple logistic regression analysis of biomarkers

	Odds ratio	Confidence interval	p-value
PAI-1	17.91	4.89–64.36	<0.001
ADAMTS13*	6.40	1.85–22.03	0.003
Protein C*	4.58	1.14–18.28	0.027
Plasminogen*	7.86	1.23–49.83	0.02
Soluble fibrin†	0.60	Not applicable	0.60
Soluble E-selectin†	0.60	Not applicable	0.65
Thrombomodulin†	0.421	Not applicable	0.42

*Decrease of marker values from the previous time point was adopted.
†Increase of marker values from the previous time point was adopted.

Discussion

The prevention and treatment of vascular thrombosis and immunological rejection of the transplanted liver during the post-operative period is a keystone of patient management. The present study analyzed the coagulation and fibrinolysis system following liver transplantation in pediatric patients to identify biomarkers for suspecting the occurrence of acute cellular rejection.

The present study suggests that the coagulation activity recovered rapidly once the graft liver started functioning, and that the graft liver might regenerate to the appropriate size in 21–28 days, with coincident full recovery of the coagulation and fibrinolysis system in pediatric patients undergoing liver transplantation. The present study also suggests that the hypercoagulable state persisted for 14 days after surgery, and that appropriate anticoagulation may therefore be required at least for 14 days post-transplant, even in the absence of any apparent vascular complications.

Recent advances in the management of patients with liver transplants have improved the clinical outcome of these patients. Adjustments in the doses of immunosuppressive drugs such as calcineurin inhibitors, based on their blood concentrations, are widely conducted after liver transplantation. However, immunological rejection of the transplanted liver still develops in a certain ratio of these patients, even when the blood calcineurin inhibitor concentration is within the appropriate therapeutic range (17, 18). A variety of methods for evaluating immune cell activation have been proposed as a basis for adjusting immunosuppressive therapy, and these have been shown to be useful for assessing the level of immunosuppression (19–22). Intensive treatment of acute cellular rejection with high dose methylprednisolone, with or without other medicines such as OKT3, is usually effective, though the prediction and rapid diagnosis of AR may be important for its effective treatment. In

this regard, the timely suspicion of acute cellular rejection using laboratory markers is a key indicator of the need for liver biopsy. Fluorescent-activated cell sorting analysis of CD25, CD28, and CD38 expression in peripheral lymphocytes is considered to be useful, not only for evaluation of the degree of immunosuppression, but also for the prediction of acute allograft cellular rejection (22). The present study showed that four coagulation and fibrinolysis markers, i.e., increase in PAI-1, decrease in protein C, decrease in plasminogen, and decrease in ADAMTS13, might be used as markers for suspecting the occurrence of acute cellular rejection. Statistical analysis suggested that an increase in the plasma PAI-1 level was the most reliable and sensitive marker for acute cellular rejection. Protein C, plasminogen, and ADAMTS13 are all synthesized in the liver, and their levels may therefore depend on the size and regeneration of the graft liver, and their plasma levels at any given time point might thus be less reliable as predictors of acute cellular rejection. PAI-1 is synthesized mainly in the vascular endothelial cells and its plasma level was elevated on day 1 after liver transplantation, and had returned to pretransplant levels after day 3. An increased plasma PAI-1 level at a single time point after day 1, together with a deterioration in liver function, may therefore be adopted as a predictive marker for acute cellular rejection.

Acute cellular rejection is characterized by portal inflammation, bile duct inflammation, and subendothelial cell inflammation (15, 16). Recent studies have suggested that not only T-cells, but also B-cells, are involved in acute cellular rejection, and cytokines and chemokines may also play roles in this process (23). As shown in a previous report, Toll-like receptor signaling through MyD88 may be involved in acute allograft rejection, indicating that toll-like receptors may be activated in the transplant setting causing inflammatory cytokine release (24). Therefore, the increase in PAI-1 levels seen during acute cellular rejection may be accounted for by immune cell-derived cytokine/chemokine activation of, and inflammation of, sinusoid-endothelial and portal vein endothelial cells. An increased PAI-1 level has previously been shown to be predictive for veno-occlusive disease developing after bone marrow transplantation (25), and this mechanism is thought to be responsible for busulfan-related toxic injury of sinusoidal endothelial cells (26, 27). The increase in plasma PAI-1 levels in patients with allograft cellular rejection is not as high as that seen in veno-occlusive disease, suggesting that the mechanisms and the outcomes of these PAI-1 increases may differ. Although the mechanisms of activation of endothelial cells may differ in veno-occlusive disease and in acute cellular rejection after allograft liver transplantation, both might result in increased plasma levels of PAI-1. Further studies are required to determine the precise mechanism responsible for the increase in PAI-1 levels occurring during acute cellular rejection.

Cytokines released from infiltrated immune cells in the liver, and inflammation in portal and sinusoid endothelial cells, might also inhibit the synthesis of ADAMTS13 in stellate cells, resulting in decreased plasma ADAMTS13 levels because the plasma ADAMTS13 level was significantly decreased in patients with sepsis-induced disseminated intravascular coagulation (5) and ADAMTS13 mRNA expression in the liver is decreased in endotoxin-injected mice (13). The decrease in protein C and plasminogen levels associated with acute cellular rejection might be due to their reduced synthesis by the graft hepatocytes, and a reduction in levels of these markers might therefore take time to become apparent. The decrease in plasminogen levels in patients with acute cellular rejection was less severe than that in protein C levels. These differences may be due to differences in the plasma half-lives of these molecules.

In conclusion, we have performed a comprehensive analysis of the coagulation and fibrinolysis system in pediatric patients undergoing orthotopic liver transplantation. Coagulation activity was quickly normalized by two days after liver transplantation. However, it took for 21–28 days for full restoration of the coagulation and fibrinolysis system. The post-operative thrombogenic state continued for approximately 14 days. PAI-1 may be used as predictive markers for acute cellular rejection in pediatric patients. These findings might also be applicable to adult liver transplant patients, though this needs to be confirmed by future prospective studies.

Acknowledgments

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