参考論文 1

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Three Types of Vitronectin in Human Blood

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ABSTRACT. Vitronectin is a cell-adhesive glycoprotein in serum and plasma, also termed serum spreading factor and complement S-protein. It consists of a mixture of a polypeptide of molecular weight 75 kilodalton (kDa) and its nicked product of 65 kDa plus 10 kDa. By a quantitative immunoblotting assay, human blood samples could be classified into three distinct vitronectin types; type I (58% of the population) was 75 kDa rich and 65 kDa poor, type II (35% of the population) contained approximately equal amounts of 75 kDa and 65 kDa, and type III (5% of the population) was 75 kDa poor and 65 kDa rich. The vitronectin type did not correlate with age, sex, or ABO blood type.

Vitronectin is a 75 kilodalton (kDa) glycoprotein in plasma and tissue (4), also termed serum spreading factor (2) and complement S-protein (7, 11). It promotes attachment and spreading of animal cells *in vitro* (2, 11), inhibits cytolysis by the complement C5b-9 complex (9), and modulates antithrombin III-thrombin action in blood coagulation (5, 6, 10, 12). The concentration of vitronectin in plasma and serum is 0.1-0.4 mg/ml (2, 4), and it does not correlate substantially with age or sex (13). The primary structure of vitronectin has been deduced from its cloned cDNA sequence (7, 14). Vitronectin is usually detected as a mixture of 75 kDa and 65 kDa polypeptides (2–4), in which the 65 kDa polypeptide is derived from the amino terminal portion of the 75 kDa polypeptide (15). This paper describes a very sensitive assay for individual 65 kDa and 75 kDa polypeptides and classifies human sera and plasma into three vitronectin types depending on the ratios of 65 kDa to 75 kDa polypeptides of vitronectin.

MATERIALS AND METHODS

Preparation of vitronectin and anti-vitronectin Antiserum. Human vitronectin and anti-human vitronectin rabbit antibody were prepared as described previously (1, 3) or by our recent procedure (17).

Human Serum and Plasma. Human blood was collected from 104 healthy human

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Abbreviation used: kDa, kilodalton.

volunteers, clotted at room temperature for 1 h followed by incubation at 4°C overnight, and centrifuged at 12,000 × g for 2 min in a Beckman Microfuge B. The supernatant (serum) was stored at −80°C. Blood was also collected randomly from outpatients at Jichi Medical School and immediately anticoagulated with 1/10 volume of 3.18% trisodium citrate dihydrate. Plasma was harvested by centrifugation at 3,000 × g for 30 min at room temperature and stored frozen until use.

Quantitative assay of the 75 kDa and 65 kDa vitronectin polypeptides. For quantitative assay of the 75 kDa and 65 kDa vitronectin polypeptides, the indicated amounts of human plasma and serum were subjected to SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (8) after chemical reduction with 0.1 M dithiothreitol. Immunoblotting was performed essentially according to Towbin *et al.* (16). The serum proteins separated in the SDS gel were transferred to a nitrocellulose sheet (Schleicher and Schuell, Inc., type BA-85, 0.45 μm pore size, Keene, New Hampshire) in 5 mM sodium tetraborate at 4°C for 90 min at 90 V in a Trans-blot cell (Bio-Rad Japan, Tokyo). The nitrocellulose sheet was blocked with 0.2% (w/v) skim milk (Yuki-jirushi Milk Co., Tokyo) in phosphate-buffered saline. Vitronectin on the sheet was allowed to react with rabbit anti-vitronectin antiserum at 1/3,000 dilution followed by the second antibody consisting of horseradish peroxidase-conjugated goat antibody against rabbit IgG at 1/1,500 dilution. Color for the bound peroxidase was developed with 34 μg/ml *o*-dianisidine and 0.01% H_2O_2 in phosphate-buffered saline. Two-dimensional densitometry of the 75 kDa and 65 kDa bands on the nitrocellulose sheets was performed with a graphic analyzer Shonic GA (Showa Denko Co., Tokyo) equipped with a personal computer PC-9801F2 (NEC Co., Tokyo) utilizing a custom program termed Sumiko 1. Statistical analysis was performed using the Student's t-test.

RESULTS AND DISCUSSION

Immunoblotting indicated that the patterns of vitronectin polypeptides in human serum could be readily classified into three types characterized by high, medium, or low ratios of 65 kDa to 75 kDa polypeptides (Fig. 1). The two bands migrated at 80 kDa and 72 kDa in our system, but the terms 75 kDa and 65 kDa are retained for consistency with the previous nomenclature. Individual 75 kDa and 65 kDa polypeptides of vitronectin stained by anti-vitronectin antiserum were quantitated by a two-dimensional densitometry of the polypeptide bands on nitrocellulose blots. Figure 2 shows a standard curve expressed as the sum of the 75 kDa and 65 kDa vitronectin polypeptides; the standard deviation was approximately ±10%. The densitometric value increased linearly with the amount of plasma in the range of 25–175 nl, roughly corresponding to 5–35 ng vitronectin. Since staining intensity of the band slightly varied from blot to blot, an internal vitronectin standard of 20 ng was included in every blot in the following experiments.

Systematic quantitative analyses were performed with aliquots of 100 nl of human sera from 104 healthy volunteers as well as 111 nl of human plasma from 100 outpatients. Samples of 202 out of 204 contained detectable amounts of the 65 kDa and/or 75 kDa polypeptides. They were readily classified into three distinct groups according to 65 kDa/(65 kDa + 75 kDa) ratios: ≥ 0 and < 0.35 (type I), ≥ 0.35 and < 0.85 (type II), and ≥ 0.85 and ≤ 1.0 (type III) comprising 58%, 35%, and 5% of the samples tested, respectively (Figs. 1 and 3). This population substantially differed neither between serum and plasma nor between healthy volunteers and outpatients. One of the remaining 2 lots of blood did not contain the 65 kDa or 75 kDa vitronectin, but rather 60 kDa and 52 kDa polypeptides stained by anti-

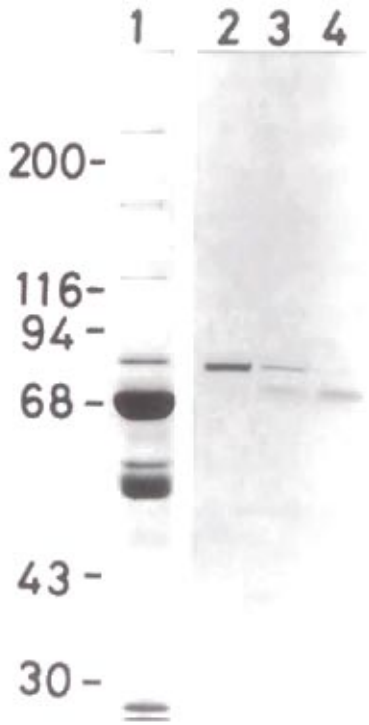

Fig. 1. Immunoblotting of human serum with anti-vitronectin antiserum. Different lots of human sera were immunoblotted and stained with anti-vitronectin antiserum. Samples were chemically reduced with 0.1 M dithiothreitol; typical type I (lane 2), type II (lane 3), and type III (lane 4). Lane 1 shows a typical serum protein pattern stained by Coomassie blue using the same amount of serum protein as for immunoblotting. Size markers (in kilodaltons) are shown at the left.

vitronectin antibody. The other lot of blood contained an approximately 25-fold lower concentration of vitronectin, and the vitronectin pattern was similar to type III. The three vitronectin types did not correlate with age (subjects were between 21 and 65 years), sex, or ABO blood type.

The total concentration of vitronectin in serum and plasma was estimated as the summation of the amounts of 75 kDa and 65 kDa polypeptides. The concentration differed neither between serum and plasma nor between healthy volunteers and outpatients. The means and standard deviations for vitronectin concentrations were 0.206 ± 0.079 mg/ml for type I, 0.196 ± 0.069 mg/ml for type II, and 0.176 ± 0.060 mg/ml for type III, respectively (Fig. 4). However, statistical analysis using the Student's t-test indicated that the difference in the three concentrations was not significant at $p < 0.1$.

The biological importance of the vitronectin type in human serum and plasma is not known, and no functional differences between 75 kDa and 65 kDa polypeptides have been reported. Since the 65 kDa polypeptide appears to be a post-translational cleavage product of the 75 kDa polypeptide (14, 15), it is assumed that the 75 kDa vitronectin is degraded to 65 kDa by some protease in the blood. The ratio of

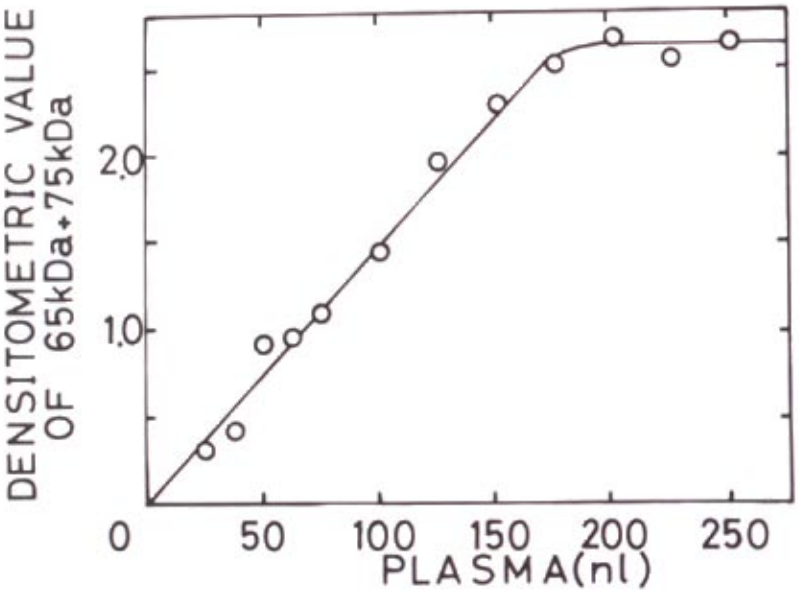

Fig. 2. Standard curve for the vitronectin assay. The indicated amounts of human plasma were immunoblotted. Bands stained by anti-human vitronectin antibody were measured by two-dimensional densitometry and expressed as a summation of the 75 kDa and 65 kDa polypeptides.

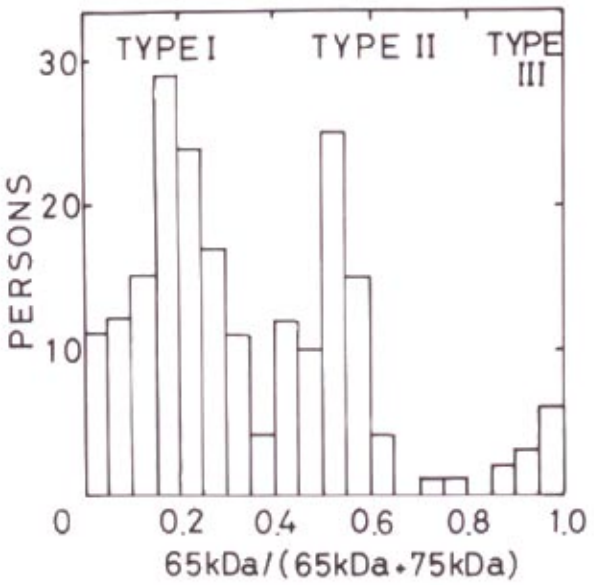

Fig. 3. The three vitronectin blood types. The ratios of 65 kDa/(65 kDa + 75 kDa) polypeptides of vitronectin were measured for 104 healthy human sera and 100 outpatient human plasma samples.

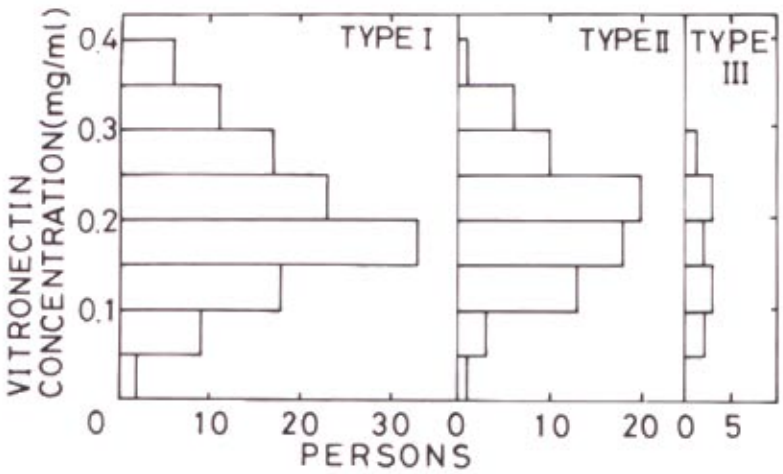

Fig. 4. Total vitronectin concentrations in each of the three blood types. Vitronectin concentrations were determined as a summation of the 65 kDa and 75 kDa polypeptides.

65 kDa/(65 kDa + 75 kDa) polypeptides did not, however, change during storage of serum at 4°C for 3 days, nor at −20°C for at least 6 months with 6 cycles of freezing and thawing.

The vitronectin blood type may reflect the activity of a blood protease cleaving vitronectin 75 kDa to 65 kDa, or the presence of two nonidentical 75 kDa polypeptides, *i.e.*, protease-susceptible and nonsusceptible populations. Future biochemical and biological studies of vitronectin should take into account this potential source of variability.

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参考論文 II

ビトロネクチンの構造と機能

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綜 説

ビトロネクチンの構造と機能

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Structure and function of vitronectin

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Key words: vitronectin, S-protein, blood type, blood coagulation, complement

はじめに

ビトロネクチン（vitronectin）は血漿中の糖タンパク質である．血漿・血清中での濃度は0.3 mg/mlと決して低くないが，精製が難しかったために他の血漿タンパク質に比べ，研究の進行は遅かった．1983年頃から漸く，アメリカのRuoslahtiグループによって分子レベルで解析されるようになった．その後，またたくまに研究が進展し，現在，ビトロネクチンの研究は大きく発展している。ビトロネクチンの機能は，「1細胞を細胞外マトリックスに接着させる」ことが中心に研究されているが，「2血液凝固系の調節」と「3補体作用の調節」にも機能していることが知られている．これら3つの機能が1つの分子に担われている必然性は十分理解できていない．本総説では，ビトロネクチンの構造と機能について，上記2と3の最先端の知識を整理し，かつ，筆者らの研究の取り組みも記述した．なお，「はじめに」ではあるが，お忙しい先生方のために「ビトロネクチンとは何か？」の要点を**表1**にまとめた．

I. ビトロネクチンの発見

1967年，アメリカのHolmes[1]がヒト血清を材料に，ガラスビーズカラムを用いて細胞伸展活性をもつα_1タンパク質を部分精製した．この報告がビトロネクチン研究のはじまりである．α_1タンパク質は，細胞伸展活性ばかりでなく，細胞増殖活性もあり，組織培養用試薬を扱う米国のGIBCO社から「ホルメスα-1プロテイン」として市販された．

ところが，ビトロネクチンの研究はその後約15年，ほとんど発展しなかった．その理由は，1973年頃，箱守仙一郎[2]，Hynes[3]，Yamada[4]，らにより発見された細胞接着性糖タンパク質フィブロネクチン（fibronectin）の陰に隠れてしまったためである．フィブロネクチンは，ビトロネクチンと同じく血清中に含まれる細胞接着性糖タンパク質で，活性がビトロネクチンとよく似ている．また，活性ばかりでなく，血清中濃度も0.2 mg/mlとよく似ている．がん細胞の特性と強く関係し，精製も容易であったため，研究が脚光を浴びて大きく発展した．日本では，1970年代後半，自治医大の松田道生教授[5]が研究の発展に大きく貢献した．

ビトロネクチンの研究は，フィブロネクチン研究の大発展のあおりを受けて，1982年までほとんど注目されなかった．この間，アメリカのGrinnellやBarnes，イギリスのKnoxらのグ

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表1 ビトロネクチンの要点

物　　質	糖タンパク質
別　　名	S-protein, serum spreading factor, epibolin
分 子 量	75,000（一部 65,000 と 10,000 に切断）
存在部位	血漿・血清中に約 0.3 mg/ml，皮膚結合組織
結合分子	ヘパリン，コラーゲン，補体 C5b 7， トロンビン・抗トロンビン III 複合体， β エンドルフィン， インテグリン（$\alpha_v\beta_3$，IIb/IIIa，$\alpha_v\beta_x$）， プラスミノーゲン活性化因子阻害因子-1（PAI-1）
生理活性	1 細胞の基質への接着 2 血液凝固系の調節 3 補体作用の調節
酵素基質	1 プロテインキナーゼ 2 トランスグルタミナーゼ
一次構造	1985 年決定
cDNA 塩基配列	1985 年決定
遺伝子塩基配列	1987 年決定
合　　成	肝臓，肝細胞
疾　　患	肝疾患と相関
医 薬 品	角膜上皮障害の治癒効果あり
市 販 品	精製品，抗体，測定キット

ループの論文が発表されているが，どの論文もビトロネクチンの精製と細胞伸展特性の解明に主力を注いでいる．しかし，どれも結果はシャープではない．したがって，1982 年以前は，ビトロネクチンはまともに精製できていない状況にあったといえる．まともに精製できていないタンパク質に興味深い活性があったとしても，活性がコンタミンのためであることは否定しにくく，信頼度が低かった．

状況の急変は 1983 年に生じた．アメリカの Ruoslahti グループ[6]が，ガラスビーズカラム法で部分精製したヒト血清ビトロネクチンを抗原に，ビトロネクチンのモノクローナル抗体を作成した．この抗体で，抗体-セファロースカラムを作り，ヘパリン-セファロースカラムと組み合わせて，ヒト血漿ビトロネクチンを大量にしかも高純度に精製した．そして，この 1983 年の論文で，ビトロネクチン（vitronectin）と初めて命名した．名前の由来は，このタンパク質が「細胞培養容器のガラス（つまり in vitro の vitro）に細胞を結合させる（つまり nectin）」という特性に基づいている．同年，Barnes のグループ[7]も 4 つのカラムの組み合わせで，ビトロネクチンを精製し，モノクローナル抗体を作成した．なお，Barnes は serum spreading factor と呼び続け，決して vitronectin と呼ばない．一方，Ruoslahti グループは，1983年以後，現在までの短期間のうちに，ドメイン構造の解明，cDNA 塩基配列と一次構造の決定，レセプターインテグリンの発見と cDNA 塩基配列の決定，コラーゲン結合能の発見など，ビトロネクチン分子の基本的特性を次々と発表してきている．したがって，今では，このビトロネクチンという名称が，ほぼ一般的な名称として定着した．

II. ビトロネクチンの構造

1985 年，アメリカの Ruoslahti グループ[8]と西ドイツの Jenne グループ[9]により，ビトロネクチンの cDNA 塩基配列が独立に発表され，ビトロネクチンの全アミノ酸配列が判明した（**図1**）．アミノ酸配列は，ヘモペキシン（hemopexin），コラゲナーゼ（collagenase），トランシン（transin）とホモロジーがあり，ビトロネクチンはこれらのタンパク質と進化的に類縁であると報告された[10][11]．

ビトロネクチンは，表1に示したように，ヘ

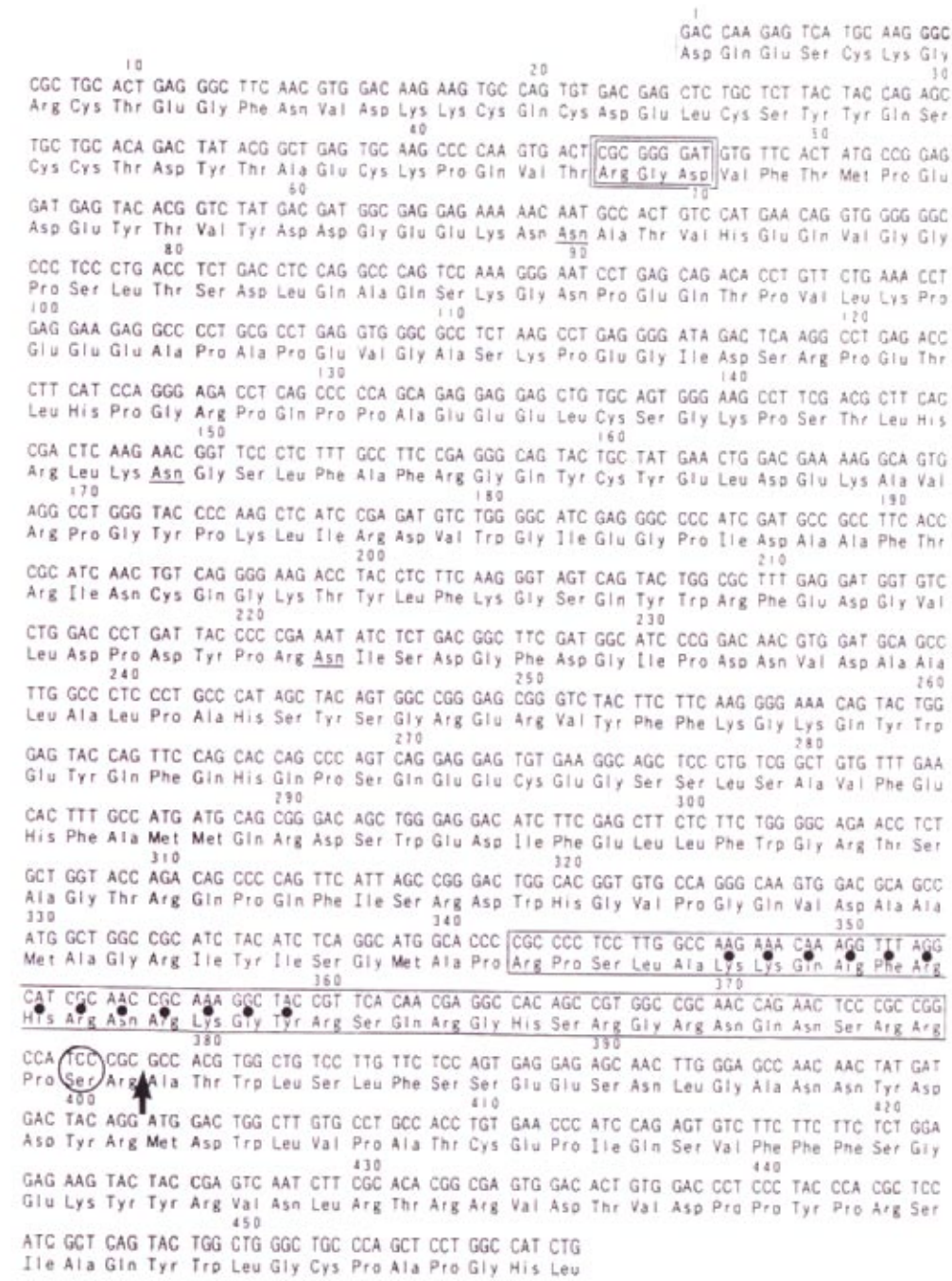

図1 ビトロネクチンの cDNA 塩基配列とアミノ酸配列[9]
N末端から順に表記。二重枠でかこんだ部分は細胞接着に働く RGD 配列，一重枠でかこんだ部分はヘパリン結合部位。詳細は本文参照。

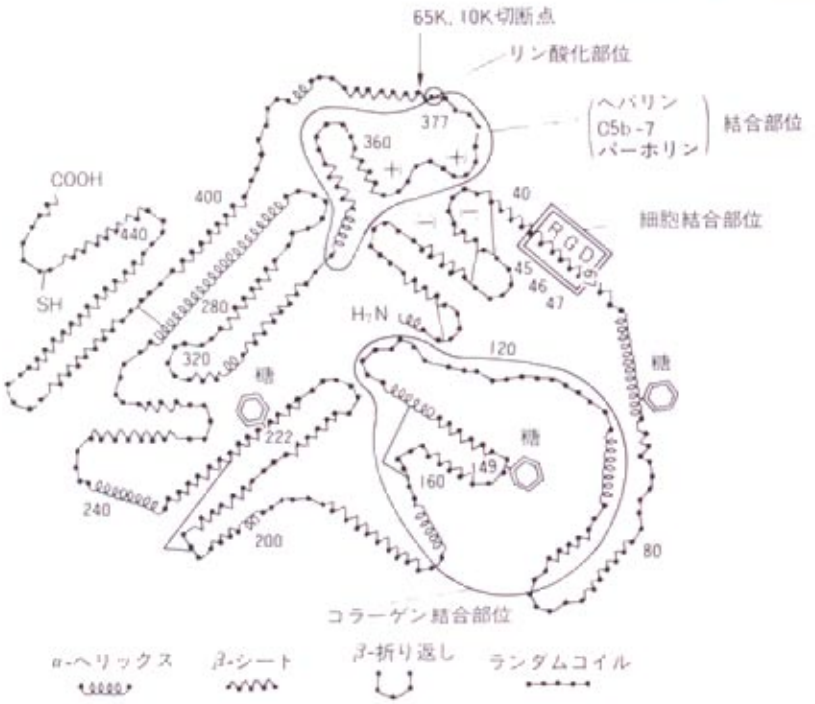

図2　Preissner[12]によるビトロネクチンの二次構造モデル

パリン，コラーゲン，トロンビン-抗トロンビンIII複合体，補体C5b-7，β-エンドルフィン，インテグリン，プラスミノーゲン活性化因子阻害因子-1（PAI-1）などと結合する．その結合部位の分子中での位置を，図1のRuoslahtiグループの一次構造[8]と**図2**のPreissnerグループの二次構造モデル[12]に示す．

ビトロネクチンは，前章で述べたように細胞接着因子である。細胞接着活性を担う部位のアミノ酸配列は，Arg-Gly-Asp（アミノ酸一文字表記でRGD）である．このRGD配列は，図1・図2の二重枠でかこんだ45～47番に位置しており，細胞膜上のビトロネクチンレセプターと相互作用する．ビトロネクチンレセプターを含め，いろいろな細胞接着因子のRGD配列を認識するレセプターは，細胞接着因子の数とほぼ同数の約10種発見されている．このレセプター群はインテグリンファミリー（integrin family）と呼ばれ，現在，研究のホットな分野である[13][14]．

ビトロネクチンには，図1の下線のひいてある67，149，222番のアスパラギンに糖鎖が結合している．Barnesによれば，この糖鎖は，細胞接着に不要である[15]．

図1と図2の一重枠でかこんだ342～375番の部分がヘパリン結合部位である[16]．この部位は，補体複合体C5b-7や細胞障害因子パーホリン（perforin）の結合部位でもある[17]．しかし，不思議なことに，血漿中のビトロネクチンのほとんどはヘパリンに結合しない．この理由は，ヘパリン結合部位が分子中に隠されているためであることを，筆者ら[18]は1985年につきとめた．尿素やグアニジン塩酸でビトロネクチンを処理すると，この結合部位が露出し，ヘパリンと相互作用するようになる．ヘパリン結合部位は，塩基性アミノ酸の含量が高く，正に荷電している．そこで，負に荷電しているN末端側と対を成し，分子の内側に隠れていると予想される（図2）．このヘパリン結合部位の潜在性の意味は，十分理解されていないが，ビトロネクチンの重要な機能調節機構と考えられる．

また筆者ら[19]は，コラーゲン結合部位がRGD配列とヘパリン結合部位の間に位置することを明らかにした．このコラーゲン結合部位も潜在

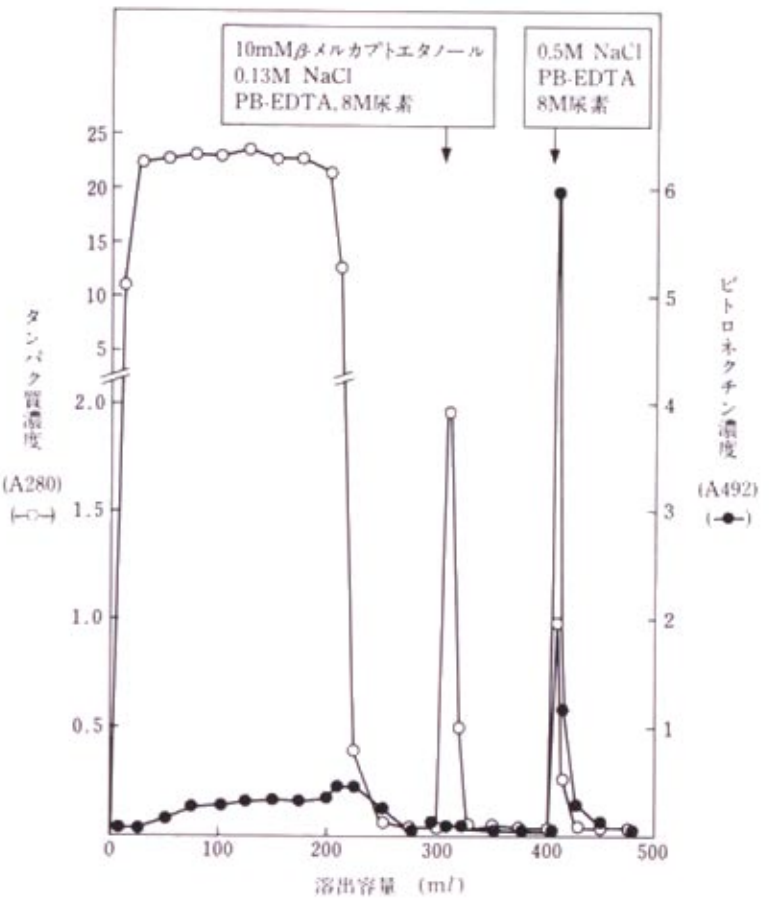

図3 ヒト血漿のヘパリンセファロースカラムによるビトロネクチンの精製[23]
PB-EDTA：10mMリン酸緩衝液(pH7.7)，5mMEDTA

性と思われ，図2にその推定位置を示した．さらに，McGuire[20] によると，図1の丸でかこんだ377番のセリンがリン酸化される．また，図1・図2ともに矢印で示した378，379番の間に，ポリペプチド鎖の切断点があり，C末側10Kのフラグメントができる[16]．IV章に示すビトロネクチン血液型は，この切断点と関係深い．トロンビン-抗トロンビンIII複合体，プラスミノーゲン活性化因子阻害因子-1，セロトニン，β-エンドルフィンなどの結合部位は，まだ決定されていない．今後，決定され，この構造図中に書き加えられるだろう。

III. ビトロネクチン精製法

ビトロネクチンの研究の流れの中で，精製法の確立が重要であったことをI章で述べた．1983年のRuoslahtiグループ[6]，Barnesグループ[7]の方法は重要ではあったが，しかし，決定的ではなかった．1985年にPodack[21]やPreissner[22]達も開発したが，それでもまだ多くの問題を抱えている．1つの問題点は，ビトロネクチンの回収率の悪さである．どの方法に依っても，ビトロネクチンは数%～10数%しか回収されない．筆者の研究室では，Barnesの方法に従い，ビトロネクチンの精製を試みたが，彼らが論文で報告している回収率さえ得られなかった．第2の問題点は，精製に多くのステップを要し，操作に時間がかかるということ

である．例えば，Podack の方法は，クエン酸バリウム沈殿，DEAE-セファセルカラム，ブルーセファロースカラムを含め，6段階を要する．第3の問題点は，Ruoslahti グループの抗ヒトビトロネクチンモノクローナル抗体カラムを用いた場合に生ずる．この方法では，操作が簡略化され，回収率が上がるという利点はある．しかし，ヒト由来品しか精製できない欠点とモノクローナル抗体が高価（例えば，より安価な国産の岩城硝子製品でも 50 μg で5万円）なため汎用性が低い欠点がある．

筆者の研究室は，このような欠点を解決すべく，新しい精製法を開発した[23]．新しい精製法は，「尿素処理していないビトロネクチンはヘパリンへの親和性を示さないが，尿素処理すると親和性を獲得するようになる[18]」というビトロネクチンの性質に着目して考え出された．この性質を利用すれば，わずか2回のカラム操作だけで，ビトロネクチンを約2日のうちに数 mg の収量（回収率は20～30%）で精製することができる．

その新精製法を以下に簡単に述べる．血清を生理条件下でヘパリンカラムに通し，ヘパリン親和性をもつタンパク質を除く．血清中ビトロネクチンの数%はヘパリン親和性を持ち[24]，この操作で除かれるが，70～98%のビトロネクチンは通過画分に回収される．その通過画分に終濃度 8 M になるよう尿素を加え，室温で2時間放置する．尿素処理した試料を再度ヘパリンカラムにかけると，**図3**のような溶出パターンが描ける．図3は，ヒト血清 100 ml（尿素処理時 300 ml）から出発したヘパリンカラムの溶出パターンである．タンパク質の溶出を○印で，ビトロネクチンの溶出を●印で示した．添加した試料タンパク質のほとんどは，ヘパリンに結合しない．さらに 10 mM β-メルカプトエタノール入り緩衝液でカラムを洗う．最後に，0.5 M NaCl を含む緩衝液でビトロネクチンを溶出する．この方法に従って精製したヒトビトロネクチンの SDS 電気泳動像を図4の一番左のレーンに示す．75K と 65K の2本のバンドが見られ，高純度の精製ビトロネクチンであることがわかる．

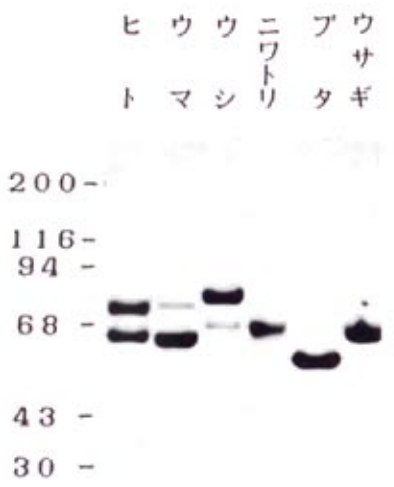

図4 精製動物ビトロネクチンの SDS 電気泳動像[25]
左側の数字と棒は分子量マーカーの分子量（K）と移動位置．

上記の精製法は，操作の煩雑さを克服しただけでなく，どのような動物種にも適用できるという利点を兼ね備えている．回収率も20～30%とかなり向上した．しかし，回収率は 100% 近くが望ましいし，今後，8 M 尿素や還元剤を使わない方法の検討も必要であろう．動物種に関しては，ヒトを含め，ウマ，ウシ，ニワトリ，ブタ，ウサギの6動物種のビトロネクチンがこの方法により精製できた．精製した各種動物ビトロネクチンの SDS 電気泳動像を**図4**に示す．驚いたことにビトロネクチンは動物種により分子量が顕著に異なる．また，ブタとウサギではバンドの数が1本であった．分子量は図4の左より，ヒト 75K と 65K，ウマ 75K と 64K，ウシ 78K と 68K，ニワトリ 78K と 68K，ブタ 56K，ウサギ 68K である．これらの分子のアミノ酸組成，N 末端側 20 残基のアミノ酸配列，糖組成を調べた．予想通りペプチド部分に高いホモロジーが見られた．しかし，意外なことに糖組成は動物種特異的に多様であった[25]．

IV. ビトロネクチン血液型

ヒトビトロネクチン分子の一部は，図1，図2に示したカルボキシル末端側 10K の位置で切れている．つまり，血漿中のビトロネクチン

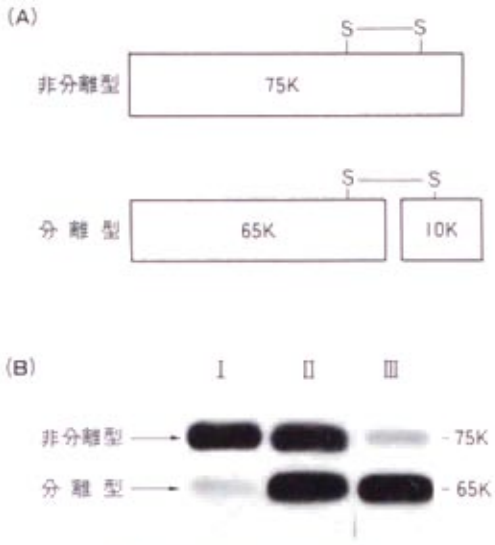

図5 ピトロネクチン血液型

（A）ピトロネクチンの非分離型と分離型のモデル，（B）I，II，III型のヒト血漿をピトロネクチン抗体でイムノブロットした像．

は，**図5**(A)に示すように切断のおこっている分離型と切断のおこっていない非分離型の2つのタイプの分子が混在している．そのため，ヒト血漿を還元剤存在下で電気泳動し，イムノブロットすると，ピトロネクチンは，75Kと65Kの2本のバンドとして検出される．この時，10Kは小さすぎるためか検出されない．このシステムで，健常者104人の血漿をイムノブロットすると，どの人のピトロネクチンも75Kと65Kの2本のバンドとして検出されるが，分離型を多く持つ人もいれば，非分離型を多く持つ人もおり，75Kと65Kの量比が一定ではない．そして，分離型と非分離型の量比により，統計的に3つのグループに分類できた．図5(B)の左端のような分離型の多いI型，中央のような分離型と非分離型がほぼ同量のII型，右端のように分離型の多いIII型という3つのピトロネクチン血液型である．I型は58%と最も多く，続いてII型が35%，III型は5%であった[26]．自治医大・松田道生教授の協力により，自治医大付属病院への外来患者100人についてピトロネクチン血液型を調べたが，疾患との関係は否定的であった．

では，ピトロネクチン血液型を生み出すものは何だろうか．2つの可能性が考えられる．第1は，ピトロネクチンを非分離型から分離型へ変化させるプロテアーゼがあり，その酵素量により型が決まるとする考え．第2は，ピトロネクチンの対立遺伝子として，非分離型と分離型の2種があり，この2種のピトロネクチン遺伝子の組み合せが人により異なっているために3つの型ができるとする考えである．

表2 ピトロネクチン血液型の分布

型	筆者ら[26]	Mosher 研[27]	Mosher の手紙
I	58%	18%	54%
II	35	59	39
III	5	22	6
対象人種	日本人	白人	米国在住中国人

筆者らより少し遅れてアメリカのMosherグループ[27]も筆者らと全く同じピトロネクチン血液型の存在を報告した．彼らの報告した血液型の分布は，筆者らの報告とは異なり，II型が一番多く59%で，I型は18%，III型は22%であった（**表2**）．Mosherらは，集団遺伝学的な解析から，この血液型は遺伝的に決まっているものであるとしている．筆者らの型分布との相違を気にして，Mosherは，1988年夏に筆者に手紙を書いてきた．その手紙によると，アメリカのウィスコンシン州マジソン市に住む中国人大学院生を対象に型を調べたところ，I型54%，II型39%，III型6%となり，筆者らの結果とよく似ているという．この結果は，ピトロネクチン血液型の分布が，人種に依存しているということを示している．

Mosherの結果も合せて考えると，ピトロネクチン血液型が生じる原因は，ピトロネクチン遺伝子が多型であるためと思われる．しかし，75Kと65Kは遺伝子塩基配列上の意味のない多型を反映しただけと言い切れるだろうか？というのは，75Kと65Kの分子の間で，トリプトファンとの結合[28]やリン酸化[29]に違いがみられ，機能的な差が示唆されている．今後，2つの型の機能的な差がより明確になれば，ピトロネクチン血液型に生理的意味があるのかどうかも明らかになるだろう．

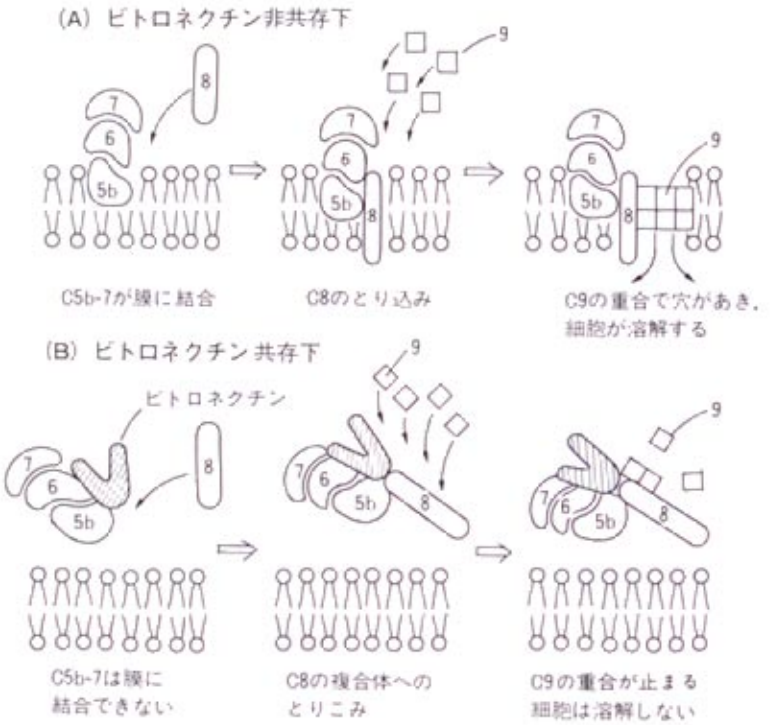

図6　ビトロネクチンによる補体 C5b-7 の細胞溶解の阻害[30]
図中の番号は，補体成分の番号．

V．補体とビトロネクチン

補体系は，9種の血清タンパク質が担う生体防御反応系の1つである．補体は，抗体などの活性化分子を表面に持つ異物や老化細胞に細胞融解をひきおこす．その融解過程を**図6**(A)に示す．細胞融解は，補体成分の C5b，C6，C7，C8，C9 からなる膜侵襲複合体（membrane attack complex：MAC）が細胞膜に穴を形成して生ずる．C5b，C6，C7 から成る複合体 C5b-7 は疎水性が高く，膜に強く結合する．C5b-7 は C8 を結合し，さらに12～18個の C9 を重合して MAC を形成する．1977年，Podack ら[29]により，MAC 形成を阻害するタンパク質としてSタンパク質（S-protein）が発見された．Sタンパク質は，ビトロネクチンと同一分子であることが1985年に判明した[9]．

ビトロネクチンは，図6(B)に示すように MAC 形成の中間産物である C5b-7 に結合し，ビトロネクチン・C5b-7 複合体を作る[29]．ビトロネクチン・C5b-7 は親水性であるため，膜に結合できず，液相にとどまる．さらにビトロネクチンは，C9 の重合も阻害し，2～3分子の C9 しか重合をおこさせない[30]．このようにして，ビトロネクチンは MAC 形成を阻害する．

一方，補体系とは異なるが，C9 の重合に似たメカニズムで働くタンパク質にパーホリンがある．パーホリンは，細胞障害性Tリンパ球の細胞障害因子で，C9 のように重合して膜に穴をあける．ビトロネクチンは，このパーホリンの重合も阻害することが，Tschopp ら[17]により報告された。

ビトロネクチンの補体に対する阻害作用は，ヘパリン結合部位が担う．最近，ヘパリン結合部位の中のさらに13残基のアミノ酸（図1の●印で示した347～359番）が重要であることが報告された[17]．13残基から成る合成ペプチド

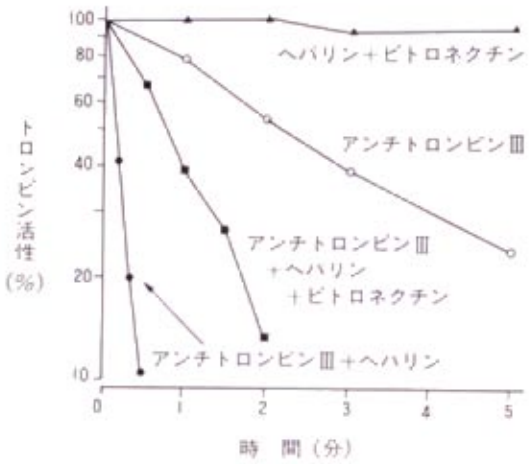

図7 ビトロネクチンによるトロンビンの保護[27]．
0分での凝固活性を100%とした時のトロンビン凝固活性の変化を経時的に示してある．グラフの傾きが大きい程，トロンビンへの阻害が強い．アンチトロンビンIII・ヘパリンは，グラフの傾きを大きくし，強く阻害をかける．ビトロネクチンが存在すると，その傾きは，緩くなり，ビトロネクチンが，アンチトロンビンIIIの作用を中和していることがわかる．

が，パーホリンやC9の重合を阻害する．この13残基のアミノ酸のうち，7残基は塩基性で，正に帯電している．逆に，C9やパーホリン分子中には，酸性アミノ酸に富む領域があり，そこがビトロネクチンとの結合に関係しているらしい．

VI．ビトロネクチンと血液凝固

血液凝固系は，血液中の約10種のプロテアーゼの活性化と阻害により複雑に進行する．そして最終的には，トロンビンによるフィブリノーゲンの分解でフィブリンが形成される．アンチトロンビンIII（ATIII）は，トロンビン阻害因子の1つである．**図7**に示したように，トロンビンのみでは，トロンビンの凝固活性は維持されるが，ATIIIが共存すると，トロンビンの活性は阻害される．さらに，ヘパリンが共存する場合には，ATIIIの阻害作用が顕著に促進される．ところが，ビトロネクチンが存在すると，ATIIIによる阻害が中和され，トロンビンの活性は維持される[21)31)32)]．このビトロネクチンの中和活性は，ヘパリン共存下でのみ見られ，ヘパリンのない時には，ビトロネクチンは何の影響も与えない．ビトロネクチンの作用の仕組みは，ビトロネクチンが溶液中のヘパリンを奪い，ヘパリンによるATIIIの活性化をおこさせないためである．つまり，ATIIIが活性化されないので，トロンビンは活性を失わない．Preissnerら[12)]は，さらに，血液凝固因子 X^a のATIIIによる阻害もビトロネクチンが中和することを明らかにした．ビトロネクチンの中和活性は，補体と同様，ビトロネクチン分子中の347～359番に相当するアミノ酸13残基の合成ペプチドにより阻害される．つまり，ヘパリン結合部位がこの中和活性を担っていると結論された．

阻害活性とは別に，ビトロネクチンは，ビトロネクチン・トロンビン・ATIII複合体(STAT)を形成する[32-35)]．STATは，血清中に存在するが，血漿中には存在しない．つまり，血液凝固の過程で生ずる複合体である．しかし，ヘパリンが存在しない条件下でも，STATは形成されるので，前述したATIIIの阻害中和活性とは別の事象としてとらえられている．この複合体形成の生理的意味は不明であるが，以下のような可能性がある．トロンビン・ATIII複合体を血液から回収する際に，STATを形成していると，ビトロネクチンの部分で細胞に結合できるため，効率良く回収できるのかもしれない．また損傷のおこった場所にSTATが，ビトロネクチンを介して接着し，トロンビンにより細胞増殖をひきおこすという，凝固系とは異なる作用をしているのかもしれない．さらに，STATは，トロンビンをATIIIから保護し，内皮細胞表面のトロンボモジュリンに活性トロンビンを引渡す可能性もある．トロンビン・トロンボモジュリン複合体は，プロテインCを活性化し，その結果，Va因子とVIIIa因子を不活性化することで，強い凝固反応の停止と線溶系の開始をもたらすことが知られている．

一方，ビトロネクチンは，プラスミノーゲン活性化因子阻害因子-1（PAI-1）結合タンパク質であることが，最近，いくつかの研究室から相次いで報告された．線溶系の中心的タンパク質分解酵素プラスミンは，プラスミノーゲン活

性化因子（PA）により活性化される．PAを阻害する生体因子は，4種類あり，プラスミノーゲン活性化因子阻害因子（PAI）-1，2，3とタンパク質分解酵素ネキシンである．これらの中で，中心的役割を担うPAI-1は，血漿，血小板，胎盤，血管内皮細胞に存在する．このPAI-1に結合し，その活性を制御するタンパク質の存在が示唆され，注目を集めていた．そして，1988年末から1989年にかけて5つのグループにより[36)~40)]，このタンパク質がビトロネクチンであると同定された．PAI-1に結合すると，ビトロネクチンはPAI-1を活性化し[40)]，また，PAI-1の活性を安定化する[36)40)]．ビトロネクチンとPAI-1の複合体形成で，PAI-1はビトロネクチンの細胞接着活性を阻害せず，ビトロネクチンはPAI-1のPA阻害活性を阻害しない[39)]．したがって，PAI-1はビトロネクチンと複合体を作ることで，ビトロネクチンを足場に細胞外マトリックスに取り込まれる[38)]．つまり，この複合体形成はPAI-1を血中から速やかに回収することに役立っているのかもしれない．Pöllänenら[41)42)]やHébertら[43)]は，細胞の接着部域にPAが局在することを発見しており，ビトロネクチン・PAI-1複合体は，組織内でプラスミンの活性を調節することができそうである．その場合，細胞外マトリックスの分解，血栓溶解，がんの浸潤，細胞移動などをビトロネクチンが制御している可能性が高い．

おわりに

ビトロネクチンの生化学的研究の現状を紹介した．ビトロネクチンという名前の由来である細胞接着機能に関しては余りふれなかったが，ビトロネクチンを含め，「細胞接着因子」研究は，急成長の研究分野として大きな注目を集めている．和文[44)]，英文[45)]の雑誌特集号や総説も出版されている．精製品，抗体，定量キット，特異的な阻害ペプチド，レセプター抗体などは数社から市販されており，下記の番号に電話し，カタログを入手されるとよいだろう．

岩城硝子（03-214-6221），和光純薬（03-270-8571），ケミコン Chemicon（米国）（コスモ・バイオ 03-663-0723とフナコシ薬品 03-295-5548が取扱），テリオス Telios（米国）（東洋紡 03-660-4819が取扱）．

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POLYMORPHISM OF THE HUMAN VITRONECTIN GENE CAUSES VITRONECTIN BLOOD TYPE

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Summary: Human blood plasma/sera are classified into three distinct vitronectin types based on the relative amount of the 75 kDa polypeptide to its cleavage product of 65 kDa. We asked whether the vitronectin blood types correlated with the polymorphism of the vitronectin gene. A portion of the vitronectin gene was amplified by using polymerase chain reaction and digested with a restriction enzyme PmaC I which may distinguish the base sequence causing the polymorphic change at the amino acid position 381. Amplified DNAs of the blood type I (75 kDa-rich), II (75/65 kDa-even), and III (65 kDa-rich) were shown to be resistant, moderately sensitive and completely sensitive to PmaC I, respectively. These results suggest that Thr at position 381 is essential for the cleavage of the vitronectin 75 kDa polypeptide and that three possible combinations of two codominant alleles of vitronectin determine three vitronectin blood types.

Vitronectin (1) also termed S-protein (2 - 4) and serum spreading factor (5) is a blood glycoprotein having cell-adhesion activity and modulating blood coagulation and complement action. It is frequently observed as a mixture of 75 kDa and 65 kDa bands in SDS-polyacrylamide gel electrophoresis under reducing conditions (1, 3 - 7). The 65 kDa polypeptide is an endogenous fragment of the 75 kDa polypeptide probably cleaved between Arg-379 and Ala-380 (6). In our previous report (7), human blood plasma/sera are found to be classified into three distinct vitronectin types concerning the relative amount of the 75 kDa polypeptide to the 65 kDa polypeptide. Type I is 75 kDa-rich and 65 kDa-poor, type II contains approximately equal amount of 75 kDa and 65 kDa polypeptides, and type III is 75 kDa-poor and 65 kDa-rich. Conlan

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et al (8) independently found the same fact and suggested that vitronectin blood type is inherited and appears to be due to two codominant alleles on an autosome. It is likely that the two vitronectin alleles encode the protease-sensitive 75 kDa (producing 65 kDa) and resistant 75 kDa polypeptide (not producing 65 kDa), respectively. No physiological or clinical difference in the vitronectin blood type has been reported.

Suzuki et al (9) and Jenne and Stanley (2) independently determined cDNA sequence of human vitronectin. Their sequence data are almost identical, but some minute discrepancies are observed. One discrepancy is the amino acid residue (and base sequence) at position 381, which is reported to be Thr-381 (ACG) by Suzuki et al (9) or Met-381 (ATG) by Jenne and Stanley (2). Since the position 381 is the nearest neighbor to the possible cleavage point, we examined whether the position 381 (the nucleotide position 4056) was really polymorphic and related to the vitronectin blood type. This paper describes for the first time that the nucleotide position 4056 is really polymorphic and determines vitronectin blood type.

MATERIALS AND METHODS

Western Immunoblotting. Western immunoblotting was performed essentially according to Towbin et al (11) as described previously (7). Plasma proteins electrophoresed in SDS-polyacrylamide gel were transferred to a nitrocellulose sheet with 5 mM sodium borate for 60 min at 150 mA using a semi-dry blotter Horize blot (No. AE-6670, Atto Inc., Tokyo, Japan). The nitrocellulose sheet was blocked with 0.2 % (w/v) skim milk in phosphate-buffered saline. Vitronectin on the sheet was detected with anti-vitronectin antiserum and horseradish peroxidase-conjugated second antibody. Each vitronectin band was quantitated by two dimensional densitometry as described (7).

Polymerase Chain Reaction (PCR). PCR was essentially performed as described by Saiki et al (12). Genomic DNA was isolated from human blood leukocytes as described (13). One μg of DNA was amplified by using the DNA amplification reagent kit in 100 ul solution containing 2.5 units Taq polymerase and each primer of 30 pmole. The primers possessed the nucleotide sequence of the number 3693 - 3712 and 4222 - 4203 for human vitronectin gene from Jenne and Stanley (10). DNA in the PCR mixture of 50 ul was digested with PmaC I for 1 h at 37°C. The digest was electrophoretically separated in 4 % NuSieve GTG agarose gel for 1.5 h at 100 mA, and DNA was visualized with ethidium bromide. Hap II fragments of pUC18 DNA and Sty I fragments of λ phage DNA were used as molecular size markers.

RESULTS

Blood plasma were obtained from healthy donors showing three vitronectin blood types, I, II, and III, respectively. Ratios of vitronectin 65 kDa/(75

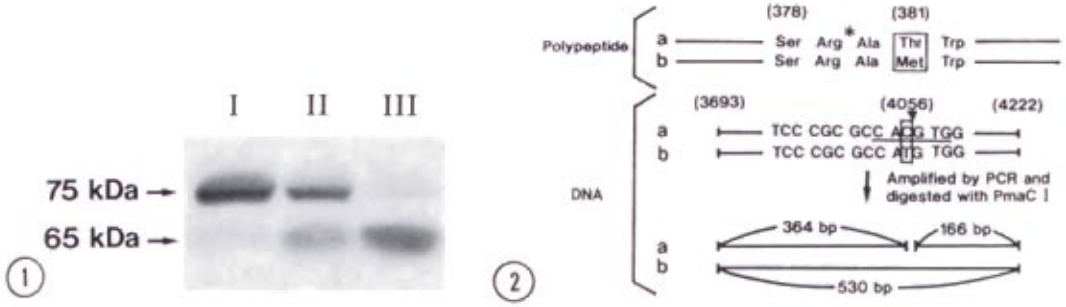
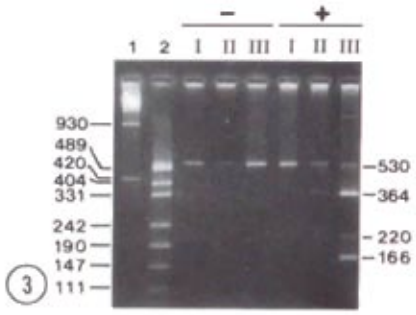


Fig 1. Western immunoblotting of human plasma of each vitronectin blood type. Human plasma was electrophoresed in SDS-polyacrylamide gel, transferred onto a nitrocellulose sheet, and stained for vitronectin with anti-vitronectin antibody. Lanes I, II, and III were showing vitronectin blood type I, II, and III, respectively.

Fig 2. The strategy to test whether polymorphism of vitronectin gene corresponds to vitronectin 75 kDa and 65 kDa polypeptides. Suzuki et al (9) and Jenne and Stanley (2) reported DNA (and polypeptide) sequences a and b, respectively. The nucleotide at position 4056 and corresponding amino acid at position 381 were different between their data as enclosed by rectangles. An asterisk indicates the possible site at which 75 kDa vitronectin polypeptide is cleaved to 65 kDa and 10 kDa polypeptides by an unknown endogenous protease(s) (6). PmaC I specifically recognizes CACGTG (underlined) but not CATGTG and cleaves it at the site indicated by an arrowhead. The presence of 364 bp and 166 bp in the PmaC I digests indicates the presence of DNA a, and in consequence polypeptide a.

kDa + 65 kDa) were 0.11 for type I (Fig 1, lane I), 0.51 for type II (Fig 1, lane II), and 0.92 for type III (Fig 1, lane III). Approximately 10 % of total vitronectin was 75 kDa in type III and conversely 65 kDa in type I. Total concentrations of vitronectin in their plasma did not considerably differ among the three types in agreement with our previous report (7).

Vitronectin blood type is inherited and possibly due to two codominant vitronectin alleles (8). Since the 65 kDa polypeptide is a cleavage product of the 75 kDa one, it seems likely that the polymorphic change of the amino acid residues at or near the cleavage site affects the sensitivity of vitronectin to a protease(s) and causes different blood types. Sequence data of vitronectin cDNA by Suzuki et al (9) and Jenne and Stanley (2) were different each other at position 381, the nearest neighbor to the cleavage site, suggesting that the position 381 is polymorphic. We thus asked whether there was a correlation between the polymorphic change at position 381 and the vitronectin blood types. Leukocyte DNA was isolated from the same lot of blood used for Fig 1, and analyzed as illustrated in Fig 2. Polynucleotides



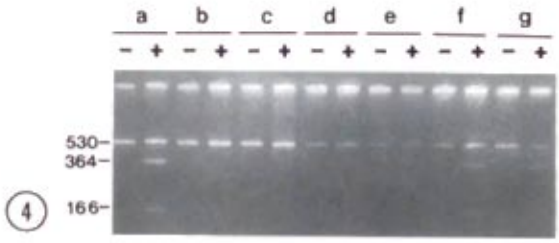


Fig 3. PmaC I digestion of typical vitronectin DNA amplified by PCR. Vitronectin DNA of 530 bp from 3693 to 4222 in the nucleotide number from Jenne and Stanley (10) was amplified by PCR. The amplified DNA was treated with or without PmaC I for 1 h at 37°C as indicated by + or -. Lanes I, II, and III were PmaC I digests prepared from the same blood showing vitronectin blood type I, II, and III in Fig 1, respectively. Size of the digests in bp was shown at the right. Molecular size markers of Sty I fragments of λ DNA (lane 1) and Hap II fragments of pUC18 DNA (lane 2) were shown at the left.

Fig 4. PmaC I digestion of vitronectin DNA from further seven individuals. PmaC I treatment is indicated by + or -. Size of the digests in bp was shown at the left. The ratio of 65 kDa/(75 kDa + 65 kDa) and vitronectin blood type of seven individuals were a (0.58, II), b (0.29, I), c (0.23, I), d (0.29, I), e (0.29, I), f (0.57, II), and g (0.44, II).

of 530 base pairs (bp) spanning the nucleotide numbers 3693 - 4222 were amplified by PCR using two 20-mer polynucleotides of the nucleotide numbers 3693 - 3712 (ATTCGGACAGAGTCAAAGGC) and 4222 - 4203 (TGGGTTCGATCTGGTCGAAG) as primers. A restriction enzyme PmaC I which recognizes CACGTG but not CATGTG cleaved extensively the amplified type III DNA into 364 bp and 166 bp, moderately the type II DNA, but not at all the type I DNA (Fig 3). Digested fragments in Fig 3 fitted the scheme in Fig 2, except a band of about 220 bp. These results indicate that vitronectin having Thr-381 was protease-sensitive but vitronectin having Met-381 was protease-resistant, and that the combination of the two polypeptides determined the vitronectin blood type. The 220 bp band may be an artifact of PCR, because the band was often but not always found in the amplified samples, regardless of PmaC I digestion. We further analyzed seven individuals having the blood type I or II (the type III is rare). As shown in Fig 4, the results were well consistent with those of Fig 3. The polymorphism at position 381 was thus correlated with the vitronectin blood type. Thr-381 derived from the codon ACG at the nucleotide number 4055 - 4057 (allele VIN Thr-381) should make the 75 kDa polypeptide

protease-sensitive, while Met-381 from the codon ATG (allele VIN Met-381) makes the polypeptide protease-resistant. From these results, we concluded that the combination of vitronectin codominant alleles VIN Thr-381 and VIN Met-381 determined the vitronectin blood type.

DISCUSSION

Vitronectin blood type was suggested to be due to two codominant vitronectin alleles, one of which (VIN Thr-381) has C and the other (VIN Met-381) T at the nucleotide position 4056. The former base has been reported by Suzuki et al (9) and the latter by Jenne and Stanley (2, 10). The base change from C to T causes the amino acid change of Thr to Met at the position 381. Allele VIN Thr-381 encodes vitronectin 75 kDa which is protease-sensitive while allele VIN Met-381 encodes protease-resistant vitronectin. The 75 kDa vitronectin may be efficiently cleaved at the position between Arg-379 and Ala-380 when the residue at position 381 is Thr, but not when it is Met. The sensitivity of the Thr-381 type to a protease(s) and the resistance of the Met-381 type seem, however, not to be absolute, because approximately 10 % of vitronectin 75 kDa was found in type III and conversely 11 - 29 % of the 65 kDa in type I. The protease(s) responsible for the cleavage is still unknown.

McGuire et al (14) reported that a protein kinase in human plasma preferentially phosphorylates 75 kDa vitronectin in the presence of 75 kDa and 65 kDa vitronectin. They determined the amino acid sequence around the phosphorylated site as [PO_4]Ser-Arg-Ala-Thr, in which the last Thr is Thr-381. It is unclear at this time why the 75 kDa vitronectin having Thr-381 is not cleaved in their preparation.

Many preparations of vitronectin contain equal amount of 75 kDa and 65 kDa polypeptides (1, 3 - 7), even it is purified from 75 kDa-rich plasma. It has been thought that 65 kDa vitronectin is selectively recovered during purification, because 65 kDa vitronectin preferentially binds to tryptophan-Sepharose and heparin-Sepharose (15, 16). We have, however, frequently observed that 75 kDa vitronectin is apt to be cleaved to 65 kDa during

chromatographic purification. Polymorphism of vitronectin gene is the most important factor to determine the vitronectin blood type, but the relative amount of the 75 kDa to the 65 kDa might be also affected by other unknown factors such as conformational change or modifications of vitronectin.

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ビトロネクチン

淀田　歴林　正男

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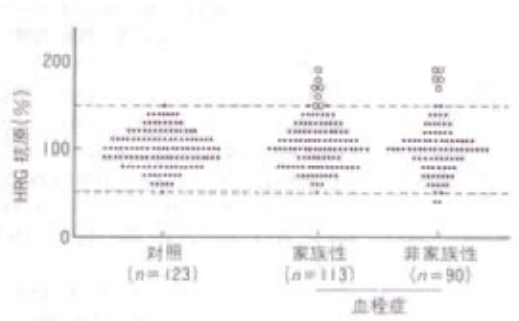

図4　血漿 HRG 濃度と血栓症

血漿中の HRG 抗原量(●)を 10 %間隔で示してある．破線は正常値範囲(56～145 %)を示す．正常値範囲の上限よりも高値を示した血栓症患者の値を○印で示してある．(文献 4 より和訳して引用)

究の結果からも支持されるものであり，HRG の生理機能確立の糸口として今後の研究に期待したい．

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ビトロネクチン

窪田　歴[*1]・林　正男[*2]

はじめに

ビトロネクチン(vitronectin)[1]は，フィブロネクチンと同様の細胞接着活性を持つ血漿糖蛋白質である．1985 年，一次構造が決定され，免疫補体系の調節蛋白質 S プロテインと同一分子であることが明らかとなった．血漿中に 0.3 mg/m*l* とかなり高濃度で存在しているほか，結合組織，細胞外マトリックス，血小板にも存在する．ビトロネクチンの分子量は 75,000 であるが，通常，分子の一部が切断された，分子量 65,000 の分子種と混在している[2,3]．

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■ 分子的性状

ビトロネクチンは，さまざまな分子と結合することが知られている．例えば，細胞膜上レセプターのインテグリン(integrin)，血液凝固系に関与するヘパリン，トロンビン・アンチトロンビンIII 複合体(TAT)，線溶系に関与するプラスミノゲンアクチベーターインヒビター(活性化因子阻害因子)-1(PAI-1)，免疫補体系の膜侵襲複合体(membrane attack complex；MAC)，内因性オピオイドペプチドの β-エンドルフィンなどと結合する．これらの結合によって，ビトロネクチンはさまざまな機能を果たしていると思われる．

■ 機　能

現在までにわかっているビトロネクチンの三つの機能について述べる．

1．細胞の接着・伸展

ビトロネクチンの細胞接着・伸展活性は，N 末端近くにある RGD 配列と細胞膜上のインテグリンとの相互作用によるものである[4]．RGD 配列とは，アルギニン-グリシン-アスパラギン酸のペプチド配列をアミノ酸一文字表記したもので，ビトロネクチンをはじめ多くの細胞接着性蛋白質の接着活性を担う共通配列として知られている．細胞がインテグリンを介して基質上のビトロネクチンと結合すると，そこを足場に細胞内のアクチン繊維が配向し，細胞伸展形態をとる．

また，血小板が活性化されると，血小板中のビトロネクチンが放出され，血小板膜上のインテグリン(糖蛋白IIb-IIIa)と相互作用をする．ビトロネクチンは，おそらく血小板凝集にも機能しているであろう．

2．免疫補体系の調節

補体系は，九つの補体成分が異物や老化細胞を認識して細胞融解を引き起こす生体防御機構の一つである．細胞融解の最終段階では細胞膜に C5b，C6，C7，C8，C9 から成る MAC が結合する．ビトロネクチンが MAC に結合すると，MAC は膜に結合できなくなり，細胞融解が阻害される．さらに，C9 やパーホリンが膜上に重合して穴を開ける過程も，ビトロネクチンが阻害する．これらの阻害には，ビトロネクチンのヘパリン結合部位が機能している[5]．

3．血液凝固系・線溶系の調節

ヘパリンは，アンチトロンビンIII(ATIII)のトロン

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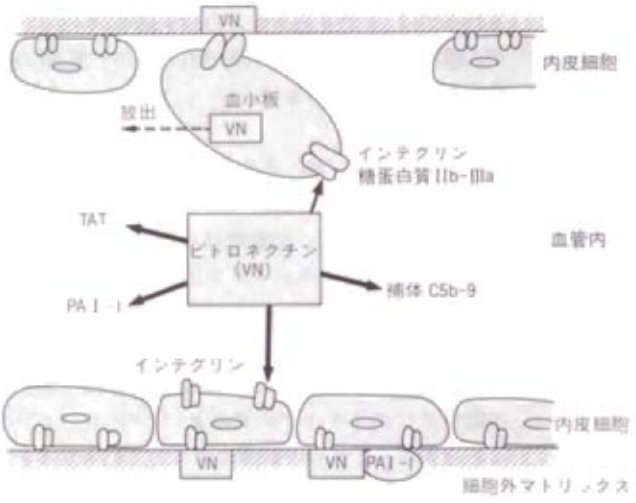

図5　血管内でのビトロネクチン機能モデル

ビン阻害を促進する抗凝固因子である．ビトロネクチンはヘパリン結合能があり，ATIIIと競合してヘパリンを奪い合う．その結果，ATIIIへのヘパリンの効果を減少させ，ATIIIの活性を阻害する[6]．

また，ビトロネクチンはプラスミン生成を阻害する因子PAI-1とも結合する．この結合は，PAI-1の活性化と安定化に役立っており，ビトロネクチンによる線溶系の調節を示唆している[7]．さらに，PAI 1の細胞外マトリックスへの取り込みは，ビトロネクチンを介したプラスミンの作用調節系かもしれない．

おわりに

いままで述べた機能を中心に，ビトロネクチンが血管という場で働く際のモデルを図5に示した．ビトロネクチンは，一つの分子で三つの機能を併せ持つ．これは炎症などの場で三つの機能を同時に引き起こす必要があるときに，効率よく働くための仕組みに違いない．

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トロンボスポンジン

安藤泰彦*

■ 正体の解明

トロンボスポンジン(thrombospondin)は血小板 α 顆粒に大量に貯蔵されている糖蛋白で，血小板総蛋白量の3％を占めている．血漿中の濃度は微量(20 ng/ml)であるが，血小板刺激により放出されて，局所的には比較的高濃度になる(2 μg/ml)．

1971年，洗浄血小板をトロンビンで刺激すると，血小板から190 kDaのポリペプチド・バンドが消失して，浮遊液中に105 kDaのポリペプチド・バンドが新たに出現することが発見され，当初は，トロンビンの酵素作用の血小板における基質であろう，と考えられて，thrombin sensitive proteinと命名されていた．しかし，間もなくトロンボスポンジンは血小板 α 顆粒に

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Yolk Vitronectin

PURIFICATION AND DIFFERENCES FROM ITS BLOOD HOMOLOGUE IN MOLECULAR SIZE, HEPARIN BINDING, COLLAGEN BINDING, AND BOUND CARBOHYDRATE*

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This is the first report on a unique vitronectin molecule, yolk vitronectin, which is similar to its blood homologue in cell spreading activity but different in molecular size, bound carbohydrate, and heparin and collagen binding activity. Yolk vitronectin was purified 2,500-fold from chick egg yolk by a combination of hydroxylapatite, DEAE-cellulose, and anti-vitronectin-Sepharose column chromatographies. In SDS-polyacrylamide gel electrophoresis under reducing conditions, yolk vitronectin was separated into 54- and 45-kDa bands, which are 16 and 25 kDa smaller, respectively, than the 70-kDa major band of chick blood vitronectin. The 54-kDa band shares the same NH_2-terminal sequence as chick blood vitronectin. In contrast, the NH_2-terminal sequence of the 45-kDa band is somewhat homologous with the internal sequences of mammalian vitronectins beginning at the 50th amino acid from the NH_2 terminus. The bound carbohydrate of the 54- and 45-kDa species of yolk vitronectin is similar to, but distinct from, that of blood vitronectin. Unlike blood vitronectin, yolk vitronectin cannot bind to either heparin or collagen.

Vitronectin is a multifunctional glycoprotein present in human blood plasma at a concentration of approximately 0.2 mg/ml (for reviews, see Preissner (1991) and Tomasini and Mosher (1990)). It is also present in blood from a variety of animals (Kitagaki-Ogawa *et al.*, 1990; Nakashima *et al.*, 1992) and in human amniotic fluid, urine, platelets, monocytes, macrophages, and extracellular matrix (for reviews, see Preissner (1991) and Tomasini and Mosher (1990)). Vitronectin binds to specific receptors on fibroblasts, endothelial cells and platelets (Pytela *et al.*, 1985, 1986), antithrombin III-thrombin complex (Jenne *et al.*, 1985; Ill and Ruoslahti, 1985; Preissner *et al.*, 1987), complement C5b-7 complex (Podack *et al.*, 1978), heparin (Hayman *et al.*, 1983; Barnes *et al.*, 1985; Hayashi *et al.*, 1985), collagen (Gebb *et al.*, 1986; Izumi *et al.*, 1988; Preissner *et al.*, 1990; Ishikawa and Hayashi, 1992), β-endorphin (Hildebrand *et al.*, 1988), and plasminogen activator inhibitor I (Wiman *et al.*, 1988; Declerck *et al.*, 1988; Seiffert and Loskutoff, 1991). Vitronectin can be phosphorylated (McGuire *et al.*, 1988; Korc-Grodzicki *et al.*, 1988), sulfated (Jenne *et al.*, 1989), and cross-linked by transglutaminase (Sane *et al.*, 1988). Its physiological functions are based on the above interactions and/or modifications. Vitronectin allows cells to adhere to a substrate, promotes haptotaxis of cells (Basara *et al.*, 1985; Naito *et al.*, 1991), modulates thrombin and plasmin activity in fibrinolysis, and prevents the cell lytic action of the membrane attack complex. But, the role, if any, of vitronectin in animal development and even the existence of vitronectin in embryos had not been established.

Fibronectin, a plasma glycoprotein similar to but distinct from vitronectin, also promotes cell spreading and cell migration *in vitro* (Yamada *et al.*, 1976; Ali and Hynes, 1978; Rovasio *et al.*, 1983). Extending this observation, Thiery and his colleagues have presented evidence that one of the major functions of fibronectin *in vivo* is related to migration of neural crest cells and gastrulation in the early development of embryos (for reviews, see Thiery *et al.* (1985, 1989)). Many other cell adhesion proteins, such as cadherin, laminin, and tenascin, play a role in morphogenesis in the early stages of animal development (for reviews, see Takeichi (1988), Ekblom *et al.* (1986), and Erickson and Bourdon (1989)). Particular combinations of these adhesion proteins and their cell surface receptors may be the key to the mechanisms by which cells are arranged to build the fine architecture of tissues during development.

We have, therefore, started to study the role of vitronectin in early development using chick embryos and anti-chicken blood plasma vitronectin. In an early stage of the experiments, surprisingly, we found that one of the egg yolk proteins reacted with anti-vitronectin. Yolk proteins are expected to influence the development of the embryo. So, we set out to characterize the yolk protein with the aim of obtaining new insight into vitronectin function in development. The yolk protein reacting with anti-vitronectin is called yolk vitronectin. In this paper, we describe its purification and a comparison of its heparin binding, collagen binding, bound carbohydrate, and molecular size with those of blood vitronectin of the same species.

EXPERIMENTAL PROCEDURES

Materials—Chick eggs were used on the day of laying or purchased commercially. Chicken blood serum and plasma were obtained from the same hen with or without the addition of a 1/7 volume of 3.18% sodium citrate as an anticoagulant and centrifuged immediately at 3,000 rpm for 10 min. For preparation of chicken blood vitronectin, pooled chicken blood plasma was obtained from Ichirei Inc. (Saitama, Japan) and stored at −20 °C until use. Chicken blood vitronectin was purified from pooled chicken blood plasma as described previously (Yatohgo *et al.*, 1988; Kitagaki-Ogawa *et al.*, 1990). Antibody to chicken blood vitronectin was raised in a rabbit, purified by ammonium sulfate precipitation followed by DEAE-cellulose column chro-

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matography, and conjugated with horseradish peroxidase by a conventional procedure (Harlow and Lane, 1988). Antibody to chicken blood fibronectin was similarly prepared. Anti-vitronectin-Sepharose 4B was prepared by the coupling of 13 mg of anti-chicken blood vitronectin with 1.3 g of CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology Inc.) according to the manufacturer's manual.

Fractionation of Yolk Protein and Purification of Yolk Vitronectin—Chick eggs were divided into egg yolk and egg white. Egg yolk (18–22 g each) was suspended in an equal volume of cold 0.16 M NaCl, 2 mM phenylmethanesulfonyl fluoride, and 10 mM sodium phosphate (pH 7.4) and centrifuged at 12,000 rpm at 4 °C for 20 min (Belitz and Grosch, 1987). The precipitate (yolk granules) was washed twice with the above solution. The supernatant (yolk plasma) was dialyzed extensively at 4 °C against 1 mM sodium phosphate (pH 7.4) containing 5 mM β-mercaptoethanol and centrifuged at 12,000 rpm at 4 °C for 20 min. Yolk plasma was separated into an upper solid layer (low density lipoprotein (LDL)[1] fraction) and a lower soluble layer (livetin fraction). The LDL fraction was solubilized with 15 ml of 0.16 M NaCl and 10 mM sodium phosphate (pH 7.4).

Yolk vitronectin was purified from the LDL fraction by means of three chromatographic procedures on columns of hydroxylapatite, DEAE-cellulose, and anti-vitronectin-Sepharose at 4 °C as follows. The LDL fraction was applied to a hydroxylapatite column (10-ml bed volume) that had been pre-equilibrated with 0.5 M NaCl and 10 mM sodium phosphate (pH 7.4). The column was washed with 100 ml of 0.5 M NaCl and 10 mM sodium phosphate (pH 7.4) followed by 50 ml of 10 mM sodium phosphate (pH 7.4). Yolk vitronectin was eluted with 200 mM sodium phosphate (pH 7.4) from the hydroxylapatite column. The eluate was diluted with an equal volume of distilled water to decrease the ionic strength in the eluate and applied directly to a DEAE-cellulose column (2-ml bed volume). The column was washed with 20 ml of 0.15 M NaCl, 5 mM β-mercaptoethanol, and 10 mM sodium phosphate (pH 7.4). Yolk vitronectin was eluted with 0.25 M NaCl, 5 mM β-mercaptoethanol, and 10 mM sodium phosphate (pH 7.4). The eluate from the DEAE-cellulose column was mixed and incubated with a 2-ml slurry of anti-vitronectin-Sepharose 4B at room temperature for 1 h. The slurry was packed in a column and washed with 20 ml of 0.25 M NaCl, 5 mM β-mercaptoethanol, and 10 mM sodium phosphate (pH 7.4) followed by 10 ml of 0.5 M NaCl, 5 mM β-mercaptoethanol, and 10 mM sodium phosphate (pH 7.4) and then 10 ml of 0.1 M sodium acetate (pH 4.4) containing 0.25 M NaCl and 5 mM β-mercaptoethanol. Yolk vitronectin was eluted with 0.25 M glycine HCl (pH 2.5) containing 0.25 M NaCl and 5 mM β-mercaptoethanol. The pH of the eluate was immediately adjusted to neutrality by adding an appropriate amount of 0.5 M sodium phosphate (pH 7.7). The antibody affinity chromatography described above was repeated several times.

Quantitation of Vitronectin and Fibronectin—Vitronectin and fibronectin in crude preparations were determined by using a sandwich ELISA with their specific antibodies. For the determination of vitronectin, the wells of 96-well microtiter plates were coated with rabbit anti-chicken blood vitronectin (10 μg/ml) in 0.01 M sodium carbonate (pH 9.6) at 37 °C for 30 min, and then the plates were washed four times with phosphate-buffered saline (PBS) followed by 0.025% Tween 20 in PBS. As egg yolk and egg yolk granules contained insoluble materials, a final concentration of 0.01% SDS was added to all samples to solubilize them. This amount of SDS was confirmed not to interfere with the ELISA system. These samples (50 μl each) were incubated on the antibody-coated plates at 37 °C for 1 h. The plates were washed four times with PBS, and then the second antibody, horseradish peroxidase-conjugated anti-vitronectin, in PBS containing 0.025% Tween 20 was added. After seven cycles of washing with PBS, the bound enzyme was measured using *o*-phenylenediamine and H_2O_2 as substrated. The estimated concentrations of yolk vitronectin were relative values since purified chicken blood vitronectin was used as a standard protein. The relative values seem to reflect a possible difference in the antibody reactivity toward these molecules. For the determination of fibronectin, the same procedure was used with rabbit anti-chicken blood fibronectin as the antibody and purified chicken blood fibronectin as the standard protein. Concentrations of pure blood vitronectin and fibronectin were estimated by means of absorbance measurements at 280 nm with 1-cm path length cells, using absorption coefficients of 1.38 for vitronectin (Dahlbäck and Podack, 1985) and 1.28 for fibronectin (Mosesson and Umfleet, 1970) at 1 mg/ml.

Determination of Amino-terminal Sequence—A mixture of polypeptides was separated by SDS-polyacrylamide gel electrophoresis (PAGE) using polyacrylamide gels that had been polymerized the day before and pre-electrophoresed with an electrode buffer containing 0.1 mM sodium thioglycolate to scavenge radicals (Moos *et al.*, 1988). Proteins separated on the gels were transferred onto a polyvinylidene difluoride membrane (Bio-Rad). The amino-terminal sequence of each polypeptide on the membrane pieces was determined with a protein sequenator model 477A (Applied Biosystems) (Matsudaira, 1987). Cysteines were not identified.

SDS-PAGE, Western Blotting, Cell Blotting, and Lectin Staining—Proteins were separated by SDS-PAGE according to Laemmli (1970) and stained with Coomassie Blue. For Western blotting, cell blotting, and lectin staining, proteins separated by SDS-PAGE were transferred to nitrocellulose sheets (Schleicher and Schuell) essentially according to Towbin *et al.* (1979). Protein bands on the nitrocellulose sheets were observed by staining with 0.1% Amido Black 10B, 45% methanol, and 10% acetic acid for 30 s followed by washing with 90% methanol and 2% acetic acid for 30 s.

For Western blotting, the sheet was incubated with 0.2% skim milk in PBS for 30 min and then allowed to react with anti-chicken blood vitronectin antiserum at a 1/1,500 dilution for 1 h. Bound antibody was visualized by means of sequential incubations with horseradish peroxidase-goat antibody against rabbit IgG at a 1/2,000 dilution for 1 h followed by 25 μg/ml *o*-dianisidine and 0.01% H_2O_2 for 20 min. Densitometry of the stained bands was performed as described by Kubota *et al.* (1988).

Cell blotting was performed according to the original report by Hayman *et al.* (1982) except that 0.1–0.03 μg of vitronectin/lane was used instead of 30–60 μg. BHK cells were attached to the nitrocellulose sheet at a concentration of 5×10^6 cells/ml in Grinnell's adhesion medium at 37 °C for 90 min.

For lectin staining, proteins transferred onto separate nitrocellulose sheets were stained with 0.06–10 μg/ml each of horseradish peroxidase lectins for 1 h as described by Kitagaki-Ogawa *et al.* (1986). The amounts of horseradish peroxidase lectins used were sufficient to stain 6 μg of porcine vitronectin strongly as a positive control. This staining was considered to be sensitive enough to detect 1 mol of carbohydrate chain/mol of protein. The lectins used were concanavalin A (ConA), wheat germ agglutinin (WGA), *Lens culinaris* agglutinin (LCA), *Phaseolus vulgaris* leukoagglutinin L (PHA-L), peanut agglutinin (PNA), and *Ulex europeus* agglutinin I (UEA-I), which were purchased as conjugates with horseradish peroxidase from Seikagaku Kogyo Inc. (Tokyo, Japan). Horseradish peroxidase-*Allomyrina dichotoma* agglutinin (Allo A) was from EY Laboratories (San Mateo, CA). Desialyzation of vitronectin with *Vibrio cholerae* neuraminidase (Calbiochem), deglycosylation of *O*-linked asialosaccharides with endo-α-*N*-acetylgalactosaminidase (Seikagaku Kogyo, Tokyo, Japan), and deglycosylation of *N*-linked saccharides with glycopeptidase F (Boehringer Mannheim) were carried out in 2 mM $CaCl_2$, 0.2 mM EDTA, 0.1% β-mercaptoethanol, 0.8% *n*-octyl-β-D-thioglycoside, and 50 mM sodium acetate (pH 5.6) at 37 °C for 16 h as described (Nakashima *et al.*, 1992).

Ligand Binding Assay—Collagen binding activity was determined by ELISA. Polystyrene microtiter plates (Sumitomo Bakelite, MS-3496F) were coated with 50 μl of native type I collagen from porcine skin (Cellmatrix I-P, Nitta Gelatin Co., Osaka, Japan) or gelatin at 10 μg/ml in 0.1 M sodium carbonate (pH 9.6) at 37 °C for 1 h. Gelatin was prepared by boiling 0.3 mg of type I collagen/ml in phosphate-buffered saline for 5 min. After being blocked with 0.2% skim milk and 10 mM sodium phosphate (pH 7.4), the wells were washed four times with a washing solution of 0.05% Tween 20 and 10 mM sodium phosphate (pH 7.4). Various concentrations of vitronectin in 50 μl of washing solution were incubated in the wells at 37 °C for 1 h. The plates were washed with the washing solution, and vitronectin bound to immobilized protein in the wells was allowed to react with horseradish peroxidase antibody against chicken blood vitronectin diluted to 1/500 in 0.2% skim milk and 10 mM sodium phosphate (pH 7.4) at 37 °C for 1 h. The wells were washed and incubated with 100 μl of 0.4 mg/ml *o*-phenylenediamine, 2.5 mM H_2O_2, 0.1 M citric acid, and 0.2 M Na_2HPO_4 at room temperature for 10 min. Color development was stopped by adding 50 μl of 4 N H_2SO_4, and the absorbance at 492 nm was measured with a microtiter plate reader, Corona MTP-32

[1] The abbreviations used are: LDL, low density lipoprotein; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; BHK, baby hamster kidney; PNA, peanut agglutinin; WGA, wheat germ agglutinin; LCA, *Lens culinaris* agglutinin; PHA-L, *Phaseolus vulgaris* leukoagglutinin; UEA-I, *Ulex europeus* agglutinin I; Allo A, *Allomyrina dichotoma* agglutinin; ConA, concanavalin A.

(Corona Electric Co. Ltd., Katsuta, Japan).

Heparin binding activity was examined with a heparin-Sepharose column in the presence of 8 M urea. Pure vitronectin (10 μg) was applied to a heparin-Sepharose column (50-μl bed volume) in 0.13 M NaCl, 8 M urea, 5 mM EDTA, and 10 mM sodium phosphate (pH 7.7), and then the column was washed with the same solution and eluted with 0.5 M NaCl, 8 M urea, 5 mM EDTA, and 10 mM sodium phosphate (pH 7.7). Each fraction was diluted to 1/4 with 0.025% Tween 20 in phosphate-buffered saline to decrease the concentration of urea, and vitronectin in the fraction was quantitated by a sandwich ELISA.

Cell-spreading Assay—Microtiter plates of 96 wells (Nunc) were coated with increasing concentrations of vitronectin up to 10 μg/ml at 37 °C for 1 h. BHK cell suspension (10^4 cells in 0.1 ml) in a serum-free medium was incubated on the protein-coated wells with or without a synthetic peptide, GRGDSP or GRGESP, at the indicated concentrations. After 90 min at 37 °C, BHK cells were fixed and observed under a microscope. Cell-spreading activity was expressed as the number of spread cells/100 attached cells.

RESULTS

Identification of Vitronectin in Yolk Fractionation—A rabbit antibody to chicken blood vitronectin specifically reacted with vitronectin from chicken blood plasma in an Ouchterlony double diffusion test, ELISA, and Western immunoblotting (data not shown). Anti-chicken blood fibronectin also reacted with only fibronectin in the same tests. No cross-reaction was observed. A sandwich ELISA using these specific antibodies revealed that yolk plasma contained a fairly high concentration (0.16 mg/ml) of yolk vitronectin and a low concentration (0.03 mg/ml) of fibronectin (Table I). The vitronectin concentration in yolk was almost the same as that in the blood plasma of the same hen. Egg white and egg yolk granules contained essentially no vitronectin or fibronectin. All the vitronectin in yolk plasma was fractionated into an upper solid layer (low density lipoprotein fraction) after extensive dialysis against 1 mM sodium phosphate (pH 7.4) followed by centrifugation at 12,000 rpm at 4 °C for 20 min. The lower soluble layer after the centrifugation, called the livetin fraction, did not contain vitronectin.

Western immunoblotting showed that vitronectin in yolk plasma migrated under reducing conditions as two bands of 54 and 45 kDa (Fig. 1, *lane 6*), which are 11–25 kDa smaller than those of blood vitronectin (70 and 65 kDa) (Fig. 1, *lanes 1* and *4*). Blood plasma also contained small amounts of vitronectin migrating at 56 and 45 kDa (Fig. 1, *lane 5*), which were similar in size to yolk plasma vitronectin, but they were not detected in a pure preparation of blood vitronectin (Fig. 1, *lanes 1* and *4*). Under nonreducing conditions, pure blood vitronectin migrated as a single band at 72 kDa (Fig. 1, *lanes 7* and *10*), the same position to which the major vitronectin band in blood plasma migrated (Fig. 1, *lane 11*), suggesting that a small polypeptide of 5 kDa may be disulfide-bonded to the 65-kDa species of blood vitronectin. On the other hand, yolk plasma vitronectin migrated as several bands at positions 54, 68, and 116 kDa (Fig. 1, *lane 12*), none of which comigrated with blood plasma vitronectin of 72 kDa. These results suggest that the size of whole yolk vitronectin is different from that of blood vitronectin even if the possible existence of small disulfide-bonded polypeptides is taken into account. Yolk vitronectin may contain intramolecular disulfide bond(s) since its migration became slower under nonreducing conditions. Western immunoblotting also revealed a vitronectin aggregate at 180 kDa in blood plasma (Fig. 1, *lane 11*) and at 116 kDa in yolk plasma (Fig. 1, *lane 12*), suggesting the presence of intermolecular disulfide bond(s). The pattern of yolk vitronectin bands on Western immunoblotting was the same for chicken eggs stored at 4 °C for several days and eggs within 3 h after being laid, even when the yolk plasma from the latter eggs was prepared at 4 °C in the presence of a mixture of protease inhibitors (1,000 units/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride, 30 μg/ml soybean trypsin inhibitor, and 20 mM EDTA). Thus, the small molecular size seems to be an intrinsic property of yolk vitronectin and not an artifact resulting from degradation by yolk proteases during preparation.

TABLE I

Concentrations of vitronectin and fibronectin in egg fraction and blood plasma of chick

Egg fraction	Total protein	Vitronectin[a]	Fibronectin	Volume/egg
	mg/ml	*mg/ml*	*mg/ml*	*ml*
Blood plasma	93	0.14	0.14	
Egg yolk	(133)[b]	(0.17)[b]	(0.03)[b]	20.2
Yolk plasma	101	0.16	0.03	15.4
Livetin	44	0	0	9.2
LDL	55	0.15	0.03	6.2
Yolk granules	32	0.01	0	3.7
Egg white	160	0	0	33.0

[a] Vitronectin concentrations are relative values estimated by ELISA using chicken blood vitronectin as a standard protein.

[b] Values in parentheses are the summation of values for yolk plasma and yolk granules.

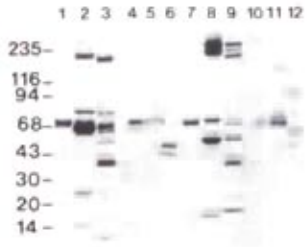

FIG. 1. **SDS-PAGE and Western immunoblotting of chicken blood plasma and egg yolk plasma.** A protein that reacted with anti-chicken blood vitronectin exists in chick egg yolk plasma as a 54- and 45-kDa species under reducing conditions. *Lanes 1–6* are reduced protein samples, and *lanes 7–12* are nonreduced protein samples. *Lanes 1–3* and *lanes 7–9* are stained with Coomassie Blue. *Lanes 4–6* and *lanes 10–12* are Western immunoblots with anti-chicken blood vitronectin of *lanes 1–3* and *lanes 7–9*, respectively. *Lanes 1* (6 μg), *4* (20 ng), *7* (6 μg), and *10* (20 ng), pure chicken blood vitronectin; *lanes 2, 5, 8,* and *11*, chicken blood plasma (6 μg each); *lanes 3, 6, 9,* and *12*, chick egg yolk plasma (60 μg each). Molecular mass (in kDa) is indicated at the *left*.

Purification of Yolk Vitronectin—Yolk vitronectin was present in the solid LDL fraction of yolk plasma after centrifugation. The LDL fraction was solubilized in 0.16 M NaCl and 10 mM sodium phosphate (pH 7.4), and yolk vitronectin was purified from the soluble LDL fraction (Fig. 2, *lane 3*) by a sequence of three types of column chromatography: hydroxylapatite, DEAE-cellulose, and anti-vitronectin-Sepharose (Fig. 2; Table II). Yolk vitronectin was 77-fold enriched in the fraction bound to the hydroxylapatite column (Table II; Fig. 2, *lane 4*), and this fraction was further applied to the DEAE-cellulose column. The fraction bound (Fig. 2, *lane 5*) to the DEAE-cellulose column contained most of the vitronectin, giving 410-fold purification (Table II). The vitronectin preparation after the final step of antibody column chromatography was purified 2,500-fold from yolk plasma (Table II). In SDS-PAGE, pure yolk vitronectin was detected by staining with Coomassie Blue as two major bands at 54 and 45 kDa with slight contamination by small polypeptides at around 10 kDa (Fig. 2, *lane 6*). Only the 54- and 45-kDa bands reacted with anti-blood vitronectin on Western blotting at all stages from the starting egg yolk to the purified yolk vitronectin. The contaminating small polypeptides did not react (Fig. 2,

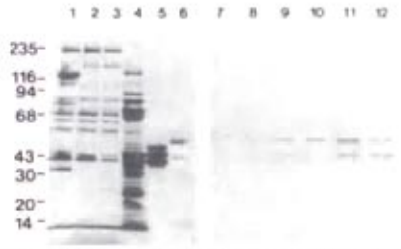

FIG. 2. **SDS-PAGE and Western blotting at each step in the purification process of yolk vitronectin.** Yolk vitronectin was purified sequentially from chick egg yolk (see Table II and "Experimental Procedures" for details) and subjected to protein composition analysis (*lanes 1–6*) by SDS-PAGE under reducing conditions and to Western blotting (*lanes 7–12*). Egg yolk (*lanes 1* and *7*) was centrifuged at 12,000 rpm for 20 min. The supernatant (yolk plasma, *lanes 2* and *8*) was centrifuged again after extensive dialysis against a low ionic strength solution. The upper solid layer (LDL fraction, *lanes 3* and *9*) was solubilized with 0.16 M NaCl and 10 mM sodium phosphate (pH 7.4). Yolk vitronectin in the LDL fraction was purified on hydroxylapatite (*lanes 4* and *10*), DEAE-cellulose (*lanes 5* and *11*), and anti-chicken blood vitronectin-Sepharose (*lanes 6* and *12*) columns. Samples used for Western blotting (*lanes 7–12*) were different lots from those used for protein staining (*lanes 1–6*). Molecular mass (in kDa) is indicated at the *left*.

TABLE II

Purification of yolk vitronectin

Purification stage	Total volume	Total protein	Total vitronectin[a]	Purification
	ml	*mg*	*mg*	*-fold*
Yolk plasma	140	7370	3.33	1
LDL	26	2960	3.12	2.3
Hydroxylapatite	35	34[b]	1.19	77
DEAE-cellulose	4.0	3.4[b]	0.63	410
Anti-vitronectin-Sepharose	4.2	0.19[b]	0.22	2500

[a] Vitronectin concentrations are relative values estimated by ELISA using chicken blood vitronectin as a standard protein.

[b] These values were estimated by multiplication of the total volume by the absorbance at 280 nm.

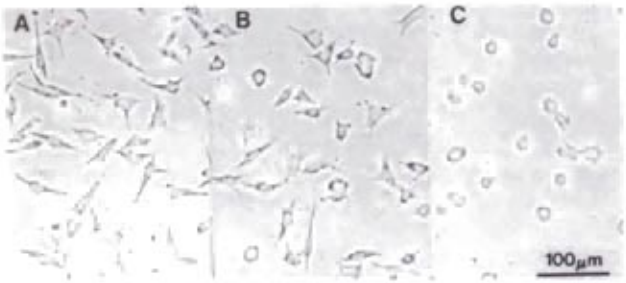

FIG. 3. **Spreading of BHK cells on vitronectin-coated wells.** BHK cells were incubated at 37 °C for 90 min on wells precoated with 3.4 μg/ml chick blood vitronectin (*A*), chick egg yolk vitronectin (*B*), or bovine serum albumin (*C*).

lanes 7–12). The relative amount of 45-kDa band/54-kDa band was estimated from the Western blots by densitometry. The 45/54 kDa ratio ranged from 0.3 to 1, depending on the preparations, from crude egg yolk to pure vitronectin. It tended to be high in older or purer preparations, suggesting some conversion to the 45-kDa polypeptide.

Cell-spreading Activity of Yolk Vitronectin—BHK cells spread on yolk vitronectin-coated microtiter plates (Fig. 3*B*). The shape of the spread cells was similar to that on blood vitronectin-coated plates (Fig. 3*A*). The dose-response curve of the cell-spreading activity was identical for blood and yolk vitronectins, giving a half-maximal concentration of 0.03–0.1 μg/ml (Fig. 4*A*). A synthetic peptide GRGDSP, but not

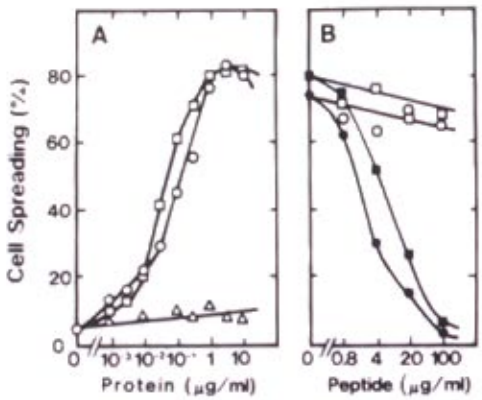

FIG. 4. **Dose-response curve of vitronectin for cell spreading (*A*) and its inhibition by synthetic peptides (*B*).** *A*, BHK cells were allowed to spread at 37 °C for 90 min on wells precoated with blood vitronectin (□), yolk vitronectin (○), or bovine serum albumin (△) at the indicated concentrations. *B*, BHK cell suspension containing a synthetic peptide, GRGDSP (■, ●) or GRGESP (□, ○), at the indicated concentrations was incubated on wells precoated with 1 μg/ml blood vitronectin (□, ■) or yolk vitronectin (○, ●).

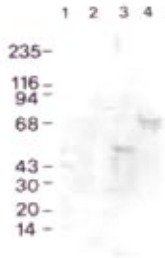

FIG. 5. **Cell blotting of vitronectin.** Two pairs of pure chick yolk vitronectin (*lanes 1* and *3*) and blood vitronectin (*lanes 2* and *4*) were subjected to SDS-PAGE and transferred from the gel onto nitrocellulose sheets. One sheet was stained with Amido Black 10B for protein (*lanes 1* and *2*). Another sheet was examined by cell blotting using BHK cells at 37 °C for 90 min (*lanes 3* and *4*). Molecular mass (in kDa) is indicated at the *left*.

GRGESP, completely inhibited spreading of BHK cells on a yolk vitronectin-coated plate as well as on a blood vitronectin-coated plate (Fig. 4*B*). These results indicate that the cell-spreading properties of yolk vitronectin are essentially the same as those of blood vitronectin.

Yolk vitronectin is a mixture of 54- and 45-kDa proteins. We examined the cell attachment activity of each yolk vitronectin band using a so-called "cell-blotting" analysis developed by Hayman *et al.* (1982). On nitrocellulose sheets, BHK cells were attached to the 54-kDa yolk vitronectin (Fig. 5, *lane 3*) as well as 70- and 65-kDa bands of chicken blood vitronectin (Fig. 5, *lane 4*). BHK cells, however, were not attached to 45-kDa yolk vitronectin (Fig. 5, *lane 3*). Protein staining of the nitrocellulose sheet (Fig. 5, *lanes 1* and *2*) indicates that the failure of cell attachment was not caused by a lower efficiency of transfer of the 45-kDa band to the nitrocellulose sheet. The contaminating small polypeptides at around 10 kDa also lacked cell attachment activity (Fig. 5, *lane 3*).

Amino-terminal Sequence of Yolk Vitronectin—Yolk vitronectin was separated into 54- and 45-kDa bands by SDS-PAGE under reducing conditions. These bands were transferred onto a polyvinylidene difluoride membrane, and their amino-terminal sequences were determined. We compared the results with those reported previously (Table III). Only the NH_2-terminal sequence of chicken blood vitronectin is available (Nakashima *et al.*, 1992), whereas the whole sequences

TABLE III

NH_2-terminal amino acid sequences of chick yolk vitronectin polypeptides

Amino acid sequences were determined with a protein sequenator. Cysteines were not identified. Boxes indicate homology among vitronectins.

	1				5				10								15				20			
Yolk 54 kd band	A	E	D	S	?	E	G	R	?	D	E	G	F	N	A	M	K	K	?	Q	G	D		
Chick blood vitronectin[a]	a	E	D	S	k	E	G	R	?	D	E	G	F	N	A	M	K	K	L	Q	?	D		

	50					55				60					65						70				
Yolk 45 kd band	A	L	P	E	D	D	Y	L	D	Y	D	L	S	I	D	T	G	?	V	G	R	P	E	?	NS
Human vitronectin[b]	T	M	P	E	D	E	Y	T	V	Y	D	D	G	E	E	-	K	N	N	A	T	V	H	E	Q
Rabbit vitronectin[c]	T	M	P	E	D	E	Y	G	P	Y	D	Y	I	E	Q	T	K	D	N	A	S	V	H	A	Q
Mouse vitronectin[d]	T	M	P	E	D	D	Y	W	S	Y	D	Y	V	E	E	P	K	N	N	T	N	T	G	V	Q

[a] Nakashima *et al.*, 1992. Small letter indicates residue identified with some ambiguity.
[b] Suzuki *et al.*, 1985; and Jenne and Stanley, 1985.
[c] Sato *et al.*, 1990.
[d] Seiffert *et al.*, 1991.

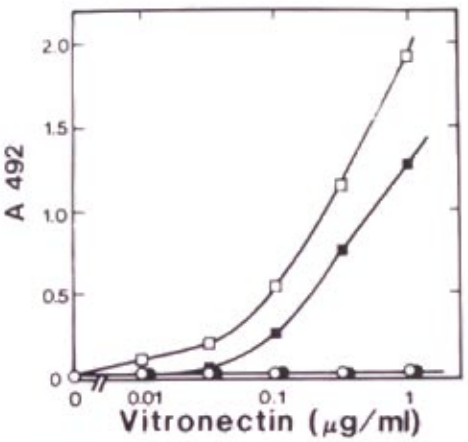

FIG. 6. **Collagen binding of vitronectin.** Blood vitronectin (□, ■) or yolk vitronectin (○, ●) was incubated at the indicated concentrations on wells precoated with 10 μg/ml native type I collagen (□, ○) or heat-denatured gelatin (■, ●) at 37 °C for 1 h. Blood vitronectin on the collagen- or gelatin-coated wells was measured in terms of absorbance at 492 nm by means of ELISA assay with horseradish peroxidase-conjugated anti-vitronectin.

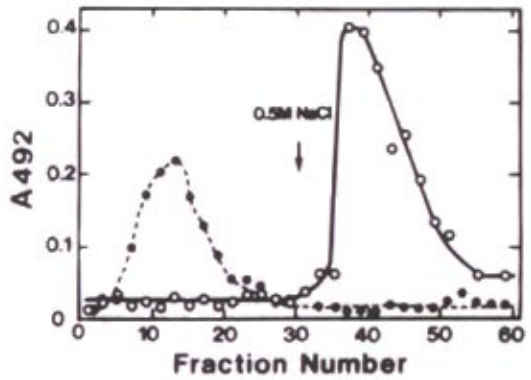

FIG. 7. **Heparin binding of vitronectin.** Blood vitronectin (○) or yolk vitronectin (●) amounting to 10 μg was applied to a small column of heparin-Sepharose (100-μl bed volume) in 0.13 M NaCl, 8 M urea, 5 mM EDTA, and 10 mM sodium phosphate (pH 7.7). The column was washed with the same solution, and bound proteins were eluted with 0.5 M NaCl in the same solution from the fraction indicated by the *arrow*. Vitronectin in the fractions was measured in terms of absorbance at 492 nm by means of a sandwich ELISA assay using anti-vitronectin.

of human, rabbit, and mouse vitronectins are known (Suzuki *et al.*, 1985; Jenne and Stanley, 1985; Sato *et al.*, 1990; Seiffert *et al.*, 1991). The NH_2-terminal sequence of the 54-kDa band is the same as that from chicken blood vitronectin. In contrast, the NH_2-terminal sequence of the 45-kDa band is completely different, though it does have some homology with the internal sequences of mammalian vitronectins beginning at the 50th amino acid from the NH_2 terminus. These results suggest that the 45-kDa band may be derived from the 54-kDa band by cleavage of the NH_2-terminal 49-amino acid peptide.

Heparin and Collagen Binding Activity of Yolk Vitronectin—Fig. 6 shows that blood vitronectin binds to native type I collagen and heat-denatured gelatin. In contrast, yolk vitronectin was bound to neither collagen nor gelatin. Neither of the vitronectins bound to bovine serum albumin, which was used as a negative control protein (data not shown).

Heparin binding activity was assayed after treatment of vitronectin with 8 M urea, since 8 M urea appears to prevent nonspecific aggregation of vitronectin and also strongly activates the heparin binding of pure vitronectin (Hayashi *et al.*, 1985; Barnes *et al.*, 1985) as well as endogenous vitronectin in blood serum (Yatohgo *et al.*, 1988). Fig. 7 shows that pure blood vitronectin was bound to heparin-Sepharose in 8 M urea, 0.13 M NaCl, 5 mM EDTA, and 10 mM sodium phosphate (pH 7.7) and eluted with 0.5 M NaCl. In contrast, pure yolk vitronectin was not bound to heparin-Sepharose. Further, none of the vitronectin in a crude sample of yolk plasma was bound to heparin-Sepharose in the presence of 8 M urea (data not shown). Thus, the yolk vitronectin molecule seems to lack a heparin-binding site as well as a collagen-binding site.

Carbohydrate of Yolk Vitronectin—Through chemical analysis and examination of the reactivity to several kinds of horseradish peroxidase lectins, we previously showed that chicken blood vitronectin contains both *O*- and *N*-linked saccharides with sialic acids (Kitagaki-Ogawa *et al.*, 1990). In agreement with our previous results, chicken blood vitronectin of 70 and 65 kDa reacted with ConA, WGA, Allo A, UEA-I, PHA-L, and PNA, but not with LCA (Fig. 8, *lane B*). Yolk vitronectins of 54 and 45 kDa were stained similarly to blood vitronectin (Fig. 8 *lane Y*). PNA reacted more strongly with

FIG. 8. **Lectin binding of vitronectin.** Blood vitronectin (*B*) or yolk vitronectin (*Y*) amounting to 6 μg was subjected to SDS-PAGE and transferred from the gel onto nitrocellulose sheets, and then stained with several kinds of horseradish peroxidase-conjugated lectins as indicated on the *top*. The amount of horseradish peroxidase lectins was sufficient to strongly stain 6 μg of porcine vitronectin as a positive control. *CB*, the same gel stained for protein with Coomassie Blue before the transfer. The asialo-type vitronectins (*aB*, *aY*) were compared with intact vitronectin for PNA staining. Molecular mass (in kDa) is indicated at the *left*.

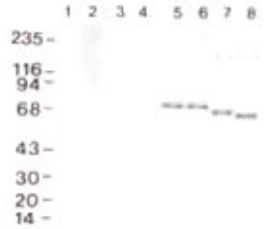

FIG. 9. **Deglycosylation of vitronectin.** Yolk vitronectin (*lanes 1–4*) and blood vitronectin (*lanes 5–8*) amounting to 3 μg were deglycosylated and stained for protein with Coomassie Blue. *Lanes 1* and *5* are intact yolk vitronectin and blood vitronectin, respectively. Sialic acids (*lanes 2* and *6*), *O*-linked asialosaccharides (*lanes 3* and *7*), and *N*-linked saccharides (*lanes 4* and *8*) were sequentially removed with neuraminidase, endo-α-*N*-acetylgalactosaminidase, and glycopeptidase-F (see "Experimental Procedures" for details). Deglycosylation was confirmed by an increment or complete loss of lectin binding activity. The band of high molecular mass in *lanes 3*, *4*, *7*, and *8* is endo-α-*N*-acetylgalactosaminidase. Molecular mass (in kDa) is indicated at the *left*.

both bands of both blood and yolk vitronectins after treatment with neuraminidase, suggesting the existence of terminal sialic acids in at least some of their *O*-linked saccharides. None of the small polypeptides contaminating yolk vitronectin reacted with any of the lectins, suggesting that they have no bound carbohydrates. On the basis of the above lectin reactivities and the previous carbohydrate analysis (Kitagaki-Ogawa *et al.*, 1990), the *O*-linked saccharides of both chicken vitronectins are considered to include a (SAα2–3)Galβ1–3GalNAc-Ser/Thr structure, a PNA receptor (where SA is sialic acid). The *N*-linked saccharides probably have a core structure of Manβ1–4GlcNAcβ1–4GlcNAc-Asn with many Man and GlcNAc residues, as well as a Fuc1–2βGal1–4βGalNAc sequence.

Quantitatively, ConA, Allo A, and PNA stained yolk vitronectin similarly to blood vitronectin, but WGA stained yolk vitronectin more strongly and UEA-I and PHA-L stained it less strongly than blood vitronectin. These differences suggest that yolk vitronectin contains more NeuNAc, less Fuc, and less terminal Gal than blood vitronectin.

To examine the amount of bound carbohydrates, yolk and blood vitronectins were sequentially deglycosylated: sialic acids by neuraminidase, *O*-linked asialosaccharides by endo-α-*N*-acetylgalactosaminidase, and finally *N*-linked saccharides by glycosidase F according to a procedure for sequential deglycosylation of mammalian and avian blood vitronectins (Nakashima *et al.*, 1992). The mass of the two bands of yolk vitronectin decreased during sequential deglycosylation from 54/45 (Fig. 9, *lane 1*) to 52/43 (Fig. 9, *lane 2*), 47/38 (Fig. 9, *lane 3*), and finally 44/35 kDa (Fig. 9, *lane 4*). The high molecular mass band in *lanes 3*, *4*, *7*, and *8* is endo-α-*N*-acetylgalactosaminidase. The difference in mass between the two vitronectin bands did not vary, suggesting that the two bands contain the same amount and the same composition of bound carbohydrates. Thus, yolk vitronectin contained an approximately 10-kDa mass of carbohydrate: a 2-kDa mass of sialic acids, a 5-kDa mass of *O*-linked asialosaccharides, and a 3-kDa mass of *N*-linked saccharides. Similarly, the molecular mass of the major band of blood vitronectin decreased from 70 (Fig. 9, *lane 5*) to 70 (Fig. 9, *lane 6*), 65 (Fig. 9, *lane 7*), and finally 63 kDa (Fig. 9, *lane 8*) during sequential deglycosylation. Blood vitronectin thus contained an approximately 7-kDa mass of carbohydrate, a less than 1-kDa mass of sialic acids, a 5-kDa mass of *O*-linked asialosaccharides, and a 2-kDa mass of *N*-linked saccharides. These results suggest that yolk vitronectin contains more sialic acids and similar amounts of *N*- and *O*-linked saccharides compared with blood vitronectin.

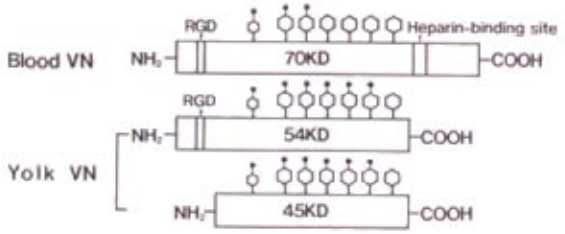

FIG. 10. **Hypothetical structural models of chick blood and yolk vitronectins.** Chick blood vitronectin predominantly consists of a 70-kDa species with a cell-binding RGD sequence near the NH_2 terminus and with a heparin-binding site near the COOH terminus. Yolk vitronectin consists of both 54- and 45-kDa glycoproteins. The 54-kDa vitronectin shares the same NH_2 terminus as blood vitronectin and contains the RGD sequence but not the heparin-binding site. The 45-kDa vitronectin seems to lack both the RGD sequence and the heparin-binding site. The bound carbohydrates are similar, consisting of *O*- (*small hexagons*) and *N*-linked (*large hexagons*) saccharide chains with more sialic acid (*dots*) in yolk 54- and 45-kDa vitronectins than in blood vitronectin.

DISCUSSION

This is the first report of the existence and biochemical characterization of a distinct vitronectin molecule in chick egg yolk. The yolk vitronectin is composed of 54- and 45-kDa glycoproteins, incorporating an approximately 10-kDa mass of carbohydrate. Yolk vitronectin has cell-spreading activity but lacks heparin and collagen binding activity.

Since vitronectin was first isolated from human plasma (Hayman *et al.*, 1983; Barnes and Silnutzer, 1983), almost all structural and functional studies of the vitronectin molecule have been concerned with human plasma vitronectin (for reviews, see Preissner (1991) and Tomasini and Mosher (1990)). Human plasma vitronectin separates into two bands of 75 and 65 kDa in SDS-PAGE under reducing conditions, and it has a heparin-binding site toward the COOH terminus, a collagen-binding site possibly toward the NH_2 terminus, and an RGD-dependent cell-spreading site near the NH_2 terminus. It contains *N*- but not *O*-linked saccharides in an amount of 10% (w/w). Its functions include modulation of the activity of membrane attack complement and hemostatic enzymes as well as promotion of cell spreading. Vitronectins from human placenta (Hayman *et al.*, 1983), HepG2 human hepatoma cells (Barnes and Reing, 1985; Nakashima *et al.*, 1992), human yolk sac carcinoma cells (Cooper and Pera, 1988), and human platelets (Preissner *et al.*, 1989) seem to be similar to plasma vitronectin. Blood plasma vitronectins from

13 other animal species have similar properties, except for some variation in apparent molecular mass, number of bands in SDS-PAGE, and carbohydrate composition (Hayman *et al.*, 1983; Kitagaki-Ogawa *et al.*, 1990; Nakashima *et al.*, 1992). Therefore, the binding activity to heparin and collagen and the cell-spreading activity are considered to be common properties of the vitronectin molecule. Vitronectin-like proteins have recently been reported to exist in a flowering plant, *Physarum*, brown algae, and a variety of invertebrates (Sanders *et al.*, 1991; Nakashima *et al.*, 1992; Miyazaki *et al.*, 1992; Wagner *et al.*, 1992). Among them, the vitronectin-like proteins from *Physarum* and brown algae have been examined and have heparin binding activity (Miyazaki *et al.*, 1992; Wagner *et al.*, 1992). However, we have found in this study that yolk vitronectin, surprisingly, lacks binding activity to heparin and collagen (Figs. 6 and 7). This makes it unique among the vitronectins so far isolated.

The 54-kDa molecule of yolk vitronectin shares the same NH_2-terminal sequence as the 70-kDa blood vitronectin molecule. The NH_2-terminal sequence of the 45-kDa molecule of yolk vitronectin is possibly homologous with the intramolecular sequence beginning at the 50th amino acid from the NH_2 terminus (Table III). These results suggest that the 45-kDa band is an NH_2-terminally truncated product of the 54-kDa vitronectin molecule. This interpretation is supported by the fact that 45-kDa yolk vitronectin lacks cell-spreading activity (Fig. 5). The site required for cell-spreading activity in all vitronectins sequenced so far (Suzuki *et al.*, 1985; Jenne and Stanley, 1985; Sato *et al.*, 1990; Seiffert *et al.*, 1991) is the NH_2-terminal Arg^{45}-Gly^{46}-Asp^{47} sequence, which should be located in the missing 49-amino acid segment of 45-kDa yolk vitronectin. Similarity in the carbohydrate compositions of the 54- and 45-kDa bands (Figs. 8 and 9) also supports this interpretation and indicates that the NH_2-terminal 49-amino acid peptide of the 54-kDa molecule does not contain any carbohydrate. The difference of 9 kDa between the 54- and 45-kDa molecules is seemingly larger than would be expected for 49 amino acids, which would correspond to roughly a 6-kDa mass. Vitronectin molecules, however, are known to behave abnormally in SDS-PAGE (Nakashima *et al.*, 1992), and therefore the sizes of 54 and 45 kDa were possibly overestimated. Thus, the apparent difference of 9 kDa is probably derived only from the NH_2-terminal truncation, not from additional COOH-terminal truncation or from intramolecular deletion in the 54-kDa molecule.

In comparison with the major 70-kDa molecule of blood vitronectin, 54-kDa yolk vitronectin is 16 kDa smaller. Considering the carbohydrate masses of 10 and 7 kDa, the polypeptide portion would be 19 kDa smaller. Human blood vitronectin of 75 kDa cleaves to 10- and 65-kDa polypeptides at Ala^{380}, the two-chain cleavage site. The domain adjacent to this site, spanning 32 amino acids toward the NH_2 terminus, Lys^{348}-Arg^{379}, is a heparin-binding site. The 54-kDa yolk vitronectin shares the same NH_2-terminal sequence as chicken blood vitronectin of 70 kDa (Table III), suggesting that 54-kDa yolk vitronectin lacks the COOH-terminal 19-kDa polypeptide of the 70-kDa blood vitronectin molecule, which contains the heparin-binding site and the two-chain cleavage site. These interpretations are summarized in a tentative structural model (Fig. 10), which compares yolk vitronectin of 54 and 45 kDa with blood vitronectin of 70 kDa.

Yolk vitronectin is present in the solid LDL fraction at low ionic strength during the purification procedure. Because purified yolk vitronectin becomes soluble in physiological salt solutions, the insolubility seems to be caused by complex formation through ionic bonding with some components in the LDL fraction. This association may depend on the lack of the heparin-binding domain, a highly charged domain.

The collagen-binding site has been suggested to be located in the NH_2-terminal half of the human blood vitronectin molecule (Izumi *et al.*, 1988). The yolk vitronectin 54-kDa molecule seems to span this domain, judging from its molecular mass (Fig. 10). This interpretation seems to be inconsistent with the lack of collagen binding activity in yolk vitronectin, unless there is an inhibitory modification of the yolk vitronectin molecule. Further characterization of the collagen binding property should enable us to elucidate this discrepancy in the future.

Our research on yolk vitronectin was initiated to study the role of vitronectin in the early development of the chick embryo, spurred by the work of Thiery and his colleagues on the role of fibronectin, a similar cell-spreading protein, in early development (Thiery *et al.*, 1985, 1989). In egg-laying species, the developing embryo depends completely on the egg components for its physiological and nutritional requirements. Cell adhesion is important during early development, and the abundance of vitronectin but not fibronectin suggests that yolk vitronectin may serve as a main cell adhesion protein in early embryogenesis of the chick. There are other reports on cell adhesion proteins in yolk including 30- and 108-kDa proteins in the newt (Komazaki, 1987) and a 160-kDa protein in the sea urchin (Noll *et al.*, 1985). However, they are not yolk vitronectins. The newt 30-kDa protein was reported to be composed of lipovitellin 2 and phosvitin by Komazaki (1987). Phosvitin from chick egg yolk purchased commercially was examined and found to have cell-spreading activity.[2] Lysozyme from chick egg white has also been reported to have cell adhesion activity (Satta *et al.*, 1980). Thus, the early development of the chick embryo probably requires the interplay of several kinds of cell adhesion proteins.

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