Immunohistochemical Demonstration of Age-related Deposition of Vitronectin (S-protein of Complement) and Terminal Complement Complex on Dermal Elastic Fibers

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Immunoreactivity of vitronectin was investigated in 100 skin specimens from different body regions in 87 individuals of different ages using monoclonal and polyclonal anti-vitronectin antibodies in an avidin–biotin–peroxidase complex technique. Vitronectin immunoreactivity was found in conjunction with dermal elastic fibers in all subjects older than 13 years. No vitronectin immunostaining was detected in subjects younger than six years, suggesting deposition of vitronectin during late childhood or early adolescence. Using an immunogold staining procedure, vitronectin immunoreactivity was ultrastructurally localized to the periphery of elastic fibers. The blood level of vitronectin in 20 healthy newborns was 67% of the adult level, suggesting active biosynthesis already in the fetus. To investigate whether vitronectin is deposited as part of the SC5b-9 complex or as an uncomplexed protein, the immunoreactivity of vitronectin was compared with that of C9, using monoclonal and polyclonal antibodies against the C9 neoantigen. Distinct C9 neoantigen immunoreactivity was demonstrated in association with dermal elastic fibers in adult subjects but only in subjects older than 30 years. The intensity of C9 neoantigen immunoreactivity appeared to increase with age and was found to be stronger in sun-exposed skin than in sun-protected skin. These findings indicate that uncomplexed vitronectin is deposited during childhood or early adolescence and that terminal complement complexes (SC5b-9 and/or SC5b-9) are deposited on elastic fibers later on in life. Hypothetically, the tissue form of vitronectin may be involved in the prevention of tissue damage in proximity to local complement activation. In addition, it may be physiologically important as substratum for cells, stimulating cell migration and anchorage. J Invest Dermatol 92:727–733, 1989.

Vitronectin, also called serum spreading factor, epibolin, and S-protein of complement, is a multifunctional glycoprotein present in plasma and in the extracellular matrix [1–5]. It belongs to a group of cell adhesion molecules, all of which mediate adhesion by a common Arg–Gly–Asp sequence [6]. Several structurally related cell surface receptors for these molecules have been identified [6]. Immobilized vitronectin induces spreading of cultured fibroblasts and stimulates migration of tumor cells [7,8]. Soluble vitronectin inhibits the membrane attack complex (MAC) of the complement pathway [9]. The MAC is formed through the assembly of the complement proteins C5, C6, C7, C8, and C9 (SC5b-9) on a cell surface and results in membrane damage [10]. In the fluid phase, a water soluble, nonlytic complex (the SC5b-9 complex) is formed as a result of the association of vitronectin (S-protein of complement) with the forming C5b-9 complex [9,10]. Plasma vitronectin may also have regulatory functions in the coagulation system. It binds to heparin, inhibits the thrombin–antithrombin III reaction, and during coagulation it is incorporated into a ternary complex with thrombin and antithrombin III [11–14]. Platelets and endothelial cells adhere to vitronectin, indicating that vitronectin may play a role in platelet aggregation and thrombus formation [15,16].

Neoantigens of C9 are exposed during the formation of both MAC and SC5b-9 [17]. Several monoclonal and polyclonal antibodies specific for such neoantigens have been characterized. These reagents distinguish between native C9 and C9 in MAC or SC5b-9, but not between C9 in MAC and the SC5b-9 complex. Antibodies specific for the C9 neoantigens have been used to demonstrate C9 immunoreactivity in several diseases [18–26]. We have found vitronectin immunoreactivity in close association with normal elastic tissue in skin and kidney in adults [27,28]. It is also recently reported to be associated with elastic fibers in lamina muscularis mucosae of the human colon and in specimens of aorta with and without signs of arteriosclerosis [23,26]. Our findings of vitronectin immunoreactivity in such degenerative lesions as neph...
To determine whether tissue vitronectin is deposited as part of MAC/SC5b-9 or as uncomplexed protein, vitronectin immunoreactivity was compared with that of C9, using antisera specific for C9 neoantigen.

MATERIALS AND METHODS

Biological Tissue  Skin samples were obtained when surrounding normal skin was trimmed off excision biopsies (55 specimens), at autopsy (5 specimens), and from surgical circumcisions (14 specimens). The autopsy specimens were obtained within 2 d after death. Vitronectin immunoreactivity was unchanged during this time (unpublished observation). In addition, skin biopsies were obtained from 13 voluntary subjects ranging in age from 39 to 80 years, from both sun-exposed skin (extensor forearm or neck) and from sun-protected skin (axilla or groin). In total 100 skin specimens from several different body regions were obtained from 87 individuals, ranging in age from 24 wk in utero to 80 years (Table 1). The specimens were stained with standard elastin staining procedure using orcein. They were from nonlesional skin, except for 10, which had clinical and histologic signs of elastosis. These specimens were from sun-exposed skin regions in subjects over 58 years of age.

The skin specimens used for electron microscopy were taken from the axilla and the trunk of middle-aged subjects.

Fixation Procedure

Light Microscopy  The specimens were either instantly frozen in chloroform/methanol R22 at the temperature of liquid nitrogen or immersed in a transport medium (550 g ammonium sulphate added to 1 L 250 mM potassium citrate, 5 mM N-ethylmaleimide, 5 mM magnesium sulphate), washed within 48 h in transport medium lacking ammonium sulphate and then frozen. The two ways of handling the specimens gave the same final results. All specimens were stored at −70°C until processed. Cryostat sections, between 4–10-μm thick, were fixed in acetone for 20 min at 4°C. Some of the specimens were also processed unfixed. This gave the same results as when acetone fixation was used.

Electron microscopy  Skin specimens were fixed in 3% paraformaldehyde, 1% glutaraldehyde in 0.075M phosphate buffer, rinsed in the same buffer, dehydrated, and block contrasted. They were either instantly frozen in liquid nitrogen or immersed in a transport medium (550 g ammonium sulphate added to 1 L 250 mM potassium citrate, 5 mM N-ethylmaleimide, 5 mM magnesium sulphate), washed within 48 h in transport medium lacking ammonium sulphate and then frozen. The two ways of handling the specimens gave the same final results. All specimens were stored at −70°C until processed. Cryostat sections, between 4–10-μm thick, were fixed in acetone for 20 min at 4°C. Some of the specimens were also processed unfixed. This gave the same results as when acetone fixation was used.

Proteins and Primary Antiserum  Vitronectin was isolated from human plasma as described by Dahlbäck and Podack [31]. A specific rabbit polyclonal anti-vitronectin antiserum, earlier characterized in detail, was used [27]. A mouse monoclonal antibody against vitronectin (called S-protein at Cytotech) was a kind gift of Dr. Ta-merius at Cytotech, San Diego, CA.

Table 1. Age Distributions and Biopsy Sites in 87 Subjects

<table>
<thead>
<tr>
<th>Skin Region</th>
<th>0–5 (yr)</th>
<th>6–10 (yr)</th>
<th>11–20 (yr)</th>
<th>21–30 (yr)</th>
<th>31–50 (yr)</th>
<th>51–85 (yr)</th>
</tr>
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<tbody>
<tr>
<td>Prepuce</td>
<td>5</td>
<td>5</td>
<td>4</td>
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<tr>
<td>Axilla</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td></td>
<td></td>
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<tr>
<td>Groin</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
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<tr>
<td>Trunk</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td></td>
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</tr>
<tr>
<td>Chest</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
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<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Thigh</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Knee</td>
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<td>1</td>
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<tr>
<td>Neck</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Face</td>
<td>1</td>
<td>1</td>
<td>1</td>
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</tbody>
</table>

In 13 of the subjects (*) biopsies were taken from both sun-exposed (forearm, neck) and sun-protected (axilla, groin) skin.

Antiserum Control Procedures  The specificities of the anti-vitronectin antibodies were tested as described in earlier reports [27]. The C9 antisera gave a strong and specific reaction with purified C9, polymerized C9, and with C9 in plasma when analyzed with Western blotting [34]. In immunoelectrophoresis, it gave no immunoprecipitate when tested against whole human plasma or native C9, demonstrating its specificity against the C9 neoantigen. The specificities of the antibodies against C9 were assayed using orcein. Aliquots (100 μl) of the two C9 antisera and of anti-vitronectin diluted in phosphate-buffered saline (PBS) containing 2.5% bovine serum albumin to their respective working dilutions, were incubated overnight with increasing amounts of purified polymerized C9 (0.003–0.3 μg) or vitronectin (0.001–10 μg). In these adsorption experiments, no cross-reactivity was found between the anti-C9 and the anti-vitronectin antiserum. The anti-C9 staining, whether obtained with polyclonal or monoclonal antibodies, remained unaffected even by the highest amount of vitronectin tested; similarly the anti-vitronectin staining was unaffected by C9. The anti-C9 staining obtained both with the monoclonal and the polyclonal antisera was only partially and completely inhibited by 0.03 and 0.3 μg purified C9, respectively.

Immunohistochemical Techniques  Light Microscopy  All the specimens were processed with polyclonal and monoclonal anti-C9 neoantigen antibodies and with polyclonal anti-vitronectin antiserum. The specimens from subjects younger than 13 years of age and selected specimens from different body regions in adults were also processed with the monoclonal anti-vitronectin antibodies. Specific rabbit antisera against amyloid A protein was used as the negative control on all specimens.

The avidin–biotin peroxidase complex technique was used as described by Hsu et al. [35] with biotinylated goat anti-rabbit or horse anti-mouse antisera as the secondary antisera and 3,3'-diaminobenzidine as the peroxidase substrate. The alkaline phosphatase–anti-alkaline phosphatase (APAAP) complex technique, using naphthol-AS-MX phosphate/Fast Blue as the alkaline phosphatase substrate, was performed as described [36]. Chicken anti-mouse immunoglobulin was used as the secondary antisera in the procedure.

The intensity of immunoreactivity was estimated by one or two observers using an arbitrary scale of gradually increasing values from 0 (negative) to 3 (strong). Staining intensity was assigned 1.5 when
estimated to be definitely positive, 1 when weak, and 0.5 when barely detected. The mean of the two values obtained with monoclonal and polyclonal antibodies was used when analyzing the results. The polyclonal and the monoclonal antibodies generally gave similar results; the differences only in a few cases exceeding 0.5.

Some of the specimens were studied with a sequential immunostaining procedure as follows: in the first reaction, monoclonal anti-C9 neoantigen or monoclonal anti-vitronectin was used in the APAAP complex technique, producing a blue color on the immunoreactive structures. After photography, the slides were left in 50 mM Tris-HCl, 0.15 M NaCl, pH 7.5, for 12 h at 37°C to detach the coverslips. The slides were then treated with xylo for 3 min at room temperature, a procedure that removed the blue color but did not affect the immunoreactivity in the subsequent reaction. In the second reaction, the sections were incubated with either polyclonal anti-vitronectin or polyclonal anti-C9 neoantigen antibodies and developed with the avidin biotin-peroxidase technique, which stains the immunoreactive structures brown. Orcein staining was used as the second reaction. Negative controls for the second reaction were either PBS or the anti-amylloid A antiserum. This sequential staining technique made it possible to compare the distributions of the anti-C9 neoantigen and the anti-vitronectin immunoreactivities in the same tissue section.

Electron Microscopy Ultrathin sections were treated either with polyclonal anti-vitronectin (working dilution 1:1000) or with polyclonal anti-serum amyloid P component overnight, rinsed in PBS, treated with gold-coated secondary antibodies for 1 h, and carefully rinsed [37]. The specificity was checked by omitting the primary antibody. Grids were stained in 1% uranyl acetate for 20 min and with 0.5% lead citrate for 5 min before examination in a JEOL 200 CX electron microscope.

Determination of Plasma Vitronectin Concentration Immediately after delivery, blood from 20 healthy newborns was collected in 1/10 vol of 129 mM trisodium citrate. The blood was taken from the umbilical vein of the cord, which was double-clamped before placental separation. The samples were immediately centrifuged and the plasma stored at −20°C until analyzed. The plasma concentrations of vitronectin was determined by electroimmunoassay run at 80 V overnight in buffer containing ethylenediaminetetraacetic acid [38]. Pooled citrated plasma from 20 healthy adult blood donors was used as standard. The standard was diluted from 1/5 to 1/40 in 50 mM Tris-HCl, pH 7.5, 0.15M NaCl. The plasma samples from the infants were diluted 1/15.

RESULTS
Specimens from different body regions in individuals of different ages, including the very young, demonstrated a dermal fiber network stainable with orcein. Specimens from young subjects generally had fine delicate elastic fibers that were thicker and more irregular with increasing age as described by others [39]. Ten of the specimens, which were from sun-exposed skin in older subjects had distinct signs of solar elastosis with a so-called grenz zone under the dermal-epidermal junction area and curled, irregular elastic fibers.

Vitronectin immunoreactivity was invariably detected in conjunction with elastic fibers in specimens from individuals older than

Figure 1. Skin specimens immunostained in an avidin–biotin peroxidase complex technique with polyclonal anti-vitronectin antibodies (a,d,f) and with monoclonal anti-C9 neoantigen antibodies (e,j). a–f: Skin from preputium of 2-year-old. b,g: Skin from back of 17-year-old subject. c,h: Skin from back of 48-year-old subject. Epidermis visualized by staining cells with methyl green (bar: 20 μm).
Table II. Mean Score of Intensity of Vitronectin and C9 Immunoreactivity in 100 Skin Specimens

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>No biopsies</th>
<th>Vitronectin</th>
<th>C9 neoantigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–5</td>
<td>11</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>6–10</td>
<td>10</td>
<td>0.5 (0–1.5)</td>
<td>0 (0–0)</td>
</tr>
<tr>
<td>11–20</td>
<td>19</td>
<td>2.1 (0–3)</td>
<td>0.1 (0–0.7)</td>
</tr>
<tr>
<td>21–30</td>
<td>8</td>
<td>3 (2.5–3)</td>
<td>0.7 (0–1)</td>
</tr>
<tr>
<td>31–50</td>
<td>24</td>
<td>3 (3)</td>
<td>1.4 (0.5–2.25)</td>
</tr>
<tr>
<td>51–85</td>
<td>28</td>
<td>3 (3)</td>
<td>1.8 (0.5–3)</td>
</tr>
</tbody>
</table>

Range in brackets. Intensity of immunoreactivity measured by an arbitrary scale of gradually increasing values from 0 (negative) to 3 (strong).

13 years, but was absent in specimens from subjects younger than six years. In samples obtained from subjects between six and 13 years of age, vitronectin immunoreactivity was weakly detected in six of 14 cases. All specimens from subjects over 20 years of age demonstrated strong immunostaining (Fig 1 a–c and Table II). Elastic material was immunostained with anti-vitronectin in agreement with an earlier report [29]. Essentially identical results were obtained with the polyclonal and monoclonal anti-vitronectin antibodies. There was no vitronectin or C9 neoantigen immunostaining of the dermal-epidermal junction area, or dermal collagen fibers. Ultrastructurally the immunoreactivity of vitronectin was found to be localized at the periphery of the elastic fibers, corresponding with the localization of amyloid P component immunoreactivity (Fig 2).

Specific C9 neoantigen immunoreactivity was absent or faint in specimens from subjects younger than 30 years, but was found in conjunction with dermal elastic fibers in individuals over 35 years of age (Fig 1 d–f). The intensity of C9 reactivity on the elastic fibers tended to increase with age (Table II). It also tended to be stronger in specimens from sun-exposed body regions than in specimens from sun-protected regions. No C9 neoantigen immunoreactivity could be demonstrated in specimens from sun-protected areas in subjects younger than 58 years. It was detected in three of six specimens from sun-protected skin of subjects older than 58 years (Table III). When compared in the same individual, the intensity of C9 neoantigen immunoreactivity was found to be fainter in sun-protected than in sun-exposed skin (Fig 3 and Table III). Elastic material was immunostained with anti-C9 neoantigen but the staining was generally fainter than that of vitronectin. All specimens were studied with both monoclonal and polyclonal anti-C9 neoantigen antibodies and they gave essentially identical results.

Using a sequential immunostaining technique it could be demonstrated that the same fibers were stained with anti-C9 neoantigen as with anti-vitronectin, thus being elastic fibers (Fig 4).

The concentration of vitronectin in plasma obtained from the umbilical blood of 20 healthy newborns was determined to be 67.3 ± 9.3% (mean ± SD) of the adult value.

**DISCUSSION**

The detection of vitronectin immunoreactivity on the dermal elastic fibers in all specimens from subjects over 13 years of age and its absence in skin specimens from younger children indicates an age-related deposition of vitronectin on these fibers during late childhood or early adolescence. The absence of vitronectin immunoreactivity in elastic fibers in the young indicates that vitronectin is not a constituent part of the elastic fibers before the age of 6 years. In this context, a noteworthy finding was that the concentration of vitronectin in plasma from umbilical blood was approximately 67% of the adult level, suggesting active biosynthesis already in the fetus. The ultrastructural localization, corresponding with that of serum amyloid P component, suggests association of vitronectin with the plasma...
periphery of the elastic fibers. Immunoreactivity of serum amyloid component has been reported to be localized in conjunction with elastin associated microfibrils [40]. It was not possible to judge from the micrographs whether vitronectin immunoreactivity was associated with the surface of the amorphous elastin or with the elastin-associated microfibrils.

The C9 neoantigen immunoreactivity was also found in conjunction with dermal elastic fibers but only in middle-aged and older individuals, and tended to increase with age. These results indicate that uncomplexed vitronectin is deposited initially on the elastic fibers. Vitronectin immunoreactivity without co-localized immunoreactivity of C-9 neoantigen has been observed earlier in the submucosa of colon and in aorta, supporting the suggestion that uncomplexed vitronectin is deposited in normal tissue [23,26].

Immunoreactivity of C9 neoantigen in conjunction with dermal elastic fibers has not been reported previously, although immunohistochemical studies have been done on human skin using anti-C9 neoantigen antibodies in studies on lupus erythematosus, leukocytoclastic vasculitis, dermatitis herpetiformis, and pemphigoid [18–21]. This may be because the specimens studied were from sun-protected skin areas or from young subjects. Moreover, the use of immunofluorescence techniques in those studies may have influenced the results. Elastin demonstrates some autofluorescence that may be difficult to eliminate despite the use of appropriate filters. Therefore, specific C9 immunoreactivity on elastic fibers may be overlooked as being the effect of autofluorescence. The use of an avidin–biotin peroxidase technique in this study eliminated these potential problems. The use of both monoclonal and polyclonal antibodies corroborated the specificities of the reactions as did cross adsorption experiments with purified C9 and purified vitronectin.

The molecular status of the C9 deposited in middle age and later in life is unknown. Hypothetically, terminal complement complexes forming in the vicinity of elastic fibers may bind to the elastin associated vitronectin. It is at present unknown whether the tissue form of vitronectin, like its soluble counterpart in plasma, can in-
hbit the formation of the MAC. An alternative explanation for the 
elastin associated C9 immunoreactivity may be a deposition of solu-
bile SCSb-9 complexes on elastin fibers.

The findings of C9 neoantigen immunoreactivity predominantly 
in sun-exposed skin suggest that sun exposure may influence its 
deposition. The UVA-induced activation of the complement sys-
tem has been reported in cases with erythropoietic protoporphyria, 
porphyria cutanea tarda, and in hematoporphyrin and chlorproma-
zine phototoxicity, but not in healthy subjects [41,42]. Data sug-
gest involvement of the complement system in UVB-induced 
inflammation have been presented recently [43].

Elastin-associated vitronectin may be an important substratum for 
migrating cells with vitronectin receptors and may be involved in 
the anchorage of certain cells in the tissue. Presumably such cells 
would be protected from lysis due to complement activation by the 
ability of vitronectin to inhibit MAC. Several cell types (e.g., neu-
trophilic polymorphonuclear leukocytes, macrophages, and fibro-
brasts) have elastase-type proteases and thus tissue vitronectin may 
play a role in the protection of the elastin fiber [44]. Several types 
of bacteria have been demonstrated to bind vitronectin, possibly facilitat-
ing invasion in the host tissue [45]. Co-localization of invading 
bacteria with leukocytes and macrophages on the elastic fibers may, 
on the other hand, facilitate the phagocytosis of the bacteria and 
thus, the tissue form of vitronectin, may be an important factor in 
host defense.

The authors thank Vinca Filinic, Anette Larsson, Lena Sandell, 
and Ingrid Olofsson for expert technical assistance; Dr. T. E. Mollnes for his kind gift 
of the monoclonal anti-C9 neoantigen antibody, Dr. John Tamarius at Cytotech, San 
Diego, for his kind gift of the monoclonal anti-vitronectin antibody, and Dr. Anders 
Grubb for the anti-AA antibody. The valuable support of Professor Hans Rorsman is gratefully acknowledged.

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