Tissue Vitronectin in Normal Adult Human Dermis Is Non-Covalently Bound to Elastic Tissue

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Vitronectin is a multifunctional human plasma glycoprotein that is also found in constant association with elastic tissue fibers in normal adults. We have investigated the nature of the association of vitronectin with elastic tissue, and compared it to that of other elastic fiber-associated proteins, namely fibrillin and amyloid P component. Samples of normal human dermis were incubated with a variety of extraction agents, including high molar salt solution, non-ionic detergent (Nonidet P-40), the reducing agents dithiothreitol or 2-mercaptoethanol, and the chaotropic agents sodium dodecyl sulfate or guanidine hydrochloride. Vitronectin purified from serum typically migrates as two bands of 75 and 65 kD. By contrast, immunoblotting studies of residual dermal material after extraction with the various agents revealed only lower molecular weight (58, 50, 42, 35, and 27 kD) anti-vitronectin reactive bands. Although these bands may represent degradation products of vitronectin generated as a result of the extraction procedure, we cannot exclude the possibility that tissue vitronectin is distinct from plasma vitronectin. Anti-vitronectin reactive polypeptides co-migrating with the 58-, 50-, and 42-kD bands were solubilized following extraction with sodium dodecyl sulfate or guanidine hydrochloride, but not with the other extraction agents. Immunofluorescence studies using residual dermal material after extraction with guanidine hydrochloride demonstrated a marked reduction in elastic fiber staining intensity with anti-vitronectin and anti-amyloid P component, but not with anti-fibrillin. Thus the majority, if not all of dermal vitronectin, is, like amyloid P component, non-covalently associated with, and not an integral constituent of, elastic fibers.
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Dermal elastic tissue is composed of mature elastic fibers in the reticular dermis, which connect with a more superficial plexus of thinner elaunin fibers running parallel to the dermo-epidermal junction, from which, in turn, thin oxytalan fibers run perpendicularly upwards to the basal lamina [1]. Ultrastructurally, elastic fibers consist of a central amorphous elastin core surrounded by a peripheral mantle of tubular microfibrils, of approximate diameter 10 to 14 nm, termed elastic fiber microfibrils [2,3]. Elastic tissue fibers just below the dermo-epidermal junction consist of microfibrils only, while elaunin fibers are intermediate in character between these microfibrillar bundles and mature elastic fibers.

Biochemical characterization of elastic fiber microfibrils has been hampered by the difficulty experienced in extracting them in pure form. Gibson et al have extracted a 340-kD protein (MP340) from fetal bovine ligamentum nuchae, which they have proposed is the subunit of which elastic fiber microfibrils are composed; other extracted lower molecular weight proteins of 78 and 31 kD, which are immunologically related to MP340, may be constituents of this protein [4,5]. The relationship of MP340 to the 350 kD glycoprotein isolated from the medium of human fibroblast cell cultures, and reported to be a major structural component of elastic fiber microfibrils by Sakai et al [6], is uncertain. In addition to these major structural proteins of elastic fiber microfibrils, a number of other elastic fiber-associated proteins have been described. We have previously reported that tissue amyloid P component (TAP), which is identical to the normal plasma glycoprotein serum amyloid P component (SAP), is constantly present in non-covalent association with elastic fibers in normal adult humans [7–9]; TAP first appears in the skin during childhood [10].

Vitronectin, also called serum spreading factor, epibolin, and S-protein of complement, is a multifunctional glycoprotein present in plasma and tissue, which promotes spreading and attachment of cells and may have a regulatory function in the complement and coagulation systems [11–18]. We have recently reported that vitronectin binds to keratin filament aggregates, which constitute the major part of dermal keratin (colloid, cytoid, Civatte) bodies formed as a result of keratinocyte apoptosis in normal skin, or of keratinocyte necrosis in a variety of skin diseases [19]. Vitronectin binding to keratin intermediate filaments may therefore play a role in removal

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Abbreviations:

AP: amyloid P component
DTT: dithiothreitol
EDTA: ethylene diamine tetraacetic acid
FITC: fluoresceinated
HRP: horseradish peroxidase-conjugated
2-ME: 2-mercaptoethanol
MP340: 340-kD microfibrillar protein
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate-buffered saline
SAP: serum amyloid P component
SDS: sodium dodecyl sulfate
TAP: tissue amyloid P component

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of keratin bodies; phagocyte recognition of apoptotic cells is mediated by the macrophage vitronectin receptor [20]. Tissue vitronectin has recently been reported to be constantly associated in an age-related manner with elastic fibers, appearing in later childhood [21–24]. Soluble vitronectin inhibits the membrane attack complex of complement by binding to nascent C5b-7 complexes, thereby blocking C9 polymerization and membrane lysis by formation of a non-lytic complex with C5b-9, the so-called SC5b-9 complex [16,17]. In this regard, it is of interest that another complement inhibitor termed decay accelerating factor, as well as C9 neoantigen present in both the membrane attack complex of complement and the non-lytic SC5b-9 complex, have been localized with vitronectin to the peripheral region of elastic fibers [24,25]. It has accordingly been suggested that elastic fiber-associated vitronectin may not only provide a substratum for migrating and resident dermal cells, but also, with decay accelerating factor, prevent complement-mediated local tissue damage. We have studied the biochemical nature of the association between vitronectin and elastic fibers by comparing the ability of a variety of reagents to extract tissue vitronectin, compared with TAP (a noncovalently bound associated protein) and/or fibrillin (an integral structural component of elastic fiber microfibrils), from normal human dermis.

MATERIALS AND METHODS

Preparation of SAP and Vitronectin SAP and vitronectin were isolated from normal human plasma or serum as previously described [17,26].

Antisera Fluoresceinated (FITC) sheep anti-human SAP, the IgG fraction of rabbit anti-human SAP, polyclonal rabbit anti-human vitronectin, and monoclonal mouse anti-fibrillin (the kind gift of Dr. L. Sakai, Shriners Hospital for Crippled Children, Portland Oregon) antibodies were prepared as previously described [6,21,27]. Other antibodies were obtained as follows: murine monoclonal anti-human S-protein (vitronectin) (Cytotech, San Diego, CA), FITC-sheep $F(ab')_2$ anti-mouse IgG and IgM (Grub, Vienna, Austria) and horseradish peroxidase-conjugated (HRP) rabbit anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark).

Preparation of Dermal Extracts The procedures used for the extraction of AP and vitronectin from normal human dermis were essentially as previously described [9]. In brief, normal human dermis prepared by ethylene diamine tetraacetic acid (EDTA) separation of whole skin obtained from surgical specimens was minced with scissors, homogenized using a ground glass homogenizer, and washed thoroughly with Dulbecco's phosphate-buffered saline (PBS; Gibco, Paisley, Scotland). One ml aliquots of the dermal suspension thus obtained were centrifuged, and the pellets were incubated for 4 h at room temperature with constant agitation with 2.5 ml of one of the extraction agents listed in Table I. Following centrifugation, individual samples were divided into an extraction supernatant and a residual dermal pellet, which were subsequently processed separately. Residual dermal pellets were washed twice with PBS and then boiled for 20 min in 1 ml of extraction buffer containing 0.0625 M Tris HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol (2-ME), 0.01% Bromophenol Blue and 1% Triton-X 100; 50-μl or 80-μl aliquots of these samples were used for SDS polyacrylamide gel electrophoresis (PAGE) or immunoblotting, respectively. Extraction supernatants were re-centrifuged for 30 min at 10,000 $\times$ g, and 0.5-ml aliquots of the supernatants were mixed with 1 ml of Laemmli's sample buffer containing 2-ME (supernatant buffer) and boiled for 10 min; 100 μl or 120 μl of these samples were used for SDS-PAGE or immunoblotting, respectively. Extraction supernatants from dermal material extracted with 2 M NaCl or 6 M guanidine hydrochloride were dialyzed against PBS before addition of the supernatant buffer. In addition, samples of three different specimens of whole skin were minced (without washing in PBS) and then boiled for 20 min directly in Laemmli's sample buffer (0.0625 M Tris HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-ME, 0.01% Bromophenol Blue); aliquots of material extracted were subjected to SDS-PAGE and immunoblotting.

Table I. Extraction of AP and Vitronectin from Normal Human Dermis

Extraction Agent	Amyloid P Component		Vitronectin	
	Pellet	Supernatant	Pellet	Supernatant
1. PBS	+[a]	−[b]	+[a]	−[b]
2. 2 M NaCl	+[a]	−[b]	+[a]	−[b]
3. 1% Nonidet P-40 in PBS	+[a]	−[b]	+[a]	−[b]
4. 0.025 M Dithiothreitol, 0.02 M Tris 0.13 M NaCl pH 7.4	+[a]	−[b]	+[a]	−[b]
5. 5% 2-Mercaptoethanol	+[a]	−[b]	+[a]	−[b]
6. 2% Sodium Dodecyl Sulfate in PBS	+[c]	+[a]	+[c]	+[a]
7. 6 M Guanidine Hydrochloride, 0.5 M Tris, 0.01 M EDTA pH 8.5	+[c]	+[a]	+[c]	+[a]
8. 6 M Guanidine Hydrochloride, 0.5 M Tris pH 8.0[d]	±[e]	+[a]	+[c]	+[a]

[a] Readily detectable.
[b] Completely absent.
[c] Quantity in residual pellet reduced.
[d] Extraction buffer changed twice; extraction for >24 h.
[e] Absent on SDS-PAGE but trace detectable on immunoblotting.

In certain experiments, one ml aliquots of the extraction supernatants were concentrated using a Minicon B 125 concentrator (Amicon Corp., Lexington, MA), and then mixed with supernatant buffer to a final volume of 250 μl. In a further experiment, aliquots of the dermal substrate were incubated in either PBS or 0.025 M dithiothreitol (DTT)/0.13 M NaCl/0.02 M Tris, pH 7.4, or 6 M guanidine hydrochloride/0.05 M Tris, pH 8.0 (Table I) for 4 h at room temperature; the samples were pelleted, incubated in fresh extraction buffer as appropriate for 17 h at 4°C, pelleted again, and incubated in a further change of appropriate extraction buffer for 4 h at room temperature. After a final centrifugation and extensive washing of the pellets in PBS, the supernatants and residual pellet samples derived from this repeated extraction procedure were then processed further for SDS-PAGE and immunoblotting as described above. In addition to the above experiments, samples of whole normal human skin were processed immediately after excision by one of two methods. In the first method, the skin was briefly rinsed in PBS and then boiled for 20 min in extraction buffer. Alternatively, the skin was minced and washed in a PBS buffer containing a battery of enzyme inhibitors, including 1 mM phenyl methyl sulfonyl fluoride, 10 mM ϵ-aminocaproic acid, 5 mM EDTA, 5 mM benzamidine, 0.1% w/v sodium azide, before boiling for 20 min in extraction buffer.

Samples of the dermal material remaining after repeated extraction with either PBS, DTT buffer, or guanidine hydrochloride buffer were embedded in O.C.T (BDH Ltd., Poole, U.K.), and stored at −20°C prior to processing for immunofluorescence studies (see below).

Incubation of Serum with Extraction Agents Five hundred-μl aliquots of normal human serum were incubated for 4 h at room temperature with constant agitation with 2.5 ml of each of extraction agents 1, 4, 6, and 7 listed in Table I. Five hundred-μl aliquots of supernatant samples obtained following centrifugation were mixed with 2.5 ml of supernatant buffer and boiled for 10 min; supernatant from serum extracted with 6 M guanidine hydrochlo-

ride was dialyzed extensively against PBS before addition of the supernatant buffer. Fifty-μl aliquots of the extraction supernatant samples boiled in supernatant buffer, and 0.3 μg (in 5 μl) of purified vitronectin (Calbiochem, La Jolla, CA), which had been stored at −70°C for 19 months, were used for immunoblotting.

Immunoblotting Immunoblotting was performed essentially as previously described [9,28,29]. Following their separation on 9% or 10.5% continuous gels, molecular weight standards and separated proteins were transferred to nitrocellulose paper and stained with 0.1% Fast Green FCF (Polysciences Inc., Warrington, PA); the position of the major protein bands was marked by punching the paper with a needle. Using this method, it was possible to calculate the approximate molecular weights of immunoreactive proteins on the nitrocellulose paper by comparison with the positions of the molecular weight standards. The presence of AP or vitronectin in the various residual dermal pellet or extraction supernatant samples was then detected using rabbit anti-human SAP (dilution 1 : 1,500) followed by HRP-staphylococcal protein A (Sigma, St. Louis, MO) (dilution 1:200), or murine anti-human vitronectin (dilution 1 : 1,000) followed by HRP-rabbit anti-mouse immunoglobulins (dilution 1 : 1,000) or polyclonal rabbit anti-human vitronectin antibody (dilution 1 : 1,000) followed by HRP-protein A (dilution 1 : 200), respectively; the peroxidase reaction products were visualized using the diaminobenzidine reaction. In control experiments, anti-vitronectin antibody was replaced by wash buffer (0.3 M NaCl, 0.01 M Tris HCl, pH 7.4, 0.5% Nonidet P40) containing 3% bovine serum albumin. Specificity controls for SDS-PAGE and immunoblotting were carried out using purified SAP (10-μl aliquots of a stock solution of 126 μg purified SAP in 600 μl supernatant buffer), purified vitronectin (2.5-μl aliquots containing 0.1 μg purified vitronectin in extraction buffer), and fresh normal human serum (10-μl aliquots of a stock solution of 100 μl serum in 0.5 ml extraction buffer). The samples of purified vitronectin and human serum, prepared as above, were boiled for 20 min prior to SDS-PAGE and immunoblotting.

Immunofluorescence Staining of Extracted Dermal Material Immunofluorescence staining of the detection of SAP, vitronectin, and fibrillin in the O.C.T-embedded residual material remaining after repeated extraction of the dermal substrate with either PBS, DTT buffer, or guanidine hydrochloride buffer, was also carried out. Five-micrometer cryostat sections were stained with FITC-rabbit anti-SAP (dilution 1 : 30), or murine monoclonal anti-human vitronectin (dilution 1 : 50) or murine monoclonal anti-fibrillin (dilution 1 : 10), followed by FITC-sheep $F(ab)_2$ anti-mouse IgG and IgM (dilution 1 : 20). These antibody dilutions were shown in preliminary pilot studies to produce the bright fluorescence associated with elastic fibers in normal human dermis.

RESULTS

Extraction of AP and Vitronectin from Normal Human Dermis When PBS was incubated with the dermal suspension, a great many polypeptides remained in the residual dermal pellet, to be released for analysis by SDS-PAGE following boiling of the sample in extraction buffer (Fig 1, *lane 2*). The majority of the other extraction agents (e.g., 5% 2-mercaptoethanol; Fig 1, *lanes 6* and *7*) also failed to solubilize or extract a significant number of dermal proteins, such that numerous proteins remained in the residual pellet sample, and few proteins appeared detectable on SDS-PAGE of the extraction supernatants, even after the supernatants had been concentrated. By contrast, when buffers containing guanidine hydrochloride (Fig 1, *lanes 4* and *5*) or SDS (Fig 1, *lanes 8* and *9*) were used as extraction agents, dermal proteins were solubilized and readily detectable in the extraction supernatant samples, especially when these were concentrated, whereas the number of polypeptides remaining in the residual pellet samples was correspondingly reduced.

As previously reported, AP (identified as such on immunoblotting and by comigration with purified SAP as a single band of 30 kD) was extractable from normal human dermis and solubilized

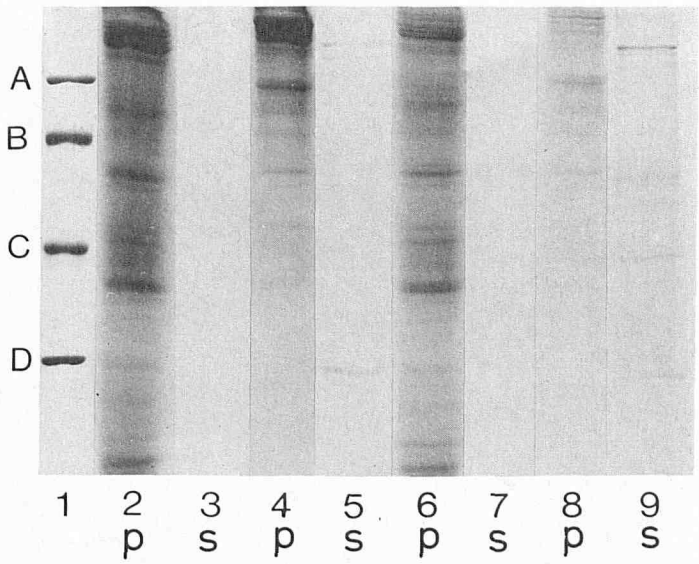

Figure 1. SDS-PAGE of residual dermal pellets and extraction supernatants stained with Coomassie blue. *Lane 1,* molecular weight markers (*A*: rabbit muscle phosphorylase b, 97.4 kD; *B*: bovine serum albumin, 66.2 kD; *C*: hen egg white ovalbumin, 42.7 kD; *D*: bovine carbonic anhydrase, 31 kD); pellet *(lane 2)* and supernatant *(lane 3)* after extraction with PBS; pellet *(lane 4)* and supernatant *(lane 5)* after extraction with 6 M guanidine hydrochloride, 0.5 M Tris, 0.01 M EDTA, pH 8.5; pellet *(lane 6)* and supernatant *(lane 7)* after extraction with 5% 2-ME; pellet *(lane 8)* and supernatant *(lane 9)* after extraction with 2% SDS. p, pellet; s, supernatant.

into the extraction supernatant only by buffers containing guanidine hydrochloride (Fig 2, *lanes 4* and *5*) or SDS (Fig 2, *lanes 8* and *9*), with a corresponding decrease in the amount of AP detectable by immunoblotting in the residual pellet sample (Table I). When dermal material was repeatedly extracted with several changes of guanidine hydrochloride buffer, virtually no AP remained in the residual dermal pellet (data not shown).

Serum vitronectin, and vitronectin purified from normal human serum, migrated as two bands of molecular weight 75 kD and 65 kD (Fig 3, *lane 1*). Immunoblotting of residual dermal pellet material with monoclonal anti-vitronectin antibody, following attempted extraction with the various extraction agents listed in Table I, revealed in all samples the presence of five polypeptide

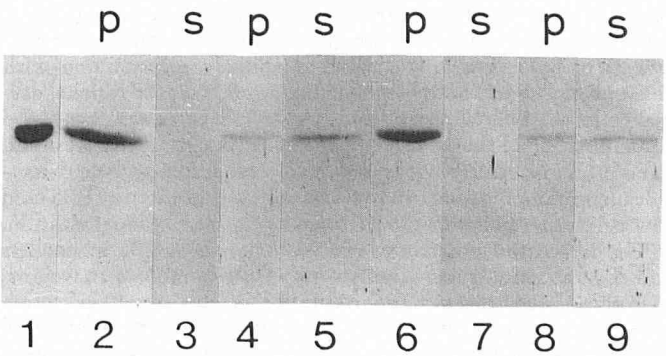

Figure 2. Immunoblot analysis of residual dermal pellet material and extraction supernatants with anti-SAP. *Lane 1,* purified SAP; pellet *(lane 2)* and supernatant *(lane 3)* after extraction with PBS; pellet *(lane 4)* and supernatant *(lane 5)* after extraction with 6 M guanidine hydrochloride, 0.5 M Tris, 0.01 M EDTA, pH 8.5; pellet *(lane 6)* and supernatant *(lane 7)* after extraction with 5% 2-ME; pellet *(lane 8)* and supernatant *(lane 9)* after extraction with 2% SDS. p, pellet; s, supernatant.

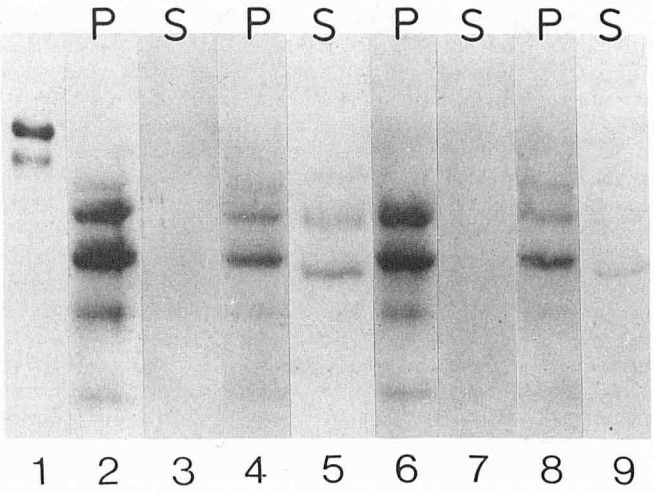

Figure 3. Immunoblot analysis of residual dermal pellet material and extraction supernatants with monoclonal anti-vitronectin (composite of several different experiments). *Lane 1,* purified vitronectin; pellet *(lane 2)* and supernatant *(lane 3)* after extraction with PBS; pellet *(lane 4)* and supernatant *(lane 5)* after extraction with 6 M guanidine hydrochloride, 0.5 M Tris, 0.01 M EDTA, pH 8.5; pellet *(lane 6)* and supernatant *(lane 7)* after extraction with 5% 2-ME; pellet *(lane 8)* and supernatant *(lane 9)* after extraction with 2% SDS. P, pellet; S, supernatant.

bands of approximate molecular weights 58, 50, 42, 35, and 27 kD (Fig 3, *lanes 2, 4, 6,* and *8*; Table I). Staining was absent in the control experiments when the monoclonal anti-vitronectin was replaced by wash buffer containing 3% albumin. When polyclonal anti-vitronectin antibody was used for immunoblotting, only the 58-, 50-, and 42-kD bands were visualized; this may have been because a less sensitive method involving HRP-protein A as the second step, rather than HRP–anti-immunoglobulin, was used. The quantity of the polypeptides remaining in the residual dermal pellet samples, as reflected by the staining intensity of the bands, varied somewhat according to the particular extraction agent used, but was particularly reduced following extraction of the dermal material with guanidine hydrochloride (Fig 3, *lane 4*) or SDS (Fig 3, *lane 8*). In contrast to the findings with anti-SAP antibody, all five protein bands that reacted with monoclonal anti-vitronectin antibody were still readily detectable in the residual dermal pellet sample even following repeated extraction with guanidine hydrochloride (data not shown). Polypeptides of molecular weights 58, 50, and 42 kD that reacted with anti-vitronectin antibody, and which corresponded to and comigrated with the anti-vitronectin reactive bands of identical molecular weight in the residual dermal pellets, appeared in both unconcentrated and concentrated extraction supernatant samples following use of guanidine hydrochloride (Fig 3, *lane 5*) or SDS (Fig 3, *lane 9*). It is unlikely that staining with anti-vitronectin antibody of polypeptides in either the residual dermal pellet or extraction supernatant samples was the result of blood contamination of the dermal suspension despite extensive washing, as no proteins reactive with anti-vitronectin antibody were detectable on immunoblotting of a fourfold concentration of the PBS used to wash the dermal preparation immediately after homogenization.

The anti-vitronectin reactive bands in the residual dermal pellets and the extraction supernatants were of lower molecular weights than those found on immunoblotting of serum vitronectin, which had been processed (including boiling for 20 min) in an identical manner, and of purified vitronectin. A number of studies were carried out to determine whether these lower molecular weight anti-vitronectin reactive bands in dermal extracts might represent degradation products of vitronectin, resulting from enzymatic cleavage or chemical destruction during the extraction procedure. Samples of all three specimens of normal skin extracted directly in Laemmli's sample buffer contained anti-vitronectin–reactive bands of lower molecular weight (approximately 58, 50, 48, 46, and 42 kD) as shown in Fig 4, *lane 5*. The 65-kD band also seen in Fig 4, *lane 5* is most likely derived from serum as the whole skin specimens were not washed before extraction; the absence of a 75-kD band here does not negate this suggestion, because it is well recorded that sera may contain a great preponderance of only one of the typical bands [13,17]. Immunoblotting with monoclonal anti-vitronectin antibody was also performed on specimens of washed whole skin, processed immediately after excision either by boiling in extraction buffer for 20 min or in the presence of a battery of enzyme inhibitors, to minimize the possibility of vitronectin degradation by tissue enzymes. Lower molecular weight anti-vitronectin–reactive bands of 58, 50, and 42 kD were still detectable in these specimens, although the amount of the corresponding proteins, as judged by the intensity of staining of the bands, was markedly reduced (data not shown). Similar lower molecular weight anti-vitronectin–reactive bands, in addition to the characteristic 75- and 65-kD bands, were seen on immunoblotting of samples of serum extracted with 6 M guanidine hydrochloride (Fig 4, *lane 2*) or 2% SDS (Fig 4, *lane 4*), when the gels were deliberately overloaded with the samples. These bands were, however, also detected on immunoblotting of purified vitronectin that had been stored at −70°C for over 19 months (Fig 4, *lane 3*). This latter finding of lower molecular weight anti-vitronectin–reactive bands even in a sample of purified vitronectin,

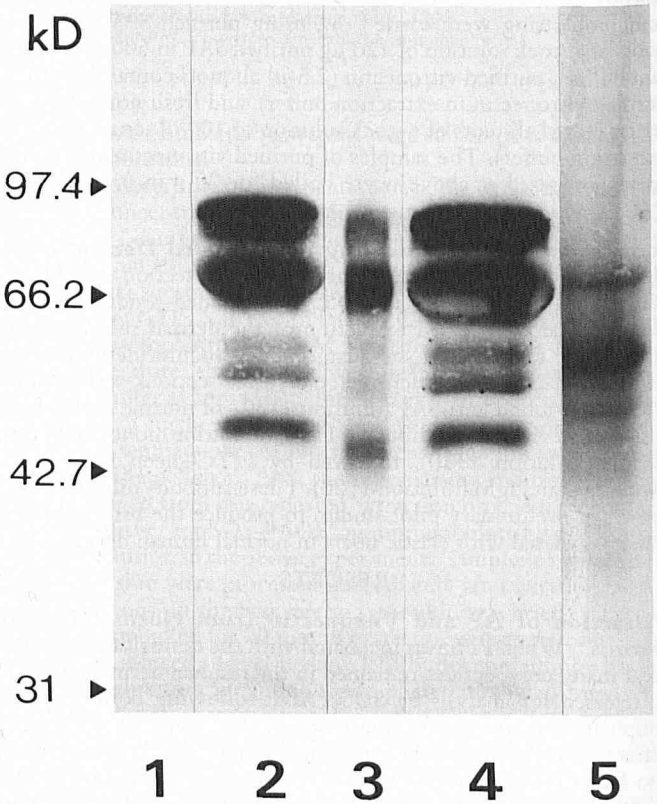

Figure 4. Immunoblot analysis with monoclonal anti-vitronectin (composite of several different experiments). *Lane 1,* position of molecular weight markers after transfer to nitrocellulose paper following SDS-PAGE, as determined by needle perforation of paper at sites of staining with 0.1% Fast Green FCF, prior to immunostaining. Normal human serum samples preincubated with 6 M guanidine hydrochloride *(lane 2),* or 2% SDS *(lane 4);* samples deliberately overloaded on the gel during preliminary SDS-PAGE. *Lane 3,* purified vitronectin which had been stored at −70°C for over 19 months. *Lane 5,* material extracted following boiling whole (unwashed) skin in Laemlli's sample buffer. Note lower molecular weight bands in *lanes 2* to *5,* in addition to higher molecular weight band(s) characteristic of serum vitronectin.

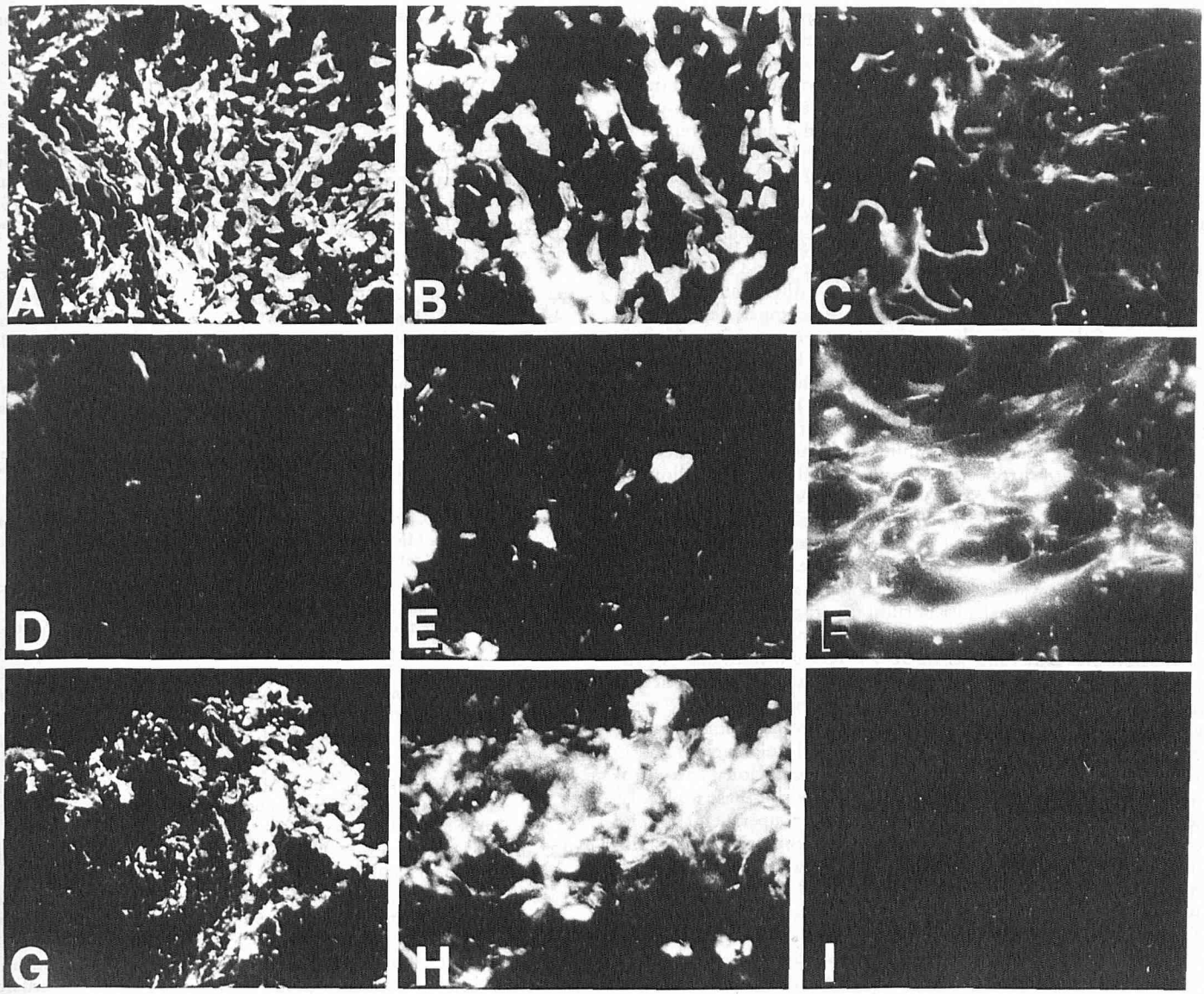

Figure 5. Bright fluorescence associated with the dermal elastic fiber network in the residual dermal material following extraction with PBS alone, on staining with anti-SAP *(A)*, anti-vitronectin *(B)*, and anti-fibrillin *(C)* antibodies. After extraction of the dermal material with guanidine hydrochloride, note considerably decreased staining with anti-SAP *(D)* and reduced staining with anti-vitronectin *(E)*, but readily detectable staining with anti-fibrillin *(F)* antibodies. After dermal extraction with dithiothreitol, staining with anti-SAP *(G)* and anti-vitronectin *(H)* is substantially unaltered, but staining with anti-fibrillin *(I)* antibody is abolished. (*A, D, G*: magnification ×100. *B, E, H*: magnification ×250. *C, F, I*: magnification ×500.)

which had not been treated with any of the agents used to extract dermal material but which might have undergone degradation during storage, tends to suggest that the corresponding bands on immunoblotting of extracted dermal pellets and supernatants do represent degradation products of vitronectin.

Immunofluorescence Staining of Extracted Dermal Material Bright fluorescence associated with the dermal elastic fiber network on staining with anti-SAP, anti-vitronectin, and anti-fibrillin antibodies was observed in the residual dermal pellet material following extraction with PBS alone (Fig 5*A,B,C,* respectively). Extraction of the dermal material with guanidine hydrochloride considerably decreased the intensity of staining with anti-SAP (Fig 5*D*) and also reduced staining with anti-vitronectin, but to a lesser extent (Fig 5*E*). Staining with anti-fibrillin antibody was still readily detectable although diminished after extraction with guanidine hydrochloride (Fig 5*F*), but was completely abolished following dermal extraction with DTT buffer (Fig 5*I*). Staining with anti-SAP and anti-vitronectin after dermal extraction with DTT buffer was substantially unchanged (Fig 5*G, H*, respectively).

DISCUSSION

We have recently shown that AP is non-covalently associated with dermal elastic tissue fibers, because it can be extracted from normal human dermis using agents that disrupt non-covalent bonds, such as SDS and guanidine hydrochloride [9]. In the present study, we have investigated the nature of the interaction of another elastic fiber-associated protein, vitronectin, with elastic fibers. We report that vitronectin, like AP [9], can be extracted from normal human dermis using chaotropic agents including SDS and guanidine hydrochloride, but not with high molarity salt solution, non-ionic detergents, or reducing agents. This finding was confirmed not only by biochemical analysis of the residual dermal pellet and the supernatant samples following extraction with a variety of agents, but also by immunofluorescence staining of residual dermal pellet material,

because the intensity of staining with anti-vitronectin antibody following extraction with guanidine hydrochloride was markedly reduced. By contrast, staining with antibodies to fibrillin, a major constituent of elastic fiber microfibrils, was substantially retained. This indicates that vitronectin, like AP, is at least in part non-covalently bound to elastic tissue. However, we did find differences in the binding characteristics of AP and vitronectin, because considerable amounts of vitronectin remained in the residual dermal pellet following extraction procedures that removed virtually all biochemical evidence of AP.

In view of their age-related deposition [10,24] and the non-covalent nature of their binding to elastic tissue, it would seem that AP and vitronectin are proteins associated with, rather than forming an integral part of, elastic fiber microfibrils. We compared the relative resistance of the anti-fibrillin-reactive protein, of AP, and of vitronectin to extraction from the dermis by PBS, and the buffers containing either DTT or guanidine hydrochloride. Gibson et al [5] found that a reductive saline buffer containing DTT caused a significant reduction in the microfibrillar component of the microfibrils of elastic fibers. We found that staining of dermal elastic tissue with anti-SAP and anti-vitronectin was substantially retained following dermal extraction with DTT; assuming that AP and vitronectin are proteins associated with elastic fiber microfibrils, this finding would suggest that the complete absence of staining with anti-fibrillin antibody of the residual dermal pellet after extraction with DTT buffer resulted from alteration of the antigenic site on fibrillin recognized by the monoclonal anti-fibrillin (as proposed by Gibson et al [5]), rather than a DTT-induced removal of microfibrillar material.

We have previously shown, on SDS-PAGE and immunoblot analysis [9], that AP extracted from normal human dermis is identical in terms of molecular weight to the 30-kD subunit of SAP. Plasma vitronectin, and vitronectin purified from normal human plasma, characteristically migrates as two bands of molecular weight 75 kD and 65 kD. It was therefore surprising that monoclonal anti-vitronectin antibody failed to identify protein bands of these molecular weights in residual dermal pellet and extraction supernatant samples on immunoblot analysis; instead, there was immunospecific staining of a series of lower molecular weight protein bands of approximately 58, 50, 42, 35, and 27 kD. For most of the experiments, whole skin was incubated at 45°C for 90 min in EDTA buffer prior to dermal extraction, in order to enable separation of dermis from epidermis. One possibility is therefore that tissue vitronectin was enzymatically degraded after excision, or during this incubation period. The fact that a similar distribution of lower molecular weight bands reacting with anti-vitronectin was seen even on immunoblot analysis of whole skin processed by boiling in extraction buffer immediately after excision, and of whole skin processed immediately after excision in the presence of a battery of enzyme inhibitors, makes this unlikely. A further possibility for this finding would certainly be that these lower molecular weight bands represent degradation products of tissue vitronectin generated during the extraction procedure, because treatment of serum with agents used to extract dermal material, namely 2% SDS, 0.025 M dithiothreitol, or 6 M guanidine hydrochloride, resulted in the appearance of such lower molecular weight anti-vitronectin-reactive bands, in addition to the typical 75-kD and 65-kD bands, on subsequent immunoblotting. Furthermore, lower molecular weight bands also appeared when large quantities of purified vitronectin, which had been stored for a prolonged period and might have undergone partial degradation, were analyzed by immunoblotting. However, if this were the case, it is curious that there was no trace of the 75-kD and 65-kD bands typical of vitronectin in the residual dermal pellets and extraction supernatants following extraction of the dermis and of washed whole skin. A third potential explanation, which cannot altogether be excluded, is that the tissue form of vitronectin may be distinct from plasma vitronectin. In this regard, it is of considerable interest that vitronectin has recently been reported to exist in two structurally and functionally distinct forms in human plasma [30]. This would not exclude the possibility that tissue vitronectin may be derived by enzymatic digestion from plasma vitronectin as a precursor.

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