Characterization of Biologically Active Domains on Elastin: Identification of a Monoclonal Antibody to a Cell Recognition Site†

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ABSTRACT: Monoclonal antibodies to bovine α-elastin were characterized with solid-phase ELISA, Western blot, immunoprecipitation, and immunoaffinity chromatography. One monoclonal antibody, BA-4, bound to insoluble elastin, α-elastin, and tropoelastin and to peptide fragments generated by proteolytic digestion of insoluble elastin. Immunoaffinity chromatography of elastin fragments released from insoluble elastin with pancreatic elastase demonstrated that BA-4 was specific for a chemotactically active epitope composed of valine, glycine, alanine, and proline in a molar ratio of approximately 2:2:1:1. This composition matches the Val-Gly-Val-Ala-Pro-Gly repeating sequence in elastin that has been shown to be a chemoattractant for fibroblasts and monocytes. Specific ablation of the chemotactic activity of synthetic Val-Gly-Val-Ala-Pro-Gly by BA-4 IgG confirmed the identity of the epitope recognized by the monoclonal antibody and suggests that, despite its hydrophobic nature, this cell recognition domain is accessible on the surface of elastin and is strongly immunogenic. BA-4 should prove useful for investigating cell surface receptors for elastin.

Accumulating evidence suggests that extracellular matrix macromolecules interact with one another and with cells through specific domains. Prototypic in this regard is fibronectin, a molecule with discrete binding sites for proteoglycans, fibrin, collagen, bacterial proteins, and receptor proteins on cells (reviewed in Ruoslahti et al., 1985).

We have been interested in the possibility that elastin has specific domains that have interactions with other matrix molecules and with cells. This possibility has support from elastin gene characterization (Yoon et al., 1984; Cicila et al., 1985) and from primary structural analysis of tropoelastin (Foster et al., 1973; Sandberg et al., 1982) which show alternating, highly specialized regions of the protein that provide elastic recoil and position lysine amino acids in the correct orientation for cross-link formation. The interaction of elastin with microfibrillar protein (Cleary & Gibson, 1983) and the demonstration that peptide segments of elastin elicit a chemotactic response by fibroblasts and monocytes (Senior et al., 1980, 1984) have suggested that elastin may have domains with biologically important activities other than ones relating to its mechanical properties.

Probes for discerning the structure–function relationships of domains of elastin have not been readily available, in part because of the difficulty of working with an insoluble protein that is extremely resistant to proteolysis. Studies with polyclonal antisera to elastin, however, have demonstrated immunologically distinct antigenic epitopes on the molecule (Mecham & Lange, 1982; Wrenn & Mecham, 1986), suggesting that monoclonal antibodies could provide unique probes for domain mapping. We report here the production and characterization of a monoclonal antibody that exhibits specificity for a hydrophobic region of elastin that includes a cell recognition site.

Materials and Methods

Production of Monoclonal Antibodies. Balb/CJ mice were immunized by intraperitoneal injection of 150 μg of bovine α-elastin prepared as previously described (Mecham & Lange, 1982) in complete Freund's adjuvant. Two booster injections of 100 μg in Freund's incomplete adjuvant were given at weeks 2 and 3, and the mice rested for 1 week. One subsequent injection of 150 μg dissolved in saline was given at week 4. Four days after the last injection, lymphocytes obtained by disruption of the splenic capsule were fused with Ag-8 myeloma cells at a ratio of 1:1 in Dulbecco's phosphate-buffered saline containing 40% w/v poly(ethylene glycol) (PEG-4000, Dutchland Laboratories, Denver, PA) and 15% v/v dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO). Fused cells were resuspended in Dulbecco's minimal essential medium supplemented with 10% horse serum, 5% fetal calf serum, 50 μg/mL gentamycin, 1.58 g/L sodium hypoxanthine, 0.017 g/L aminopterin, and 0.39 g/L thymidine (HAT medium), and 1 mL of the cell suspension was plated in each well of a 24-well tissue culture dish containing mouse peritoneal exudate cells in 1 mL of HAT medium. After 1 week, HAT medium was replaced with HT medium (as above without aminopterin), and the cultures were maintained for 1 week. Medium was then replaced twice weekly with complete medium. After 3 weeks, supernatants of growth positive wells were screened for antibody to elastin by enzyme-linked immunoadsorbent assay (ELISA) as described (Wrenn & Mecham, 1986). Antibody producing wells were cloned by limited phase dilution into 96-well plates. Subsequently, a single clone designated BA-4, producing antibody of the IgG class, was subcloned and grown to high density in tissue culture flasks. Cells were harvested from late log-phase cultures and washed with phosphate-buffered saline (pH 7.4) (PBS),[1] and 10^7 cells were injected intraperitoneally into pristane-primed mice.

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminqmethane; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis.

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The collected ascites fluid was clarified by centrifugation at 10000*g* for 30 min. Solid ammonium sulfate was added to 45% saturation, and the slurry was mixed overnight by end-over-end agitation. Precipitates were collected by centrifugation as above, resuspended in 0.02 M Tris, pH 8.0, and 0.028 M NaCl (column buffer), and dialyzed exhaustively against the same. IgG was isolated using a 2.5 × 40 cm column of DEAE Affi-Gel Blue (Bio-Rad Laboratories, Richmond, CA) equilibrated with column buffer and eluted with a linear gradient of 0.028–0.15 M NaCl. Peak fractions containing IgG were pooled, dialyzed against PBS, concentrated by ultrafiltration, and stored at −70 °C until use.

Inhibition ELISA. The specificity of the antibody for elastin was assessed by inhibition ELISA performed under nonequilibrium conditions (Rennard et al., 1980) using bovine α-elastin and collagen types I and III extracted from ligamentum nuchae as competing inhibitors. Aliquots of protein (200 μL) serially diluted in PBS containing 0.05% (v/v) Tween-20 (PBS–Tween) were incubated overnight at 4 °C with 200 μL of antibody diluted 1:2000 in PBS–Tween. Duplicate 100-μL aliquots of the mixtures were transferred to elastin coated microtiter plates (100 ng/well) and incubated for 90 min at 37 °C. The plates were rinsed twice with PBS–Tween (100 μL/well) and incubated as above with 100 μL per well horseradish peroxidase–conjugated goat anti-mouse immunoglobulin (Cappel, West Chester, PA) diluted 1:500 in PBS–Tween containing 1% (w/v) ovalbumin. The plates were rinsed 3 times with PBS–Tween and 100 μL of ABTS substrate [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), ammonium salt] was added. Reaction product was determined spectrophotometrically at 410 nm.

Electrophoresis and Electrophorectic Blotting. Sample proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as described by Matsudaira and Burgess (1978) and transferred to nitrocellulose at 100 V for 1 h at 4 °C (Tobwin et al., 1979). Following transfer, excess binding sites were blocked by incubating the blot for 30 min at room temperature in Tris-buffered saline (0.01 M Tris, pH 7.4, 0.9% NaCl) (TBS) containing 3% (w/v) nonfat dried milk (BLOTTO) as described by Johnson et al. (1984). The blot was washed twice for 15 min with TBS and incubated with BA-4 antibody diluted 1:250 in 3% BLOTTO for 1 h at room temperature. Following incubation with monoclonal antibody, the blot was washed with TBS as above and incubated with peroxidase conjugated goat anti-mouse immunoglobulin diluted 1:100 in 3% BLOTTO. Following incubation with second antibody, the blot was rinsed 4 times with TBS as above and incubated in enzyme substrate solution (10 mg of 4-chloro-1-naphthol/mL of methanol containing 0.4% H_2O_2). The reaction was terminated after 20–30 min by rinsing the blot in water.

Isolation and Characterization of Antigenic Elastin Peptides. Elastin peptides recognized by BA-4 were isolated by affinity chromatography. All procedures were performed at 4 °C. To prepare an affinity resin, approximately 30 mg of BA-4 antibody in 0.1 M sodium bicarbonate (pH 8.0) was mixed overnight by end-over-end rotation with 4 mL of Affi-Gel-10 resin (Bio-Rad Laboratories, Richmond, CA) in a siliconized glass tube. The affinity resin with bound antibody was transferred to a small glass column and washed with 5 volumes of column buffer [0.1 M potassium phosphate buffer (pH 7.4)] until the OD_{280} returned to background. Unreacted binding sites on the resin were blocked by incubation with 0.1 M ethanolamine (pH 8) for 1 h followed by a 5-column volume wash with column buffer. Efficiency of antibody coupling as determined by monitoring the OD_{280} of the resin washes was approximately 85%.

Ten milligrams of elastin peptides prepared by pancreatic elastase digestion of insoluble elastin as described by Senior et al. (1982) was resuspended in 1 mL of column buffer, and the suspension was loaded onto the column and allowed to penetrate fully into the gel. Buffer flow was terminated and the incubation was allowed to proceed for 1 h. Unbound peptides were eluted by washing the column with buffer until a stable OD_{280} base line was achieved. Elution of specifically bound peptides was achieved by washing the column with buffer containing 8 M urea. Peak fractions were pooled, dialyzed against 0.05 N acetic acid using Spectrapor 3 dialysis membrane (3500 molecular weight cutoff), and lyophilized. Lyophilized peptides were resuspended in 0.1 M phosphate buffer, pH 7.4, and aliquots were removed for amino acid analysis (Mecham & Lange, 1982) and SDS–PAGE. An additional aliquot was chromatographed by C_{18} reverse-phase HPLC (Beckman Ultraspher-ODS, 4.6 mm × 25 cm) with a Beckman Gradient Model 334 HPLC system. The column was eluted with 0.01% trifluoroacetic acid (buffer A) and 0.01% trifluoroacetic acid in acetonitrile (buffer B) by using a linear gradient starting with buffer A changing to 100% buffer B over 20 min. The column flow rate was 1 mL/min.

Immunoprecipitation of biosynthetically labeled tropoelastin from elastin-producing cells was as described (Wrenn & Mecham, 1986).

Assay of Chemotaxis. The ability of BA-4 monoclonal antibody to block the fibroblast chemotactic activity of elastin-derived peptides and of synthetic peptide VGVAPG was assessed by incubating the peptides with BA-4 for 1 h at room temperature and then testing for residual chemotactic activity. Fibroblast chemotaxis was determined as described previously by Senior et al. (1982).

Results

Monoclonal Antibody Characterization. Previous studies have shown elastin to be a weak immunogen (Mecham & Lange, 1980, 1982a,b). Therefore, we used relatively high doses of the oxalic acid solubilized form of the protein for the initial and subsequent booster immunizations of mice. With this protocol, it was important to use several independent tests to characterize the specificity of antibody binding to exclude monoclonal antibodies that may react with minor, more immunogenic contaminants which might be present in the antigen preparation. Thus, screening of hybridomas for antielastin reactivity included solid-phase ELISA, Western blot, immunoprecipitation, and immunoaffinity chromatography.

After 3 weeks in culture, medium from growth-positive hybridomas was screened for antibodies to elastin by using a solid-phase binding assay. Of 50 growth-positive wells, only two produced antibodies to elastin. These cells were cloned, recloned, and then tested again for antibody production. A single clone, designated BA-4, which remained strongly reactive with bovine α-elastin, was selected for production of mouse ascites fluid. IgG obtained from ascites fluid was used in all subsequent assays.

To assure the specificity of BA-4 IgG for elastin, inhibition ELISA assays were performed by utilizing as competing antigens collagens type I and III, which are constituents of nuchal ligament extracellular matrix and sometimes detected as minor contaminants in elastin preparations. Neither collagen type inhibited BA-4 binding to α-elastin-coated microtiter plates; however, strong inhibition of antibody binding was obtained with α-elastin peptides (Figure 1).

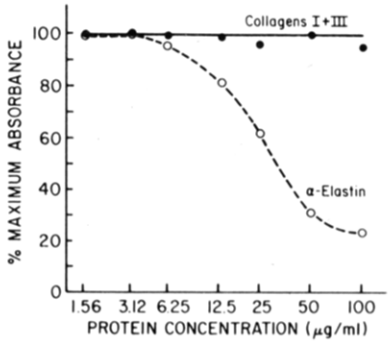

FIGURE 1: Competition ELISA demonstrating binding of BA-4 IgG to bovine α-elastin-coated polystyrene dishes following preincubation of antibody with bovine α-elastin, collagen type I, or collagen type III.

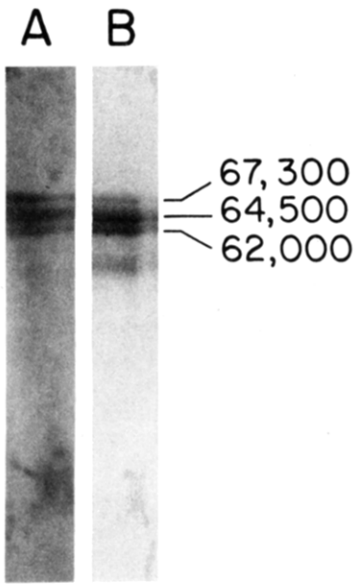

FIGURE 2: Reactivity of BA-4 IgG to bovine ligamentum nuchae tropoelastin. Lane A: Immunoblot analysis of purified tropoelastin separated by gradient SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose. Lane B: Autoradiogram of a gradient SDS–polyacrylamide gel showing [^{3}H]leucine-labeled tropoelastin immunoprecipitated by BA-4 IgG from culture medium conditioned by fetal ligamentum nuchae fibroblasts.

Evidence of BA-4 reactivity with tropoelastin, the soluble, non-cross-linked precursor of insoluble elastin, was obtained by immunoblotting and immunoprecipitation. Tropoelastin prepared by alcohol fractionation of acetic acid extracts of bovine ligamentum nuchae tissue minces was chromatographed on SDS–PAGE, transferred to nitrocellulose, and incubated with BA-4 IgG. As shown in Figure 2, three immunoreactive bands were demonstrated at 67 300, 64 800, and 62 000 daltons, corresponding to the molecular weights of three tropoelastin isoforms (Wrenn et al., submitted for publication). Lower molecular weight immunoreactive bands in the blot correspond to known proteolytic fragments that are frequently present in tropoelastin preparations (Mecham & Foster, 1977). Furthermore, BA-4 IgG immunoprecipitated radiolabeled proteins secreted into culture medium by elastin-producing cells that were resolved on SDS–PAGE autoradiograms as three distinct bands migrating at the same molecular weights as authentic tropoelastin (Figure 2).

Species Cross-Reactivity. The binding of monoclonal antibody to nonbovine elastins was tested by incubating BA-4 IgG with microtiter dishes coated with porcine, sheep, human, hamster, rabbit, and rat α-elastins. The results (Figure 3) show cross-reactivity of antibody with porcine and sheep

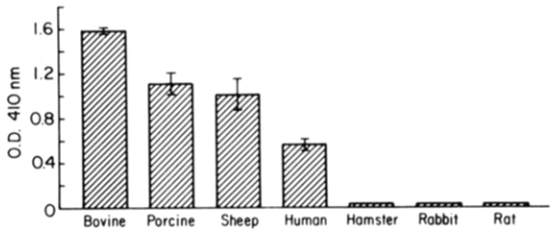

FIGURE 3: Species cross-reactivity of BA-4 monoclonal. Microtiter plates were coated with 160 ng/well bovine, porcine, sheep, human, hamster, and rat α-elastins. Shown is the relative color development per well (mean ± standard deviation of triplicate determinations) following incubation with BA-4 IgG and peroxidase-conjugated second antibody.

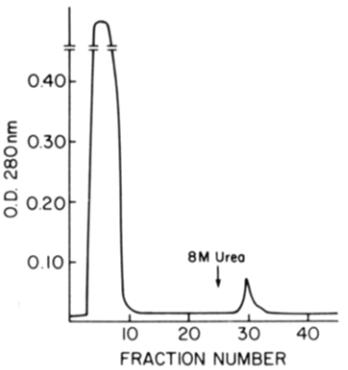

FIGURE 4: Elution profile of elastase-generated elastin peptides from an immunoaffinity column prepared by coupling BA-4 IgG to an affinity support. Specifically bound peptides were eluted from the column with 8 M urea.

elastin, lesser reactivity with human elastin, and little or no cross-reactivity with hamster, rabbit, and rat elastins.

Characterization of Antigenic Epitope. To determine the epitope on the elastin molecule that is recognized by BA-4 IgG, elastin peptides generated by elastase digestion of insoluble bovine ligamentum nuchae elastin were chromatographed over an affinity column constructed of BA-4 antibody covalently linked to Affi-Gel-10 resin. Figure 4 displays the elution profile of elastin peptides from the BA-4 affinity column. The majority of the material eluted in the column void volume. Elution of the remaining, specifically bound peptide(s) required the inclusion of 8 M urea in the column buffer as repeated attempts to elute this material with high salt (>2 M NaCl) or 2.5 M potassium thiocyanate or by lowering the pH to 2 with 0.2 M glycine–HCl were unsuccessful.

On electrophoresis through a 7.5–12.5% SDS–polyacrylamide gradient gel the eluted peptide(s) migrated as a single band near the tracking dye at a molecular weight less than 10 000. When chromatographed with reverse-phase HPLC, the affinity-purified material eluted as a single peak as shown in Figure 5 and inhibited binding of BA-4 IgG to α-elastin coated dishes (Figure 5, inset). The amino acid composition of material recovered from HPLC was valine, glycine, alanine, and proline in molar ratio of approximately 2:2:1:1, respectively, a composition identical with the hexapeptide Val-Gly-Val-Ala-Pro-Gly, which repeats six times within a stretch of 57 amino acids in the hydrophobic, non-cross-linked region of porcine elastin.

Chemotactic Activity of Immunoaffinity-Purified Peptide. Because the VGVAPG hexamer has been shown to be a chemoattractant for bovine fibroblasts and human monocytes, it was of interest to determine if the immunoaffinity-purified peptide(s) was biologically active in a chemotaxis assay. Figure 6 shows that the isolated peptide produced a chemotactic response by fibroblasts and that this response was in-

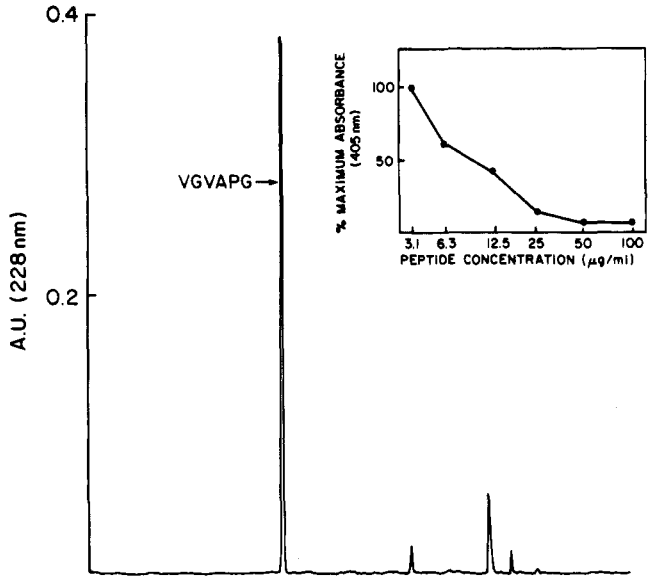

FIGURE 5: Peptide material eluted from the BA-4 affinity column (Figure 4) was dialyzed against dilute acetic acid using Spectrapor 3 dialysis membrane (molecular weight cutoff of approximately 3500), lyophilized, and resuspended in 100 mM phosphate buffer, pH 7.5. An aliquot was then chromatographed by C_{18} reverse-phase HPLC as described in Materials and Methods. Column effluent was monitored at a wavelength of 228 nm. The inset presents the results of a competition binding assay demonstrating that increasing concentrations of HPLC-purified peptide (large peak on the chromatogram) block antibody binding to α-elastin.

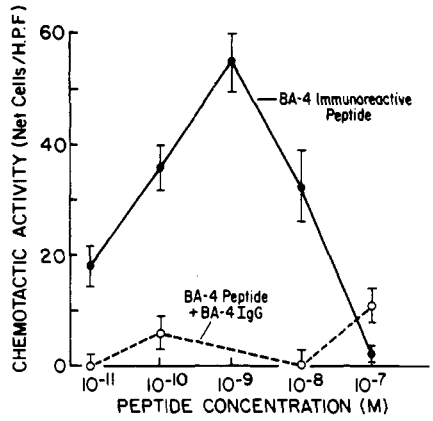

FIGURE 6: Chemotaxis of fibroblasts to immunoaffinity-purified peptide. Peptide material immunoreactive with BA-4, purified by reverse-phase HPLC (see Figure 5), was assayed for chemotactic activity for ligamentum nuchae fibroblasts by a double micropore membrane system in modified Boyden chambers. The positive chemotactic response (mean ± SEM, $n = 15$) was blocked by incubation of peptide with BA-4 IgG prior to the chemotaxis assay.

hibited when the peptide was incubated with BA-4 IgG prior to the chemotaxis assay.

To investigate the possibility that the epitope recognized by BA-4 was VGVAPG, the ability of monoclonal antibody to block chemotaxis to a synthetic peptide with the VGVAPG sequence was examined. As shown in Figure 7, preincubation with BA-4 IgG completely inhibited the chemotactic activity of synthetic VGVAPG peptide for fibroblasts but inhibited only partially fibroblast chemotaxis to the mixture of peptides present in elastase-solubilized insoluble elastin, an observation that is in agreement with our previous findings of multiple, yet distinct, chemotactic sites on the molecule (Senior et al., 1984). The specificity of inhibition of VGVAPG chemotaxis by BA-4 was confirmed by the observation that preimmune mouse serum exerted no inhibition upon the activity of

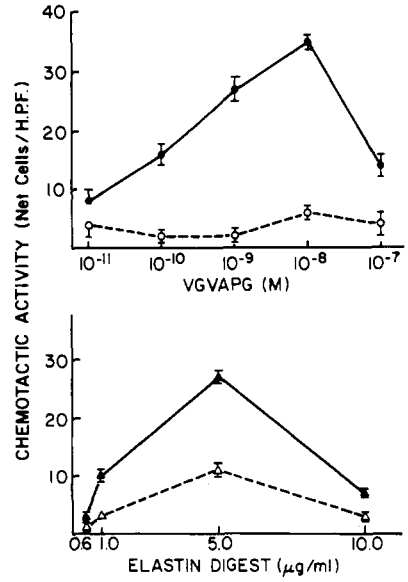

FIGURE 7: Effect of BA-4 monoclonal IgG on ligamentum nuchae fibroblast chemotaxis to synthetic VGVAPG (top) and elastase-generated peptides from insoluble elastin (bottom). BA-4 IgG completely blocked chemotaxis of fibroblasts to VGVAPG (open circles) but produced only a partial inhibition of chemotactic activity associated with elastin peptides (open triangles). Mean and standard error of the mean are shown ($n = 15$).

VGVAPG nor did BA-4 IgG alter fibroblast chemotaxis to nonelastin chemoattractants, such as platelet-derived growth factor. Furthermore, immune complexes consisting of VGVAPG and BA-4 IgG did not alter the chemotatic activity of monocytes to formylmethionylleucylphenylalanine (not shown), indicating that immune complexes do not have a general inhibitory effect upon chemotaxis. These results strongly suggest that VGVAPG is the domain on elastin that is recognized by BA-4 monoclonal antibody.

Discussion

In this study, we have described a monoclonal antibody against elastin that localizes to a chemotactically active sequence within the elastin molecule. The data support our earlier finding (Senior et al., 1980, 1984) that elastin, like fibronectin and other matrix proteins, contains a biologically active domain that interacts specifically with cells.

The antigenic epitope of elastin recognized by this monoclonal antibody (BA-4) was found to consist of the nonpolar amino acids valine, glycine, alanine, and proline. Although the amino acid sequence for this epitope was not determined, its composition is identical with the hexapeptide VGVAPG, which has been shown to have chemotactic activity for fibroblasts and monocytes (Senior et al., 1984). That BA-4 IgG reacts with synthetic VGVAPG and blocks its chemotactic activity provides strong evidence that this sequence defines a dominant cell recognition site in bovine elastin.

When enzymatically fragmented elastin is tested in chemotaxis assays, preincubation with BA-4 IgG reduces but does not ablate fibroblast movement to the elastin peptides. These results are suggestive of other cell recognition sequences on the elastin molecule that are immunologically distinct from VGVAPG. The nature of other chemotactically active sequences is unknown. Since the hydrophobic domains of elastin contain numerous repeating sequences that are similar to the VGVAPG hexapeptide repeat (Sandberg et al., 1982), it is possible that other regions of the molecule contain sufficient sequence homology to provide a chemotactic signal and yet remain antigenically unique. Our studies with synthetic

peptides have been confined to the hexamer so that we do not as yet know whether all or only a portion of the hexameric sequence is responsible for cell recognition.

The cell-recognition sequence described in this study is strikingly different from the hydrophilic tetrapeptide Arg-Gly-Asp-X (RGDX) that defines the cell-attachment domain of fibronectin (Pierschbacher et al., 1985). Yet, the strong immunogenicity of the BA-4 epitope suggests that it, like the RGDX sequence in fibronectin, is accessible at the molecular surface. According to the known preference for proteins to bury hydrophobic residues and for antigenic epitopes to be surface expressed, one would not predict the hydrophobic BA-4 epitope to be immunogenic (Hopp & Woods, 1981). Interestingly, however, biophysical studies have shown that $(VGVAPG)_n$ forms a rigid β spiral conformation that is predicted to create a hydrophobic ridge on the surface of the elastin molecule (Urry et al., 1975; Urry, 1978).

The persistence of strong immunoreactivity in peptide fragments of elastin following elastase digestion suggests that the VGVAPG sequence is resistant to proteolytic degradation. The structural and proteolytic stability of the BA-4 epitope is potentially important for regulating, or expressing immunologic, chemotactic and perhaps other biological activities associated with this site.

These results, which support earlier data of chemotaxis to elastin peptides, suggest that elastin, like collagen, fibronectin, laminin, and proteoglycans, interacts specifically with receptors at the cell surface. The reactivity of monoclonal antibody BA-4 to a cell recognition site on elastin provides a powerful research tool to investigate receptors for elastin.

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