Val-Gly-Val-Ala-Pro-Gly, a Repeating Peptide in Elastin, Is Chemotactic for Fibroblasts and Monocytes

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ABSTRACT Recent studies have demonstrated that tropoelastin and elastin-derived peptides are chemotactic for fibroblasts and monocytes. To identify the chemotactic sites on elastin, we examined the chemotactic activity of Val-Gly-Val-Ala-Pro-Gly (VGVAPG), a repeating peptide in tropoelastin. We observed that VGVAPG was chemotactic for fibroblasts and monocytes, with optimal activity at $\sim 10^{-8}$ M, and that the chemotactic activity of VGVAPG was substantial (half or greater) relative to the maximum responses to other chemotactic factors such as platelet-derived growth factor for fibroblasts and formyl-methionyl-leucyl-phenylalanine for monocytes. The possibility that at least part of the chemotactic activity in tropoelastin and elastin peptides is contained in VGVAPG sequences was supported by the following: (*a*) polyclonal antibody to bovine elastin selectively blocked the fibroblast and monocyte chemotactic activity of both elastin-derived peptides and VGVAPG; (*b*) monocyte chemotaxis to VGVAPG was selectively blocked by preexposing the cells to elastin peptides; and (c) undifferentiated (nonelastin producing) bovine ligament fibroblasts, capable of chemotaxis to platelet-derived growth factor, did not show chemotactic responsiveness to either VGVAPG or elastin peptides until after matrix-induced differentiation and the onset of elastin synthesis. These studies suggest that small synthetic peptides may be able to reproduce the chemotactic activity associated with elastin-derived peptides and tropoelastin.

Chemotactic activity has been associated with several components of the extracellular matrix including collagen (18), fibronectin (1, 5, 17), laminin (27), peptides of insoluble elastin (6, 22, 23), and tropoelastin (23). The sites of chemotactic activity on collagen (16) and fibronectin (1, 25) have been partially localized, however the chemotactically active regions of laminin and elastin have not yet been established. The present study was undertaken to identify the site(s) of chemotactic activity on elastin.

In approaching the molecular basis of the chemotactic activity of elastin peptides two points seemed important: first, the presence of fibroblast chemotactic activity in tropoelastin (23), the soluble precursor of insoluble elastin (20), suggested that the lysine-derived cross-links characteristic of insoluble elastin are not essential for the chemotactic activity of elastin peptides; and second, the presence of repeating peptide sequences in tropoelastin (4, 19) directed us to look at these peptide repeats as the possible source of the chemotactic activity. In this report, we present evidence that some of the chemotactic activity of elastin is associated with Val-Gly-Val-Ala-Pro-Gly (VGVAPG),[1] a hexamer that repeats six times in one tryptic fragment of porcine tropoelastin (20).

MATERIALS AND METHODS

Preparation of Elastin-derived Peptides: Bovine ligament elastin, obtained from Elastin Products (St. Louis, MO), was solubilized with porcine pancreatic elastase (Sigma Chemical Co., St. Louis, MO) by incubation at a substrate to enzyme ratio of 1:100 (wt/wt) for 24 h at 37°C. The elastase was removed from the elastin digest by affinity chromatography as previously described (23) and the resultant elastin peptides were lyophilized. The concentration of elastin peptides in solution was based upon the dry weight.

[1] *Abbreviations used in this paper:* Boc, tert-butyloxycarbonyl; Bzl, benzyl ester; C5fr, the chemotactic fragments of human C5; EtOAc, ethyl acetate; FMLP, formyl-methionyl-leucyl-phenylalanine; m.p., melting point; PDGF, platelet-derived growth factor; Pet, petroleum; VGVAPG, valyl-glycyl-valyl-alanyl-prolyl-glycine.

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Synthesis of VGVAPG: Amino acid derivatives were obtained from Bachem Inc. (Torrance, CA) and were of the L-configuration, except for glycine. Isobutyl chloroformate, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride, and 1-hydroxybenzotriazole were purchased from Aldrich Chemical Co. (Milwaukee, WI). Melting points were determined with a Thomas Hoover apparatus and are uncorrected. Thin layer chromatography (TLC) was performed on Whatman silica gel plates (Whatman, Inc., Clifton, NJ) with the following solvent systems: chloroform (C), methanol (M), acetic acid (A); relative flow rate (R_f), CM (5:1); R_f, CMA (95:5:3); R_f, CMA (85:15:3); R_f, CMA (75:25:3); R_f, butanol:acetic acid:water:ethyl acetate (1:1:1:1). The purity of the peptides was also confirmed by carbon-13 nuclear magnetic resonance spectra obtained on a JEOL PFT 100 pulse spectrometer operating at 25.15 MHz with proton noise spin decoupling and an internal deuterium lock. Elemental analyses were performed by MicAnal (Tucson, AZ).

(*a*) Boc-Val-Gly-OBzl: To tert-butyloxycarbonyl-valine (Boc-Val) (8.69 g, 0.04 mol) in acetonitrile (100 ml) cooled to 0°C was added *N*-methyl morpholine (4.4 ml, 0.04 mol). The solution was cooled to −15°C and isobutylchloroformate (5.24 ml, 0.04 mol) was added slowly under stirring while maintaining the temperature at −15 ± 1°C. After stirring the reaction mixture for 15 min. at this temperature, a pre-cooled (−10°C) solution of glycine-benzyl-ester p-tosylate (13.5 g, 0.04 mol) and *N*-methyl morpholine (4.4 ml, 0.04 mol) in dimethyl formamide, 35 ml, was added and stirred overnight at room temperature. Acetonitrile was removed under reduced pressure and the residual dimethyl formamide solution was poured into a cold 4% $NaHCO_3$ solution and stirred for 30 min. The precipitate obtained was filtered, washed sequentially with water, 20% citric acid solution, and water and dried to obtain 13.5 g of the product (yield: 92.6%). A sample was crystallized from ethyl acetate (EtOAc)/petroleum (Pet) ether to give a product with a melting point (m.p.) 80–82°C; TLC: R_f, 0.86; R_f, 0.53; R_f, 0.93. The analysis calculated for $C_{19}H_{28}N_2O_5$ 1/2H_2O was C, 62.44%; H, 7.80%; N, 7.65%. The observed composition was C, 62.62%; H, 7.74%; N, 7.69%.

(*b*) Boc-Val-Gly-OH: Boc-Val-Gly-OBzl (5 g, 13.72 mmol) in glacial acetic acid (50 ml) was hydrogenated at 40 psi in the presence of 10% palladium-carbon catalyst (0.5 g for 6 h. The catalyst was filtered and the filtrate concentrated under reduced pressure. The residue was taken in 4% $NaHCO_3$ solution and extracted with EtOAc (three times). The aqueous solution was cooled, acidified to pH 2 and the peptide extracted into $CHCl_3$ after saturating with NaCl. The combined $CHCl_3$ extracts were washed once with saturated NaCl solution, dried over anhydrous $MgSO_4$ and the solvent removed under reduced pressure. The peptide was precipitated from EtOAc/Pet ether, 3.83 g (Yield: 100%); m.p. 109–113°C; TLC: R_f, 0.25; R_f, 0.51, R_f, 0.7. The analysis calculated for $C_{12}H_{22}N_2O_5$ was C, 52.54%; H, 8.08%; N, 10.21%. The observed composition was C, 52.34%; H, 8.22%; N, 10.28%.

(*c*) Boc-Val-Gly-Val-Ala-Pro-Gly-OCH_3: Boc VAPG-OCH_3 (14) (6 g, 12.89 mmol) was treated with trifluoroacetic acid, 60 ml, for 1 h and the trifluoroacetic acid removed under reduced pressure. The residue was triturated with ether:Pet ether (1:1), decanted, and the oily residue was dried over P_2O_5 and NaOH in a vacuum desiccator. To II (2.92 g, 10.65 mmol) and 1-hydroxybenzotriazole (1.79 g, 11.715 mmol) in dimethyl formamide (25 ml) cooled to −15°C was added 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (2.25 g, 11.715 mmol) and stirred for 15 min. To this was added a pre-cooled (−10°C) solution of the above trifluoroacetic acid salt of the peptide (5 g, 10.65 mmol) in dimethyl formamide (25 ml). The reaction mixture was allowed to come to room temperature and stirred over night. Solvent was removed under reduced pressure and the residue taken in $CHCl_3$ and extracted with water, 4% $NaHCO_3$, water, 10% citric acid, water, dried over anhyd. $MgSO_4$ and the solvent removed. The peptide was crystallized from EtOAc/Pet ether, 3.08 g (yield: 47.2%), m.p. shrinks at 97°C and completely melts at 118°C; TLC: R_f, 0.61; R_f, 0.3; R_f, 0.8. Analysis calculated for $C_{28}H_{48}N_6O_9$ H_2O was C, 53.31%; H, 7.99%; N, 13.32%. The observed analysis was C, 53.40%; H, 7.81%; N, 13.27%.

(*d*) Boc-Val-Gly-Val-Ala-Pro-Gly-OH: Boc-Val-Gly-Val-Ala-Pro-Gly-OCH_3 (1 g, 1.62 mmol) in methanol (10 ml) was stirred with 1 N NaOH (2.02 ml, 2.023 mmol) for 1 h and methanol removed under reduced pressure. The residue was diluted with water and extracted with EtOAc. The aqueous solution was cooled, acidified to pH 2, saturated with NaCl and extracted with $CHCl_3$ (three times). The combined $CHCl_3$ extracts were washed with saturated NaCl, dried over anhydrous $MgSO_4$, and the solvent removed under reduced pressure. The peptide was crystallized from EtOAc/Pet ether, 0.95 g (yield: 98%), m.p. 125–130°C (decomp); TLC: R_f, 0.36; R_f, 0.56. Analysis calculated for $C_{27}H_{46}N_6O_9$ was C, 54.16; H, 7.74%; N, 14.03%. The observed analysis was C, 54.2%; H, 8.25%; N, 14.17%.

(*e*) HCO_2H·H-Val-Gly-Val-Ala-Pro-Gly-OH: A solution of Boc-Val-Gly-Val-Ala-Pro-Gly-OH (0.6 g, 1 mmol) in 95–97% formic acid (16.5 ml) was stirred for 2.5 h and formic acid removed under reduced pressure. The residue was dissolved in water and lyophilized to obtain the title compound in quantitative yield. TLC: R_f, 0.55.

Chemotactic Factors: Platelet-derived growth factor (PDGF), purified from outdated platelet packs (3), was kindly provided by Dr. Thomas F. Deuel, (Jewish Hospital at Washington University Medical Center, St. Louis, MO) and C5-derived chemotactic activity from human serum (C5fr) was a gift from Dr. Donald L. Kreutzer (University of Connecticut Health Center, Farmington). Formyl-methionyl-leucyl-phenylalanine (FMLP) was purchased from Sigma Chemical Corp. (St. Louis, MO).

Antielastin IgG: IgG was prepared from normal rabbit serum and from rabbit antiserum to bovine ligamentum nuchae alpha-elastin by column chromatography using DEAE Affigel-Blue (Bio-Rad Laboratories, Richmond, CA) as described (12). The specificity of the antiserum for elastin has been demonstrated previously (9, 10).

Cells: Fetal bovine ligament nuchae fibroblasts were obtained and cultured as previously described (11). Human mononuclear peripheral blood cells were separated from blood, donated by healthy volunteers, using Ficoll-Hypaque gradients (2). Undifferentiated ligament fibroblasts were induced to synthesize elastin by contact with extracellular matrix material from the ligament of a late gestation calf using procedures recently presented (13).

Assays of Chemotaxis: Chemotaxis was assayed by a double micropore membrane system in modified Boyden chambers, by previously described procedures (21, 22). Briefly, the lower compartment of the chamber, containing 240 μl of test material or basal medium, was separated from the upper compartment containing 360 μl of cell suspension (1.2×10^5 fibroblast/ml or 2.5×10^6 mononuclear cells/ml), by either a 8 μm (fibroblast) or a 5 μm (monocyte) polycarbonate membrane (Nucleopore Corp., Pleasanton, CA) which was overlying a 0.45-μm cellulose nitrate membrane (Millipore Corp., Bedford, MA). After the upper and lower compartments were filled, the chambers were incubated for either 2 h (monocytes) or 6 h (fibroblasts) at 37°C in 5% CO_2-balance air. The chambers were then disassembled and the membrane pairs removed and stained with hematoxylin. In each experiment, the data points represent the mean of cell counts of five high power (× 400) fields on each of three membrane pairs. The results are reported as the mean number of cells that migrated through the upper membrane, corrected for blanks as determined by chambers containing only medium in the lower compartment. All experiments included positive controls: for fibroblast studies, PDGF, 30 ng/ml (1 nM), was put in the lower compartment; for monocyte studies, FMLP, 10^{-8} M and/or C5fr was put in the lower compartment.

To assess the capacity of elastin peptides and VGVAPG to desensitize monocytes to the chemotactic activity of elastin peptides and VGVAPG, we incubated mononuclear cells for 30 min at 25°C with either elastin peptides, 100 μg/ml or VGVAPG, 10^{-8} M, respectively, and then washed the cells three times with basal medium. Chemotaxis was then assayed as described above.

The effects of antielastin IgG on chemotaxis to VGVAPG and elastin peptides were determined by preincubating antielastin IgG with either VGVAPG or elastin peptides for 60 min at 37°C and then assaying the mixture for chemotactic activity. In these experiments, controls included (*a*) VGVAPG or elastin peptides preincubated with preimmune IgG and (*b*) mixtures of antielastin IgG preincubated with PDGF.

RESULTS

Chemotactic Activity of VGVAPG

VGVAPG caused fibroblast and monocyte migration, with a maximal activity in the range of 10^{-9}–10^{-8} M (Fig. 1). At

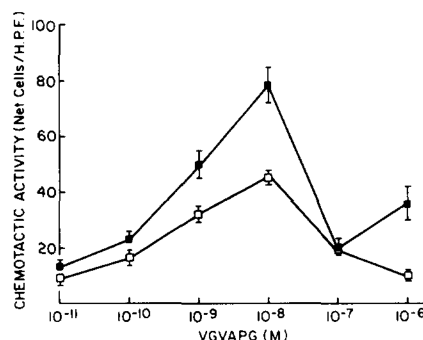

FIGURE 1 Fibroblast and monocyte migration in response to VGVAPG. Mean and standard error are shown, $n = 15$. Background cell migration was 16 for the fibroblast (□) studies and 36 for the monocyte (■) experiments.

Table I

Fibroblast Chemotaxis to VGVAPG

VGVAPG (M), lower compartment	VGVAPG (M), upper compartment			
	0	10^{-10}	10^{-9}	10^{-8}
0	(7)	1 ± 0.6	0 ± 0.7	−2 ± 0.5
10^{-10}	4 ± 1.0*	2 ± 0.7	−3 ± 0.4	1 ± 1.0
10^{-9}	15 ± 1.7	7 ± 1.3	0 ± 0.4	0 ± 0.6
10^{-8}	27 ± 1.7	13 ± 1.8	3 ± 0.6	1 ± 0.7

* Net cells per high power field ± SEM, n = 15. Positive control PDGF, 10^{-9} M = 31 cells per high power field.

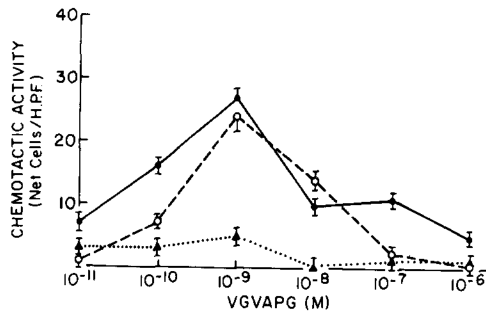

Figure 2 The effect of antielastin IgG on the fibroblast chemotactic activity of VGVAPG. In these studies background cell migration was 11. (●) No IgG. (O) Pre-immune IgG. (▲) Antielastin IgG. The error bars are as in Fig. 1.

the peak of activity, the number of cells that migrated was nearly as great as that observed with the optimal PDGF concentration for fibroblast chemotaxis, 10^{-9} M (21), and the optimal concentration of FMLP for monocyte chemotaxis, 10^{-8} M. By "checker board analysis" the fibroblast migration to VAVAPG was chemotactic, not chemokinetic (Table I).

Effect of Antielastin IgG upon the Chemotactic Activity of VGVAPG

Polyclonal antielastin IgG, previously shown to inhibit the chemotactic activity of elastin peptides for monocytes (22), abolished the chemotactic activity of VGVAPG (Fig. 2). Specificity of the inhibition was shown by demonstrating that antielastin IgG had no inhibitory effect against the chemotactic activity of PDGF and that pre-immune rabbit IgG exerted no inhibition upon the chemotactic activity of VGVAPG. Moreover, immune complexes consisting of VGVAPG plus antielastin IgG did not block the chemotactic activity of FMLP, indicating that these immune complexes do not have a generalized inhibitory effect upon chemotaxis.

Desensitization to VGVAPG

Preexposure of monocytes to elastin peptides before testing them for chemotaxis completely abolished their chemotactic responses to both elastin peptides and VGVAPG (Fig. 3*A*). The loss of chemotactic responsiveness was specific since the cells still migrated normally to two other unrelated chemotactic factors, C5fr and FMLP. Preincubation with VGVAPG completely blocked chemotaxis to VGVAPG but only partially reduced chemotactic responses to elastin peptides (Fig. 3*B*), suggesting that elastin peptides contain chemotactically active sequences other than VGVAPG.

Chemotactic Responses of Undifferentiated Ligament Fibroblasts to VGVAPG

Undifferentiated ligament fibroblasts showed no migration in response either to VGVAPG or elastin peptides, but were fully responsive to PDGF (Fig. 4, *top*). After these cells were exposed to extracellular matrix from 270-d gestation bovine ligamentum nuchae, however, they responded to both VGVAPG and elastin peptides (Fig. 4, *bottom*).

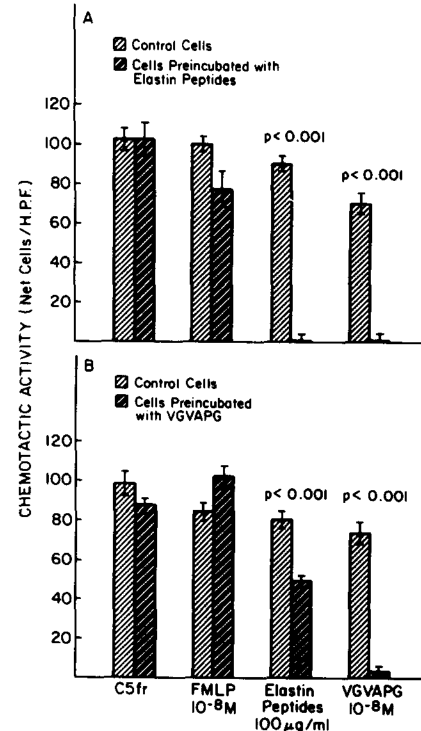

Figure 3 Monocyte chemotaxis to C5fr, FMLP, elastin peptides, and VGVAPG after preincubating the cells with either (*A*) elastin peptides or (*B*) VGVAPG. The background numbers of migrating cells for these experiments were 49 and 51, respectively. The error bars are as in Fig. 1.

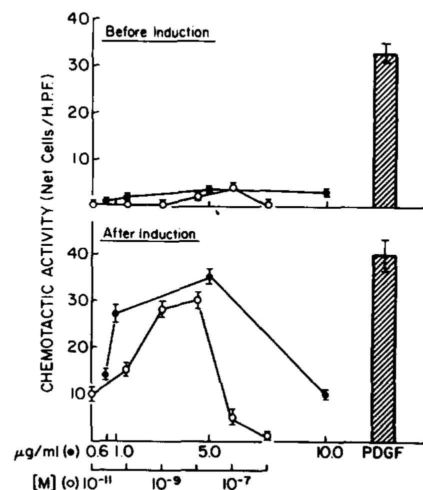

Figure 4 Chemotaxis of undifferentiated ligament fibroblasts to VGVAPG and elastin peptides before and after the cells were induced to synthesize elastin by exposing them to matrix from differentiated ligament fibroblasts. (*Top*) Before induction. (*Bottom*) After induction. Background cell migration for these studies was 19 and 25, respectively. (●) Elastin peptides. (O) VGVAPG. The error bars are as in Fig. 1. The PDGF standards were 30 ng/ml, 10^{-9} M.

DISCUSSION

Working with tryptic fragments of tropoelastin extracted from the aortas of copper-deficient pigs, Sandberg et al. (20) have succeeded in sequencing nearly all of this molecule of approximately 800 amino acid residues. The tryptic fragments of tropoelastin appear to be divisible into two groups: (*a*) small peptides of two to four amino acids, consisting mainly of alanine and lysine, and thus parts of the molecule destined to be involved in cross-links; and (*b*) more hydrophobic peptides of 17–81 amino acids, some of which contain repeating sequences (19). The principal repeating sequences are a tetrapeptide, GGVP, occurring several times in a peptide near the N-terminus, a pentapeptide, PGVGV, which repeats 11.4 times in one peptide of 81 residues, and a hexamer, VGVAPG, used in the present study, which repeats six times within a stretch of 57 amino acids. The functions served by these repeats are not yet clear, but it has been postulated that they have a specific role in the physical properties of elastin (28, 29). In addition to having chemotactic activity it is clear that the hexameric repeat is a strong antigenic epitope (10), suggesting that this region of the molecule may be an important biologically active domain of elastin.

It should be emphasized that the present work was limited to the repeating hexamer. The results therefore do not exclude the presence of chemotactic activity associated with other parts of tropoelastin or elastin peptides, especially other repeats. Indeed, the observations that the chemotactic activity to elastin peptides is only partially blocked by preexposure of the target cells to VGVAPG, strongly suggests that elastin peptides have chemotactically active sites besides VGVAPG sequences. Moreover, we have not yet determined whether all six residues are necessary for the activity of the hexamer nor whether the order of the residues is critical. It should also be noted that while there are no sequence data on bovine elastin, the source of the elastin peptides used in the present work, studies of the amino acid composition and of the immunoreactivity of elastin suggest conservation of the sequence between species (9, 10, 26).

The finding that a small hydrophobic repeating peptide in elastin is chemotactic for fibroblasts and monocytes is analogous to observations of chemotaxis to collagens, reported by Gauss-Muller et al. (5) and by Postlethwaite (16). Interstitial collagens, types I, II, and III, and basement membrane collagens types IV and V are chemotactic for fibroblasts and monocytes and the activity is retained even if the collagens are subjected to proteolytic cleavage with mammalian and bacterial collagenases or with cyanogen bromide. The chemotactic activity of isolated collagen chains matches their capacity to bind to fibroblasts and the capacity of collagen fragments to inhibit the binding of collagen chains to fibroblasts correlates with their chemotactic activity. Of particular relevance to the present report, synthetic di- and tri-peptides from collagen containing hydroxyproline also have fibroblast chemotactic activity, suggesting that cellular recognition of collagens may involve very short amino acid sequences. The cell attachment activity of fibronectin has been localized to within a 30-amino acid peptide (15). Since the chemotactic activity of fibronectin appears to be located near or coincident with the cell attachment region of the molecule (1, 7, 25), fibronectin may be another example of an extracellular matrix macromolecule with chemotactic activity that will ultimately be localized to a small peptide segment.

Hunninghake et al. (6) first demonstrated that monocytes remained chemotactically responsive to elastin peptides after exposure to activated serum, suggesting that recognition of elastin peptides by monocytes does not involve the C5a receptor. The present study confirms and extends this observation: (*a*) elastin peptides are shown not to utilize the FMLP receptors on monocytes (30); and (*b*) a peptide not previously studied for monocyte chemotaxis (VGVAPG) has been identified as a monocyte chemoattractant and to use neither the C5a or the FMLP receptors. It is also worthy of emphasis that being able to demonstrate that a synthetic analog of a repeating peptide in elastin is chemotactic for fibroblasts and monocytes strengthens the earlier reports that elastin-derived peptides are chemotactic (6, 22, 23) because it excludes the possibility that the previously reported activity was due to a contaminating substance(s) in proteolysed elastin preparations.

The physiological roles that chemotaxis to tropoelastin and elastin peptides might serve in vivo can only be speculated on at this time. Recognition by monocytes might facilitate removal of proteolysed elastin. For fibroblasts, chemotaxis to elastin peptides might serve to promote wound healing and matrix remodeling generally, however, there may be purposes for fibroblast recognition of elastin peptides and tropoelastin besides cell movement. Recognition, which we have measured in vitro as chemotaxis, might have a role in cell activities such as regulation of elastin synthesis or orientation of tropoelastin molecules on the cell surface to facilitate cross-linking.

Apart from helping to identify the sites of chemotactic activity on elastin, the results of the present study represent another illustration of changes in chemotactic responsiveness with development. We have shown elsewhere that undifferentiated ligament fibroblasts do not show chemotactic responsiveness to elastin peptides until after induction of differentiation as reflected by the onset of elastin synthesis (8). Here, we have presented a similar picture with respect to a repeating hexamer of elastin.

These results indicating that VGVAPG is an important component of the chemotactic activity of elastin peptides suggest that the hexamer will be a useful probe to explore interactions between cells and tropoelastin and elastin peptides such as binding reactions and metabolic effects. The results also indicate that the preparation of artificial elastomers using peptide repeats of tropoelastin (28, 29) may lead to materials that have biologically interesting properties as well as the physical characteristics of native elastin.

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Note Added in Proof: Recent studies indicate that tropoelastin does not completely desensitize monocytes to the chemotactic activity of elastin-derived peptides. Thus, domains unique to insoluble elastin contribute to the overall chemotactic activity of peptides generated from the insoluble molecule.

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