Appearance of Chemotactic Responsiveness to Elastin Peptides by Developing Fetal Bovine Ligament Fibroblasts Parallels the Onset of Elastin Production

ROBERT P. MECHAM, GAIL L. GRIFFIN, JUDY G. MADARAS, and ROBERT M. SENIOR

Respiratory and Critical Care Division, Department of Medicine, Jewish Hospital at Washington University Medical Center, St. Louis, Missouri 63110

ABSTRACT We studied chemotaxis to elastin peptides by bovine ligamentum nuchae fibroblasts to determine whether there is a developmental association between chemotactic responsiveness to elastin and expression of the elastin phenotype. Undifferentiated ligament cells demonstrate chemotactic responsiveness to platelet-derived growth factor and fibronectin, known chemoattractants for fibroblasts, but do not show chemotaxis to elastin peptides. After matrix-induced differentiation, however, young cells display a positive chemotactic response to elastin that persists even after the cells are removed from the matrix substratum. Matrix-induced chemotaxis to elastin could be inhibited selectively by incorporation of bromodeoxyuridine into DNA of undifferentiated cells before (but not after) contact with inducing matrix. These results show that the appearance of chemotaxis to elastin peptides parallels the onset of elastin synthesis and suggests that the acquisition of chemotactic responsiveness to elastin and expression of the elastin phenotype are affected by the same inducing elements or processes and may be closely coupled in development.

Fibroblasts exhibit directional migration (chemotaxis) to specific macromolecular components of the extracellular matrix. Recent experiments have shown that fibronectin and its cell binding domain (1, 17, 23, 27), all three interstitial collagens (22) and collagens type IV and V (7) elicit a chemotactic response in fibroblasts (for a review of fibroblast chemotaxis, see Postlethwaite [21]). In an earlier report we showed that fibroblasts from the ligamentum nuchae of a bovine fetus near term demonstrate a chemotactic response to tropoelastin and to peptides derived from cross-linked, insoluble elastin (26). Our subsequent studies have shown that undifferentiated ligament cells do not exhibit the same chemotactic response to elastin. Because undifferentiated ligament cells do not synthesize elastin in vivo or in vitro (14), we were curious as to whether chemotaxis to elastin peptides is developmentally controlled and appears in coordination with the onset of elastin synthesis. In the preceding paper (16) we showed that young fetal ligament cells can be induced to differentiate and synthesize elastin by contact with extracellular matrix (ECM)[1] from the ligament of a late gestation fetal calf. Using this in vitro induction model, we were able to test whether elastogenic differentiation is required for expression of chemotaxis to elastin. In the study described below, we show that ligament cells acquire the capacity for directed movement to elastin peptides concomitant with cell differentiation and the onset of elastin synthesis.

[1] *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; ECM, extracellular matrix; FCL, fetal calf ligament; PDGF, platelet-derived growth factor.

MATERIALS AND METHODS

Cell culture, quantification of soluble elastin production by radioimmunoassay, preparation of ligament ECM substratum, and preparation of conditioned media are as described in the preceding paper (16). Antibodies to elastin were prepared in rabbits as previously described (14).

Determination of Chemotactic Activity: Chemotactic activity was determined using modified Boyden chambers as described earlier (25, 26). A double-membrane technique, with a polycarbonate membrane (Nuclepore Corp., Pleasanton, CA), 8-μm pores, on top of a cellulose nitrate membrane (Millipore Corp., Bedford, MA), 0.45-μm pores, was used to separate each well of a multi-blind well apparatus into an upper and lower compartment (3). To facilitate cell attachment, we soaked membranes in 1% (wt/vol) poly-L-lysine HBr (>70,000 mol wt; Sigma Chemical Co., St. Louis, MO) in distilled water for 2 h at room temperature, washed for 2 h under running water, and then air dried (13). In the upper compartment was placed 0.36 ml of cell suspension containing 1.2×10^5 cells/ml in Dulbecco's modified Eagle's medium supple-

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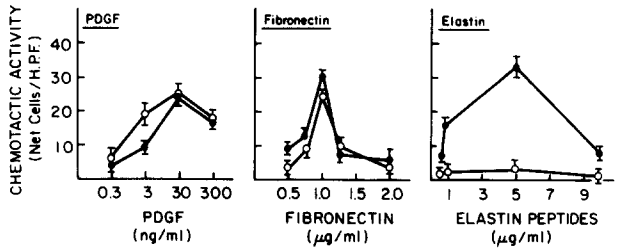

FIGURE 1 Chemotactic response of FCL-135 (open circles) and FCL-270 (solid circles) cells toward increasing concentrations of PGDF, human plasma fibronectin, and elastin peptides prepared by digesting insoluble bovine ligamentum nuchae elastin with pancreatic elastase. Mean ± SEM of 15 determinations.

mented with 1 mg/ml human serum albumin (American Red Cross Blood Services, Washington, DC). To the lower compartment was added 0.25 ml of medium containing elastin peptides derived from purified insoluble bovine ligamentum nuchae elastin by enzymatic digestion with porcine pancreatic elastase as described earlier (26). The chemotaxis apparatus was placed in a humidified incubator at 37°C for 6 h, after which it was disassembled and each membrane pair was removed and stained with hematoxylin. Cell migration was quantified under high-dry magnification (×400) by counting the cells that had moved to the interface between the two membranes and those on the lower membrane. Five fields were counted on each membrane; all samples were done in triplicate and the results from each set of three membrane pairs were pooled. Cell migration is expressed as net cell migration, that is, the number of migrated cells per high power field corrected for the number of cells that migrated in response to control medium. Chemotaxis was distinguished from chemokinesis by checkerboard analysis (26, 33). Platelet-derived growth factor (PDGF) and fibronectin were kindly provided by Thomas F. Deuel and John A. McDonald, respectively, of The Jewish Hospital at Washington University School of Medicine, St. Louis, MO.

Matrix-induced Differentiation: Acellular ECM was prepared from fetal bovine ligamentum nuchae as described in the preceding paper (16). Freshly trypsinized fetal calf ligament (FCL)[2] cells were plated on the ligament slices and cultured in Dulbecco's modified Eagle's medium–10% fetal calf serum for 3 d. Matrix-induced differentiation of young cells was assessed by the onset of elastin synthesis as determined by radioimmunoassay and immunoprecipitation of tropoelastin.

Inhibition of Differentiation by Bromodeoxyuridine: To determine whether the matrix-induced onset of chemotaxis could be blocked by bromodeoxyuridine (BrdU), we passaged FCL-270 and FCL-135 cells from explant culture at 1×10^6 cells into 100-mm^2 tissue culture dishes. Shortly before reaching visual confluency, half of the cultures were treated for 24 h with 30 μM BrdU (Sigma Chemical Company), removed from the plates using trypsin, and replated at subconfluent density on tissue culture plastic or ligament ECM from a 270-d-old bovine fetus in four groups: (*a*) cells on plastic without BrdU; (*b*) cells on plastic with BrdU; (*c*) cells on ligament ECM without BrdU; (*d*) cells on ligament ECM with BrdU. After 72 h the cultures were sampled for elastin production and cells were trypsinized and assayed for chemotaxis. In a parallel set of experiments, cells in the four groups defined above were exposed to 150 mM thymidine for 48 h before the chemotaxis assay.

The incorporation of BrdU into DNA was determined by including [^{3}H]-BrdU (2 μCi/ml, sp act 26 Ci/mmol, New England Nuclear, Boston, MA) with unlabeled BrdU in the culture medium. At the end of each incubation period, cells from one dish were collected by trypsinization and solubilized with 2% Na_2CO_3 in 0.1 N NaOH (wt/vol). Nucleic acids were precipitated with 10% trichloroacetic acid and counted for tritium radioactivity.

RESULTS

The specific chemotactic responses of undifferentiated (FCL-135) and differentiated (FCL-270) FCL cells towards platelet-derived growth factor (PDGF), fibronectin, and elastin peptides are shown in Fig. 1. Both cell strains showed positive chemotaxis to PDGF and fibronectin, but undifferentiated cells did not show a migratory response to elastin peptides.

To determine if chemotactic responsiveness to elastin was associated with expression of the elastin phenotype, we compared chemotactic activity in undifferentiated ligament cells and cells induced to differentiate by contact with ECM. Fig. 2 (*top*) shows the levels of elastin synthesis and chemotactic response to elastin peptides of FCL-135 cells before and after exposure to ECM from a 270-d-old ligament. Before exposure to the inducing matrix, the cells showed no chemotactic response to the elastin digest and were not synthesizing elastin. After contact with inducing matrix for 72 h (see preceding paper for conditions necessary for differentiation), the cells showed levels of elastin production and a migratory response to elastin equal to differentiated FCL-270 cells (Fig. 2, *bottom*). Removal of induced cells from the matrix did not affect their ability to migrate towards elastin peptides, indicating that continuous contact with the matrix substratum is not necessary to maintain their chemotactic responsiveness. Culturing undifferentiated cells on noninducing matrix (from a 140-d-old fetal ligament) did not stimulate elastin synthesis or chemotactic responsiveness to elastin, and in this regard, was similar to the response of undifferentiated cells maintained on tissue culture plastic. Growth of FCL-135 cells in medium conditioned by inducing ligament or co-culture with differentiated FCL-270 cells did not stimulate chemotactic responsiveness to elastin peptides (Fig. 2), suggesting that the acquisition of the chemotactic response requires direct cell-matrix contact.

To further substantiate that chemotaxis to elastin peptides develops concurrently with the cell's ability to synthesize elastin, we used BrdU to block induction of elastin synthesis (16). As shown in Fig. 3, incorporation of BrdU into genomic DNA of FCL-135 cells before cellular contact with inducing ECM inhibited the matrix-induced onset of elastin production

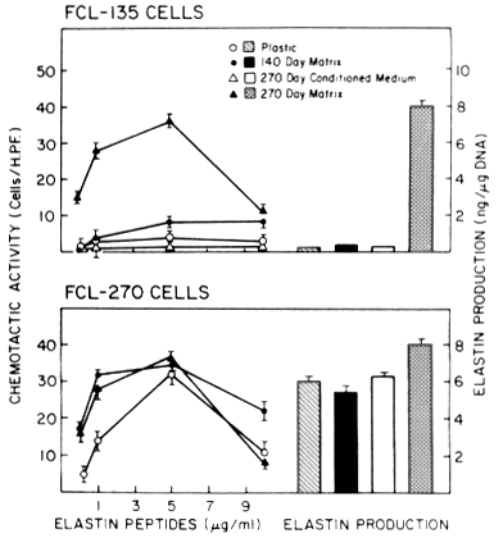

FIGURE 2 Chemotactic response (mean ± SEM, $n = 15$) of undifferentiated FCL-135 (*upper* panel) and differentiated FCL-270 (*lower* panel) cells to elastin peptides before and after culture on inducing ECM from a 270-d-old fetal ligament and noninducing matrix from a 140-d-old fetus. Also shown is the chemotactic response of FCL-135 cells cultured in medium preconditioned by inducing matrix. Elastin production, expressed as nanograms elastin per micrograms DNA, is the mean plus standard deviation of triplicate determinations.

[2] Fetal calf ligament cell strains are identified by the abbreviation FCL followed by the age of the fetus from which the cells were derived. For example, FCL-135 indicates fetal calf ligament cells from a 135-d-old fetus.

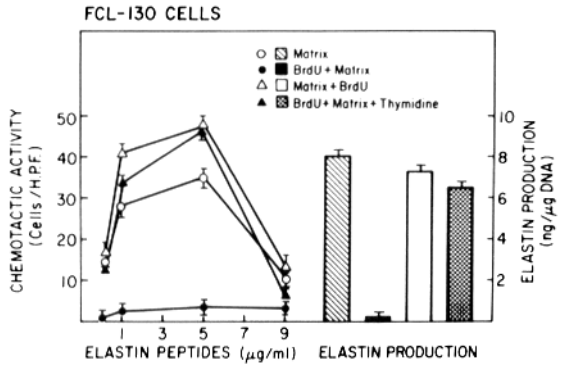

FIGURE 3 Effects of BrdU on stimulation of elastin chemotaxis by inducing ECM (mean ± SEM, $n = 15$). FCL-135 cells show positive chemotaxis to elastin peptides when grown on inducing ECM (open circles). If BrdU is added to cell culture medium 24 h before and during exposure to inducing matrix, differentiation (elastin synthesis) does not occur and the cells do not respond to elastin peptides in the chemotaxis assay (solid circles). Addition of exogenous thymidine to the culture medium or cell division on inducing ligament in the absence of thymidine reverses BrdU inhibition of elastin synthesis and chemotaxis to elastin peptides (solid triangles). BrdU added to culture medium after cells have contacted inducing matrix does not block the development of chemotactic responsiveness (open triangles). Elastin values are mean plus standard deviation of triplicate determinations.

as well as the acquisition of chemotactic activity to elastin peptides. Chemotaxis to PDGF (30 ng), however, was only slightly decreased by BrdU (47 cells per high power field for untreated cells compared to 39 cells per high power field for cells treated with 30 μM BrdU) and provided a positive control to show that BrdU did not inhibit the chemotactic response generally.

Both chemotaxis to elastin and elastin synthesis were evident when 150 mM thymidine was added to the culture medium of BrdU-inhibited cells on inducing matrix or when the cells were trypsinized and replated onto matrix in the absence of BrdU. BrdU did not inhibit elastin synthesis or the appearance of chemotactic activity to elastin peptides when added to culture medium after the cells had contacted inducing ECM (Fig. 3).

DISCUSSION

In this study we provide experimental evidence that the ability of ligament cells to show a directed migratory response (chemotaxis) to elastin peptides coincides with the cell's ability to synthesize elastin. Cells from young fetal ligaments (<180 d of gestation) that have not yet differentiated to synthesize elastin do not recognize elastin peptides in the chemotaxis assay, even though they have the capacity for chemotaxis as shown by their responses to other fibroblast chemotactic factors such as PDGF and fibronectin. Differentiated cells that synthesize elastin show positive chemotaxis to elastin peptides as well as to PDGF and fibronectin.

To test whether undifferentiated ligament cells become responsive to extracellular elastin concomitant with activation of elastin synthesis, we compared the migratory response to elastin of undifferentiated ligament cells (i.e., cells not synthesizing elastin) before and after matrix-induced differentiation and in every instance chemotaxis to elastin correlated with expression of the elastin phenotype. The close association between the development of chemotactic responsiveness to elastin and the onset of elastin production raises the possibility that the cells's ability to "recognize" elastin peptides is an important aspect of elastogenic differentiation.

Fibroblasts are known to play an important role in tissue repair and regeneration and movement of cells to sites of tissue damage is an essential feature of tissue restoration. The release of soluble elastin peptides at sites of inflammation (facilitated by elastases secreted by inflammatory cells) could serve as specific signals to attract cells to assist in remodeling damaged connective tissue.

Recognition of elastin peptides may have important functions in addition to guidance of cells to sites of tissue damage, especially in a tissue such as the ligamentum nuchae. It is now clear that the cell surface is of major importance in regulating many aspects of differentiation and cell metabolism and that many of these events are mediated by cell surface receptors. Indeed, it is generally accepted that chemotaxis results from ligand binding to specific receptors on the target cell (2, 6, 29, 32, 33) and in this regard it is intriguing to speculate that the chemotactic response of FCL fibroblasts results from the appearance or activation during differentiation of a specific elastin "receptor" or "binding site" on the cell surface. This possibility is supported by our observation that BrdU blocks matrix-induced chemotaxis to elastin peptides, suggesting that activation of chemotactic responsiveness during differentiation requires transcription of a gene(s) that is not expressed in the undifferentiated cell. There is ample precedent for the appearance of specific receptors as a result of differentiation-associated events (4, 9, 10, 19, 20, 24, 28) and there is accumulating evidence for receptors for matrix molecules on connective tissue cells. In particular, specific receptors or binding sites have been suggested for collagen (6, 8, 18), hyaluronate (31), fibronectin (12), and laminin (30). The functional relationship between these receptors and cellular metabolism has not been fully established although specific receptors have been implicated in chemotaxis of fibroblasts to collagen (6), in the binding and uptake of collagen peptide fragments by fibroblasts (11), in aggregation of platelets by collagen (5), in the attachment of cells to collagen and plastic substrata (12), as binding sites for hyaluronate (31), as binding sites for type II collagen on chondrocytes (18), and involved in the initial interaction of tumor cells via laminin with the vascular basement membrane (30).

Although the existence of an elastin receptor or binding site on the fibroblast cell surface must await further investigation, the results presented in this and the preceding studies (15, 16) demonstrate that the ligament cell is highly responsive to its extracellular milieu. The capacity of the cell to react to its external environment is critically dependent upon its ability to recognize external stimuli. The appearance of chemotactic responsiveness to elastin peptides concomitant with the acquisition of the elastin phenotype is suggestive of a highly specific mechanism whereby the ligament cell can respond to physiological factors that are important for the cell to discharge its differentiated function.

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