A simple cell motility assay demonstrates differential motility of human periodontal ligament fibroblasts, gingival fibroblasts, and pre-osteoblasts

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Abstract During periodontal regeneration, multiple cell types can invade the wound site, thereby leading to repair. Cell motility requires interactions mediated by integrin receptors for the extracellular matrix (ECM), which might be useful in guiding specific cell populations into the periodontal defect. Our data demonstrate that fibroblasts exhibit differential motility when grown on ECM proteins. Specifically, gingival fibroblasts are twice as mobile as periodontal ligament fibroblasts, whereas osteoblasts are essentially non-motile. Collagens promote the greatest motility of gingival fibroblasts in the following order: collagen III > collagen V > collagen I. Differences in motility do not correlate with cell proliferation or integrin expression. Osteoblasts display greater attachment to collagens than does either fibroblast population but lower motility. Gingival fibroblast motility on collagen I is generally mediated by α2 integrins, whereas motility on collagen III involves α1 integrins. Other integrins (α10 or α11) may also contribute to gingival fibroblast motility. Thus, ECM proteins do indeed differentially promote the cell motility of periodontal cells. Because of their greater motility, gingival fibroblasts have more of a potential to invade periodontal wound sites and to contribute to regeneration. This finding may explain the formation of disorganized connective tissue masses rather than the occurrence of the true regeneration of the periodontium.

Keywords Gingival fibroblast - Periodontal ligament fibroblast - Osteoblast - Integrin - Collagen type I - Collagen type III - Collagen type V - Human

Introduction

The periodontium is composed of the cementum overlying the dentinal root surface and the alveolar bone connected by the periodontal ligament (PDL). Together, these combine to form the structural attachment of the tooth that provides both anchorage and cushioning for the mechanical stresses of mastication (Nanci and Bossardt 2006). Encasing this structure is the gingiva, a more loosely organized mesenchymal tissue, and its overlying epithelia (Bartold et al. 2000). During wound repair and tissue regeneration, all of these tissues become reorganized (Wikesjo et al. 1992). Although maintenance of this structural unit is necessary for tooth retention, little is known about the molecular mechanisms regulating the proper formation of these structures.

The extracellular environment plays several roles in tissue formation and regeneration. Its molecules act as chemotactic factors for invading cells and provide the highways for motility. The extracellular matrix (ECM) as a whole provides structural support for tissues and scaffolding for tissue regeneration (Badylak 2002; Robinson et al. 2005; Zantop et al. 2006) and has three major components: collagen...
fibers and non-collagenous glycoproteins and proteoglycans (Hay 1981). Each of these components is represented by a variety of molecules that interact differently with the various cell types and that serve different functions. For instance, the basal lamina underlying the oral epithelium is largely composed of collagen type IV, the glycoprotein laminin, and heparin sulfate proteoglycans (Steffensen et al. 1992). The gingiva proper contains several fibrous collagens (types I and III) and the glycoprotein fibronec. The PDL contains the fibrous collagens (types I and III) and high levels of vitronectin (Steffensen et al. 1992). The calcified tissues of bone, cementum, and dentin are composed largely of type I collagen fibers and hydroxyapatite, with collagen type III fibers also being associated with Sharpey’s fibers attaching the ligamentum to bone and cementum (Rao et al. 1979; Somerman et al. 1988; Tung et al. 1985; Wang and Waco 1980). Thus, these tissues express complex and varied ECM molecules that are likely to be important in tissue remodeling and regeneration. The source of the cells involved in the regeneration of the periodontal attachment remains unclear, although these cells probably originate within either the gingiva or the PDL. Fibroblasts of the PDL can be induced to form calcified tissue by a number of factors, including bone morphogenetic protein-2 (Takikuchi et al. 1999), dexamethasone (Kuri et al. 1999), and hydroxyapatite (Urabe et al. 2000). Under these conditions, PDL fibroblasts produce a number of proteins common to bone and cementum, including osteopontin (Strayhorn et al. 1999), bone sialoprotein (Strayhorn et al. 1999), and alkaline phosphatase (Allott-Licht et al. 1997). These same processes are also downregulated by the presence of plated derived growth factor (Strayhorn et al. 1999), interleukin 1β, tumor necrosis factor alpha, and transforming growth factor beta (Takeshita et al. 1992). Thus, the differentiation of PDL fibroblasts into mineralized tissue (bone or cementum) is probably under complex regulation.

Although there appears to be dramatic reorganization of cell types and tissue in the regeneration of the periodontium, the regulation of these events is poorly understood at the molecular level. This re-organization involves the dynamic regulation of the expression of a number of ECM proteins including osteopontin and bone sialoprotein (Lekic et al. 1996; Nguyen et al. 1997). Therefore, the interaction of these cells with the ECM proteins probably has some influence on both their trans-differentiation and tissue remodeling.

Contemporary periodontal therapy seeks to reverse the plaque-induced resorption of bone and PDL by promoting the regeneration of the periodontium. This process requires the appropriate positioning of cells capable of synthesizing collagen, cementum, and bone. Although the source of competent cells in the adult for such repair remains uncertain, PDL fibroblasts have been proposed as likely candidates (Garnt et al. 1971; Tenenbaum 1990). Recognized therapeutic strategies, such as guided tissue regeneration (Caton et al. 1992) and coronally positioned flaps designed to exclude epithelial cells from regenerating areas (Martin et al. 1988), provide indirect evidence for the importance of mesenchymal cells in this process (PDL fibroblasts, cementoblasts, or osteoblasts). Since, PDL fibroblasts express alkaline phosphatase (Groeneveld et al. 1995; Yamaguchi et al. 1995), a marker for developing bone cells that is not expressed by gingival fibroblasts (Giannopoulou and Cimasoni 1996; Ohlfors et al. 1988), the source for the cells that repair bone may overlap those that repair the ligament. Several studies have presented evidence that PDL fibroblasts are capable of differentiating into osteoblasts (Bermick et al. 1989; Reddi 1981; Urist et al. 1983), are chemotactically responsive to appropriate growth factors (Nishimura and Teranowa 1996), and participate in defect repair at surgical sites (Narayan and Page 1983a,b). Thus, PDL fibroblasts are likely to play a significant role in the re-growth of bone following periodontal surgery. Although we have become more successful in regenerating bone and cementum, a notable drawback to current periodontal therapy is the failure reproducibly to regenerate the PDL. Too often osseous integration is not achieved following the use of bone graft materials. Instead, the graft particles are encapsulated into a disorganized fibrous mass (Hallinan et al. 2001). Furthermore, in those cases in which osseous integration is achieved, the connective tissue fibers are frequently disorganized, often not connecting bone to cementum but rather running parallel to the root, providing little support or stability (Akizuki et al. 2005; Haasgawa et al. 2005). Therefore, significant improvements in periodontal therapies are required in order reproducibly to regenerate functional periodontium.

One of the goals of this study has been to investigate the mobility of the cells of the periodontium, since this may influence their ability to aid in tissue regeneration. Moreover, we wish to determine whether different ECM proteins can be used to promote differential cell mobility. Therefore, cell mobility comparisons have been made between PDL and gingival fibroblasts and osteoblasts. These comparisons have been carried out on the following ECM proteins: collagen I, collagen III, collagen V, fibronec, and laminin.

Materials and methods

Cells

Periodontal and gingival fibroblasts were established from patients who had healthy gingiva but who underwent oral
surgery at the Louisiana State University School of Dentistry for the purpose of removing impacted wisdom teeth. In all cases, tissues were obtained from subjects following informed consent as prescribed in an approved Institutional Review Board protocol. PDL fibroblasts were obtained from the PDL remaining attached to extracted molars, whereas gingival fibroblasts were obtained from loose gingival tissue that was free of epithelium and associated alveolar bone (Palatalogou et al. 2001), as characterized in Laliller et al. (2005). Cells were maintained in alpha-MEM containing 10% fetal calf serum (FCS) and 200 U/ml penicillin and 200 μg/ml streptomycin (GIBCO, Grand Island, NY). PDL and gingival fibroblasts between the 5th and 12th passages were used.

Both gingival and PDL fibroblasts are stable for up to 25 passages with detectable changes in proliferation or gene expression (Laliller et al. 2005). After passage 30, these cells tend to slow their rate of proliferation, eventually becoming non-mitotic at around passage 40.

Cell lines of pre-osteoblasts (ATCC-CRL-11372) were obtained from the American Type Culture Collection. The pre-osteoblasts are a line of fetal osteoblasts (obtained from a spontaneous miscarriage) transfected with a temperature-sensitive expression vector (pUCSV8tsA58) and with the neomycin-resistance expression vector pSV2-neo (Harris et al. 1995). They were grown at 33°C to prevent their temperature-sensitive auto-differentiation to a non-mitotic mature osteoblast phenotype. Pre-osteoblasts were maintained in DMEM/F12 media (without phenol red) containing 10% FCS and 200 U/ml penicillin and 200 μg/ml streptomycin (GIBCO). Mature osteoblasts were obtained by growing pre-osteoblasts at 37°C for 5 days prior to use in subsequent assays. All of these cell types have been more fully characterized elsewhere (Laliller et al. 2005).

Pre-osteoblasts express many transcripts associated with osteogenesis, including alkaline phosphatase, osteonectin, osteomodulin, osteocalcin, osteonidogen, and peristin. Thus, pre-osteoblasts appear to represent a mitotic osteoblast precursor population of cells associated with the peristin.

Cell motility assays

Cell movement was measured on several ECM proteins (collagen I, III, IV, and V and fibronectin and laminin) coated at 10 μg/ml (Sigma, St. Louis, Mo.). T25 tissue-culture flasks or 75-cm tissue-culture flasks with a 40-μm grid etched into the bottom (E&K Scientific Products, Santa Clara, Calif.) were used to facilitate determination of cell position. ECM proteins were adsorbed at 10 μg/ml in 200 μl PBS at 4°C for 12 h. Non-specific attachment of cells to the underlying tissue-culture plastic was blocked by the adsorption of 500 μg/ml heat-inactivated bovine serum albumin (BSA) at 37°C for 1 h. Flasks coated with ECM protein were rinsed three times with PBS prior to the addition of cells.

The ECM-coated tissue-culture flasks were placed upright, and cells (PDL fibroblasts, gingival fibroblasts, pre-osteoblasts, and osteoblasts) were then added at a concentration of 40,000 cells/ml (Fig. 1). Cells were

![Fig. 1 Simplified cell motility assay. Standard T25 tissue-culture flasks were coated with various extracellular matrix (ECM) proteins. The flasks were then tipped upright, and cells allowed to adhere to the end of the flask for 24 h. After this time, the flasks were placed in their normal position (at a 20° angle), and the cells from the end of the flask were allowed to migrate onto the ECM-coated bottom of the flask for up to 10 days. Cell motility rates could be analyzed daily, by measuring the distance from the edge of the flask to the leading edge of the cells.](image-url)
allowed to adhere to the end of the flask by overnight incubation at 37°C. All flasks were subsequently positioned so that their normal bottoms (coated with ECM proteins) were lying flat, and the ends of the flask (now coated with a layer of cells) were vertical. This ensured a common starting point for the motility of each cell type on each protein and control, since cells needed to migrate off the vertical end of the flask and onto the perpendicular ECM-coated bottom of the flask. In some instances, flasks were tipped at approximately 20°, so that both the entire cell-coated surface and the ECM-coated surface were immersed in media. Both procedures resulted in comparable results; however, tipping the flasks to 20° reduced the number of randomly scattered cells along the substratum, presumably by reducing the number of cells floating off from the air-exposed end of the flask. The cells were incubated at 37°C for various periods of time (3, 5, 7, or 10 days). Media were replaced every 3 days.

Cell movement evaluation

Cell movement was measured in one of two ways. (1) At each time point, cells were fixed with 4% paraformaldehyde for 30 min to ensure complete fixation of cells and rinsed three times with phosphate-buffered saline (PBS). The cells were then stained with hematoxylin (Sigma) in order to allow for better visualization. (2) Live cells were stained with Calcein-AM (Molecular Probes, Eugene, Ore.) at 10 μM in 100 mM PBS containing 0.1% dimethylsulfoxide for 30 min at 37°C (this dye is non-fluorescent until it is internalized by cells and cleaved by endogenous esterases). Cells were visualized by using a Typhoon flat bed fluorescent scanner (Amersham, Piscataway, N.J.) that allowed the same cells to be measured throughout the duration of the experiment (Fig. 2). The two techniques produced similar results.

In both cases, the distance that the cells had traveled was determined by visually comparing the cell position with the starting position (edge of contact between the bottom of the flask and the end of the flask) by using an inverted microscope (Nikon) with a 10-μm optical reticle. Thirty cells were measured for each experimental condition, and the average was taken. All data were represented as the average distance migrated for 30 cells for each condition (±SD). Statistical significance was determined by an analysis of variance (ANOVA). An ANOVA was applied to all the values, and statistical significance was determined at P<0.01.

Cell adhesion assay

Cell attachment was measured to ECM proteins adsorbed to tissue-culture plastic. ECM proteins were adsorbed to 48-well tissue-culture plates at 10 μg/ml per well in 200 μl PBS at 4°C for 12 h. Non-specific attachment of cells to the underlying tissue-culture plastic was blocked by the adsorption of 500 μg/ml heat-inactivated BSA at 25°C for 1 h. Wells coated with fibronectin, collagens types I, III, IV,

and V, and a 1:1 mixture of collagen I and III were rinsed three times with PBS prior to the addition of cells. 20,000 PDL or gingival fibroblasts were added to the ECM proteins in a volume of 500 μL MEM containing 10% fetal bovine serum. Cells were allowed to adhere to the substrate from 45 min to 2 h. Eight samples were prepared for each ECM protein for each experiment. Non-adherent cells were removed by three washes of 500 μL PBS and used by freezing at -70°C for 1 h. Adherent cells were quantified fluorometrically by using CyQuant fluorescent dye (Molecular Probes).

The basis for this assay is the use of a proprietary green fluorescent dye, CyQUANT GR dye, which exhibits strong fluorescence enhancement when bound to cellular nucleic acids. Under these conditions, this assay has a linear detection range extending from about 50 to approximately 50,000 cells per microplate well. This dye fluoresces at 530 nm when bound to DNA. The sample fluorescence is measured by using a fluorescence microplate reader (FL600, BioTek Instruments, Winooski, VT) with filters appropriate for ~480 nm excitation and ~520 nm emission. All data were collected, each group of eight samples was averaged, and the mean and SD was compared with the control value according to the following formula:

(Experimental Value/Control Value) x 100% = cells bound

ANOVA analysis was applied to all the values, and statistical significance was determined at P < 0.01.

**Antibodies**

Anti-integrin antibodies were used to inhibit cell adhesion and motility on several ECM proteins. Cell adhesion and motility were examined as described above, with the exception that all tissue proteins were added to each cell sample. Antibodies used in this study were function-blocking antiserum directed against the α1 (Cat. no. MAB1973), α2 (Cat. no. MAB1950), and β1 (Cat. no. MAB2253Z) integrin subunits (Chemicon, Temecula, Calif.). For cell adhesion assays, antibodies were added to cells prior to exposure to the substrates, whereas for migration assays, antibodies were added 1 day after the flasks were placed in their final position.

**Cell proliferation assay**

Approximately 2 x 10^4 cells in a volume of 500 μL MEM-alpha containing 10% fetal bovine serum were plated onto 24-well tissue-culture plates. After incubation for up to 10 days at 37°C (for PDL cells, gingival fibroblasts, and mature osteoblasts) or at 33°C (for pre-osteoblasts), the relative number of cells in each sample was determined by using a fluorescent cell attachment protocol (Papalougou et al. 2001). Cell proliferation was measured with the same CyQUANT GR dye (Molecular Probes) employed to quantify cell attachment (see above).

**Enzyme-linked immunosorbent assays**

Integrin subunit antibodies (Chemicon) were used to detect integrin α1, and α2 subunit expression. Briefly, extracts of cell proteins (100 μg/ml in 50 mM PBS with 100 mM NaCl and 1 mM phenylmethylsulfonylate) were plated into 48-well plates and allowed to adhere for 24 h at 4°C. Non-specific protein binding was inhibited by using 1 mg/ml BSA. An amount representing 10 μg/ml anti-integrin-subunit antibodies was incubated with each sample for 2 h at 4°C, probed with alkaline-phosphatase-conjugated secondary antibodies, and visualized by using 1 mg/ml p-nitrophenol phosphate in 0.1 M diethanolamine (pH 8.3) with 5 mM levamisole (incubated at 25°C for 30 min with gentle agitation). The enzymatic color reaction was stopped by the addition of 500 μl 0.75 N NaOH and assayed for 405 nm absorbance in a microplate reader (FL600, BioTek).

**Results**

Differential periodontal cell motility on ECM

Cell motility was examined for several periodontal cell types on selected ECM proteins. PDL fibroblasts demonstrated the greatest motility on the ECM glycoprotein, fibronectin, invading roughly 5 mm in 10 days (Fig. 3a). Gingival fibroblasts were slower, moving about 3.5 mm in the same time. Osteoblasts (both pre-osteoblasts and mature osteoblasts) were the least motile on fibronectin, moving less than 2 mm in 10 days. In contrast, cell motility on laminin was greatest for gingival fibroblasts, showing movement of over 8 mm in 10 days (Fig. 3b). This was significantly greater than the movement of PDL fibroblasts (P < 0.01), which was less than 3 mm over the same period, and that of osteoblasts (at less that 1 mm). The movement of gingival and PDL fibroblasts was significantly greater on fibronectin or laminin substrates (P < 0.01) when compared with that on untreated tissue culture plastic (Fig. 3c). The movement of both osteoblast types on untreated tissue-culture plastic was comparable (P > 0.01) to that on either laminin or fibronectin, indicating that neither of these ECM proteins stimulated osteoblast motility. Cell movement on BSA-blocked tissue-culture plastic was virtually non-existent (data not shown).

An examination of cell motility on several interstitial collagens (collagen types I, III, and V) revealed some
characteristics of these cells (Fig. 3d,e, and f). Gingival fibroblasts were significantly more motile on these three collagen substrates than were PDL fibroblasts (P<.01).

Gingival fibroblast motility was greatest on collagen III (roughly 15 mm in 10 days) and least on collagen I (roughly 10 mm in 10 days). Motility on collagen V (roughly 10 mm in 10 days) was comparable with that on collagen I (P>.05). In contrast, PDL fibroblast motility was similar on all three collagen substrates (range: 4–6 mm over 10 days). The motility of both types of osteoblast on all three collagen substrates was minimal, with cells traversing less than 2 mm in 10 days. No significant difference was seen in the motility rates of pre-osteoblasts or mature osteoblasts, indicating that the differentiation of osteoblasts had no effect on the cellular machinery required to promote cell movement.

In order to improve our understanding of these apparent differences in motility, side-by-side comparisons of the...
motility rate of these cells on all of these substrates were made for the first 7 days in culture (Fig. 4a) and from 7 to 10 days in culture (Fig. 4b). After 7 days in culture, gingival and PDL fibroblasts moved at roughly the same rate on fibronectin and collagen I substrates and on untreated plastic. In contrast, the gingival fibroblast motility rates on collagen III and laminin were more than twice those of PDL fibroblasts (P < 0.01). The rate of gingival fibroblast motility on collagen V was also significantly greater than that of PDL fibroblasts (P < 0.01). After 10 days in culture, the pattern of motility rates for these cells was slightly different (Fig. 4b). By this time, gingival fibroblast motility rates on all collagen substrates (collagen I, III, and V) and fibronectin had increased, ranging from a two-fold increase for collagen III to a four-fold increase for collagen I and collagen V. These increases in motility rate were significantly greater for gingival fibroblasts when compared with PDL fibroblasts (P < 0.01). The rate for gingival fibroblast motility on laminin remained constant during this period. Thus, gingival fibroblasts displayed different responses to different ECM proteins, speeding up on collagenas and fibronectin over time, but moving at a constant rate on laminin. In contrast, PDL fibroblasts displayed only a modest acceleration, not rising to the level of significance (P > 0.01).

Fig. 4 Comparison of periodontal motility rates on ECM proteins. a: Statistical analysis of gingival fibroblasts (GF), PDL fibroblasts (PDLF), pre-osteoblasts (pOB), and mature osteoblasts (mOB) was examined. After 7 days (a) and after 10 days (b), on several ECM proteins: fibronectin (FN), collagen type I (Col-I), collagen type III (Col-III), collagen type V (Col-V), laminin (LN), and untreated tissue-culture plastic. Untreated chick embryo fibroblasts displayed rapid motility on collagen I, with lower motility on laminin. On all other substrates, these cells moved as rapidly as PDL cells. Between day 7 and day 10, the motility of gingival fibroblasts on all collagen substrates (and laminin and fibronectin) was significantly greater than that of PDL fibroblasts, which moved at a constant rate. Osteoblasts displayed only minimal motility. Each data point represents the mean (±SD) of the measurement of the distance moved by 30 cells along the leading edge of the cell sheet.

a Day 1-7 Rate

b Day 7-10 Rate

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Differential proliferation of periodontal cells

Because of the nature of the motility assay, differential cell proliferation may play a significant role in differential cell motility. Thus, rapid division of some cells could drive their more rapid motility, simply by causing cells to pile up upon one another.

Contrary to this postulate, our data indicated that pre-osteoblasts divided the most rapidly (Fig. 5). In contrast, mature osteoblasts displayed no distinguishable proliferation. Pre-osteoblasts proliferated at roughly twice the rate of PDL fibroblasts, whereas gingival fibroblasts proliferated at roughly half the rate of PDL fibroblasts. Thus, the most rapidly dividing cells (the pre-osteoblasts) migrated at the same rate as the least rapidly dividing cells (the mature osteoblasts). Among the fibroblasts examined, the rate of proliferation was inversely related to their rate of motility. Therefore, increased cell proliferation did not appear to drive the rapid relative motility of gingival fibroblasts.

Periodontal cell motility on mixed collagens substrates

In order to examine the greater migratory potential of gingival (and to a lesser extent PDL) fibroblasts on collagen type III which compared with collagen type I, cells were examined migrating on a mixed substratum (Fig. 6). For gingival fibroblasts, the greatest motility was detected on collagen III, with comparable motility on a mixed substratum of collagen I and collagen III. Gingival fibroblast motility on collagen type I was significantly lower than that on the other collagen substrate (P<0.01). In contrast, PDL fibroblasts moved most rapidly on collagen type I, with lower motility on substrates containing collagen III. Thus, gingival fibroblasts preferentially migrated on collagen III, independent of their interaction with collagen I.

Differential attachment to ECM proteins

An examination of cell attachment to various ECM proteins was performed in order to determine whether cell attachment correlated with cell motility. With the exception of cell attachment to collagen type XIV, pre-osteoblasts adhered to all substrates more vigorously than did either gingival or PDL fibroblasts (Fig. 7). PDL and gingival fibroblasts adhered to all substrates at similar levels, with the exception that gingival fibroblasts adherence to collagen type III was significantly lower than that of PDL fibroblasts. Thus, the lower motility of pre-osteoblasts on all substrates correlates with their greater cell adhesion. Similarly, the greater motility of gingival fibroblasts on collagen type III correlates with their notably lower adhesion. Cell motility therefore appears to be inversely related to cell attachment.

Inhibition of periodontal cell attachment to collagen

Integrins serve as cell surface receptors for the ECM. Specifically, the β1 integrin subunit pairs with the α1, α2, α10, and α11 subunits to form functional collagen receptors. Previous experiments have indicated that each of these cell types expresses transcripts for each of these integrin subunits at comparable levels, with the exception that osteoblasts express more α1 (two-fold) and α2 (eight-fold) integrin transcripts (Laliter et al. 2005).

An examination of integrin subunit protein expression by these cells revealed that they expressed comparable levels of the β1 subunit of integrin (Fig. 8). In contrast,
Fig. 6 Fibroblast motility on mixed collagen substrates. The motility of gingival fibroblasts (GF) and PDL fibroblasts (PDLF) was examined on mixed collagen substrates. Cell motility was examined on substrates of collagen I (Col 1, squares), collagen III (Col 3, circles), and a 1:1 mixture of collagen I and collagen III (Col 1+3, triangles). In general, the motility of gingival fibroblasts on collagen III was twice that on collagen I, whereas PDL fibroblasts moved at roughly half the rate of their gingival fibroblast counterparts. The addition of collagen I to collagen III matrices did not significantly alter cell motility. Each data point represents the mean (±SD) of the measurement of the distance moved by 30 cells along the leading edge of the cell sheet.

Fig. 7 Periodontal cell adhesion to ECM proteins. Adhesion of gingival fibroblasts (GF), PDL fibroblasts (PDLF), and pre-osteoblasts (pOB) to several ECM proteins: fibronectin (FN), collagen type I (Col-1), collagen type IV (Col-4), collagen type III (Col-3), collagen type V (Col-5), and a mixed collagen substrate of 1:1 collagen type I and collagen type III (Col-1+3). In general, osteoblasts adhered to all substrates with great avidity (with the exception of collagen type V). Fibroblast adhesion was generally equivalent on all collagen substrates, with the exception that gingival fibroblasts adhered less well to collagen type III. Each data point represents the mean (±SD) of eight replicate wells (asterisks statistically significantly different cell adhesion from that exhibited by PDL cells, P<0.01).
Inhibition of gingival fibroblast motility by anti-integrin antibodies on collagen substrates

Anti-integrin antibodies were also used to examine the roles of several integrin subunits in gingival fibroblast motility on both collagen I and collagen III (Fig. 10). In all cases, anti-β1-integrin subunit antibodies eliminated all cell motility (data not shown). The addition of antibodies against the integrin α2 subunit reduced cell motility on collagen I by about 50% and by 60% on collagen type III. In contrast, addition of antibodies against integrin α1 did not significantly reduce motility on collagen I and only reduced motility on collagen III by 25%–30%. A combination of the two antibodies did not enhance the inhibition of cell motility on either collagen substrate, when compared with that of anti-α2-integrin subunit antibodies alone.

Discussion

Cells of the periodontium displayed differential motility on the ECM proteins examined in this study (summarized in Table 1). Gingival and PDLC fibroblasts were significantly more motile than pre-osteoblasts and mature osteoblasts on fibronectin, laminin, and interstitial collagens. Collagen III promoted the greatest motility of gingival fibroblasts, whereas laminin and several other collagens promoted significantly more motility in gingival fibroblasts than in PDLC fibroblasts. Pre-osteoblasts and mature osteoblasts were essentially non-motile. The rate of gingival fibroblast motility significantly increased after 7 days in culture, an
Fig. 9 Inhibition of adhesion to collagen by anti-integrin antibodies (NoAB no antibodies). Function-blocking integrin antibodies were used to examine the requirement of various integrins for the adhesion of gingival fibroblasts (GF), PDLC fibroblasts (PF), and pre-osteoblasts (Col III) to collagen (Col I) or collagen type III (Col III). Anti-α1 integrin antibodies (gray bars) diminished the attachment of all cells to both collagens. Anti-α1-integrin antibodies reduced pre-osteoblast attachment to both collagens, but only minimally affected gingival and PDLC fibroblasts. Anti-α2-integrin antibodies significantly reduced the attachment of all cells to both collagens. The addition of both anti-α1-integrin and anti-α2-integrin antibodies did not significantly increase cell attachment to either collagen. Each data point represents the mean (±SD) of eight replicate wells (asterisks statistically significant inhibition of cell attachment by anti-integrin antibodies; *P<0.01)

Observation not evident for PDLC fibroblasts or osteoblasts. Cell motility rates did not correlate with cell proliferation. They were however inversely correlated with cell attachment to specific ECM proteins, with the greatest motility being observed with cell and substrate combinations that promoted the least cell attachment. β1 integrins were essential for attachment while β3 was dispensable. Cell motility was inversely correlated with the expression of integrin α1 and α2 collagen receptors, with the greater expression of these receptors by osteoblasts leading to the lowest motility rates. In osteoblasts, α1 and α2 integrins both appeared to play a role in attachment to both collagen I and collagen III. In contrast, in fibroblasts (both gingival and PDLC), α2 integrins were mostly responsible for mediating their attachment to and motility on collagen substrates. Finally, both osteoblast and fibroblast attachment and motility seemed to be partially insensitive to function-blocking α1 and α2 integrin antibodies, suggesting a role for other integrin collagen receptors, such as α10 and α11 integrins.

Different roles for different collagens

The periodontium is a complex connective tissue that expresses diverse receptors of ECM molecules. Both gingival and PDLC fibroblasts express collagen types I, III, and V (Hou et al. 1995; Hou and Yaeger 1993; Narayanan and Page 1983a,b; Romanos et al. 1991; Tardieu-Moreau et al. 1992). Whereas collagen type I is the most abundant and the major structural constituent of these tissues, collagens III and V appear to have more specialized functions. Both collagen I and collagen III are upregulated during wound healing and regeneration (Niyibizi et al. 2000; Shimomura et al. 2003; Werfully et al. 2002). During orthodontic tooth movement, the expression of collagens I, III, and V also increases within the PDLC (Burnann et al. 1997), suggesting that remodeling of the periodontium requires the synthesis of all three collagens.

Our data demonstrate that collagen type III promotes a greater motility of gingival fibroblasts in culture when compared with other interstitial collagens (types I and V). Collagen type II is an accessory collagen and makes up about one-fifth of the collagen of the PDLC, with most of the remainder being collagen I (Butler et al. 1975). Collagen III promotes the cell attachment of fibroblasts cultured from rabbit periodontium (Kerkvliet et al. 2003). An examination of mixed collagen I and collagen III substrates has revealed that collagen III dominates in promoting motility. Thus, collagen III may play a role in promoting more rapid cell invasion into wound sites. Furthermore, collagen V is
Fig. 10 Inhibition of gingival fibroblast motility on collagens by anti-integrin antibodies. Function-blocking integrin antibodies were used to examine the requirement of various integrins for gingival fibroblast motility on collagen type I (Coll-1) or collagen type III (Coll-3). Anti-α1-integrin antibodies only minimally reduced gingival fibroblast motility on either collagen. Anti-α2 integrin antibodies significantly reduced gingival fibroblast motility on both collagens. The addition of both anti-α1 and anti-α2 integrin antibodies did not significantly increase cell motility on collagen I but did further reduce motility on collagen III. Each data point represents the mean (±SD) of the measurement of the distance moved by 30 cells along the leading edge of the cell sheet.

Expressed in developing ligaments (Bland and Ashhurst 1996), and its expression within the PDL is highest adjacent to the cementum (Romano et al. 1991). Functionally, collagen V is involved in regulating the fiber size of collagen I by limiting its polymerization (Shimomura et al. 2003). This suggests a role for collagen V in PDL attachment to cementum, possibly by limiting the diameter of collagen I fibers and thereby creating an increased surface area per unit of collagen fiber mass. Thus, in periodontal tissue repair and rearrangement, collagens III and V play major regulatory roles.

Roles of integrins in periodontal cell motility

Integrins are the major family of collagen receptors, with α2β1 integrin being the first identified collagen receptor and the major receptor for many cell types (Takada et al. 1988). α1β1 integrin acts as a promiscuous collagen receptor (Elices and Henner 1989) that can also directly mediate attachment and motility on laminin (Lalier and Bronner-Fraser 1992). More recently, both α10 and α11 have been identified as collagen receptors (Camper et al. 1998; Tiger et al. 2001). In many cases, the expression
Table 1. Summary of results. Cellular motility, proliferation, and integrin subunit expression were compared with those of PDL cells as the reference point (GP gingival fibroblasts, PDL cells, osteoblasts, mOB mature osteoblasts, n.a. not analyzed).

<table>
<thead>
<tr>
<th>Character</th>
<th>ECM substrate</th>
<th>Anti-integrin subunit antibodies</th>
<th>Cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell motility</td>
<td>All ECM</td>
<td>2a 1a &lt;0.5% 0.5%</td>
<td>GF, PDL, pOB, mOB</td>
</tr>
<tr>
<td>Cell proliferation</td>
<td></td>
<td>1a &lt;0.5%</td>
<td>n.a.</td>
</tr>
<tr>
<td>Cell attachment</td>
<td>Collagen I</td>
<td>60% 60% 90%</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>Collagen III</td>
<td>60% 60% 90%</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>Collagen IV</td>
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<td>n.a.</td>
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<tr>
<td></td>
<td>Other ECM</td>
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<td>n.a.</td>
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<tr>
<td>Integrin subunit expression</td>
<td></td>
<td>1a 1a 2a 4a</td>
<td>n.a.</td>
</tr>
<tr>
<td>Integrin-dependent cell</td>
<td>Collagen I and III</td>
<td>1a &lt;0.5% &lt;10% 20% 30%</td>
<td>n.a.</td>
</tr>
<tr>
<td>attachment</td>
<td></td>
<td>2a -50% -50% -50% -60%</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3a &gt;90% &gt;90% &gt;90% &gt;90%</td>
<td>n.a.</td>
</tr>
<tr>
<td>Integrin-dependent cell</td>
<td>Collagen I</td>
<td>1a 0 a.a. n.a. n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>motility</td>
<td></td>
<td>2a -25% n.a. n.a. n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>Collagen III</td>
<td>1a -25% n.a. n.a. n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2a -15% n.a. n.a. n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

patterns of the a2 and a11 integrin subunits overlap (Popova et al. 2004), indicating that any cell interaction with collagen may involve multiple integrins and therefore be complex. The a2B1 integrins act as receptors for collagen I, II, III, and V (Seagman et al. 1992), whereas a1B1 acts as a receptor for collagen III (Kim et al. 2005) plus collagens I, IV, and V (Riikonen et al. 1999). In chondrocytes, a10 integrins act as collagen II receptors (Bengtsson et al. 2005; Camper et al. 2001). Thus, although many integrins seem to promote attachment to multiple collagens, the action of some integrins might be specific when expressed by certain cell types. In addition to promoting cell attachment, the a2B1 and a11B1 integrins aid in the polymerization of collagen I and III (Velling et al. 2002). Therefore, integrins not only mediate cell adhesion to the ECM, but also play a role in ECM assembly.

Periodontal cells express several collagen-binding integrins. Both PDL and gingival fibroblasts express a1, a2, and a10 integrin transcripts (Palaiologou et al. 2001). PDL fibroblasts express more a10 integrin transcripts than do gingival fibroblasts (Palaiologou et al. 2001) or osteoblasts (Lallier et al. 2005). Gingival fibroblasts express more a11 integrin transcripts than do PDL fibroblasts (Palaiologou et al. 2001). In addition, osteoblasts express more a2 and a10 integrin transcripts than do PDL fibroblasts (Lallier et al. 2005). Here, we present data that gingival and PDL fibroblasts express roughly equal quantities of a1 and a2 integrin proteins, whereas osteoblasts express more of both integrin subunits. Thus, periodontal cells differentially express several collagen-binding integrins, indicating that these cells interact differently with collagens based upon their repertoire of expressed integrin collagen receptors.

Several studies have shown that integrins mediate cell motility. Both carcinoma cell invasion (Lochtier et al. 1999) and endothelial cell spreading require a1 and a2 integrins (Senger et al. 1997). The invasiveness of osteogenic tumor cells (Whitten et al. 1996) and the motility of melanoma cells increase with increased a2 integrin expression (Schon et al. 1996). The cytoplasmic tail of a2 integrin is required for cell motility (Klebeck et al. 2001) and has been linked to a requirement for attachment to the cytoskeleton. Interestingly, a2B1-collagen interactions increase RhoA activity and lead to the slowing of endothelial cells (Zhou and Kramer 2005). In these cells, a2B1 binds to vimentin (Kreis et al. 2005), possibly indicating that an interaction with the more stable intermediate filament proteins, rather than the more dynamic actin cytoskeleton, directly influences the rate of cell motility. Fibroblast motility is increased in a11 knockout mice, leading to the conclusion that a11 normally slows fibroblast motility (Popova et al. 2004); a11 integrin also suppresses cell motility on collagen 1 (Tiger et al. 2001). Taken together, these data indicate that a11 promotes more stable cell attachments than do the other integrins, such as a2B1 heterodimers. Thus, integrin-mediated cell motility on collagen can be regulated directly by integrin expression and by any changes in their interactions with cytoskeletal proteins.

Implications of motility rates of periodontal cells for periodontal wound healing and regeneration

Fibroblasts from oral connective tissues are the preferred cells to repopulate the wound after traditional periodontal treatment, although epithelial cells often intervene to prevent the reformation of the normal periodontal attachment (Fernyough and Page 1983). One goal of periodontal regeneration is to persuade the proper cells to invade the wound site and form the required structures. Cell motility is
governed by interactions of the respective cells with the extracellular environment, principally through integrin receptors for ECM proteins (Paliakogou et al. 2001). Therefore, the ability of different ECM proteins selectively to promote cell motility may be useful in guiding specific cell populations into a periodontal defect. During the process of migration, cells that ingress into the wound site should ideally have the potential to form replacements for the original tissues and structures. Within the periodontium, this means that it would be optimal if osteoblasts invaded to form bone, if PDL fibroblasts invaded the cleft between the tooth and the bone, and if gingival fibroblasts were excluded from this region, in order to form the overlying connective tissue for those structures. Unfortunately, our data indicate that gingival fibroblasts migrate at barely move at all. During the early stages of migration (up until day 3), PDL fibroblasts tend to move almost as fast as gingival fibroblasts. However, gingival fibroblasts quickly begin migrating more rapidly, especially on collagen III, which is present as the framework throughout the periodontium, together with collagen I (Butler et al. 1975). Hence, gingival fibroblasts have the potential to invade the wound site much more rapidly and efficiently than PDL fibroblasts or osteoblasts. This may be the underlying cause for the creation of disorganized fibrous tissue following periodontal treatment (Hallman et al. 2001). The early invasion of gingival fibroblasts into the wound site may impede later invasion by other cell types (either PDL fibroblasts or osteoblasts) and thereby reduce the ability of this tissue truly to regenerate. A better understanding of the molecular regulators of cell movement should aid in the design of improved therapeutic treatments to enhance regeneration of the periodontium.

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