

A Novel ‘Sandwich’ Assay for Quantifying Chemo-Regulated Cell Migration Within 3-Dimensional Matrices: Wound Healing Cytokines Exhibit Distinct Motogenic Activities Compared to the Transmembrane Assay

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The extracellular matrix profoundly affects cellular response to soluble motogens. In view of this critical aspect of matrix functionality, we have developed a novel assay to quantify chemo-regulated cell migration within biologically relevant 3-dimensional matrices. In this “sandwich” assay, target cells are plated at the interface between an upper and lower matrix compartment, either in the presence of an isotropic (uniform) or anisotropic (gradient) spatial distribution of test motogen. Cell migration in response to the different conditions is ascertained by quantifying their subsequent disposition within the upper and lower matrix compartments. The objective of this study has been to compare the motogenic activities of platelet-derived growth factor (PDGF-AB) and transforming growth factor-beta isoforms (TGF- β 1, - β 2 and - β 3) in the sandwich assay and the commonly employed transmembrane assay. As previously reported, dermal fibroblasts exhibited a motogenic response to isotropic and anisotropic distributions of all tested cytokines in the transmembrane assay. In contrast, only PDGF-AB and TGF- β 3 were active in the sandwich assay, each eliciting directionally unbiased (symmetrical) migration into the upper and lower type I collagen matrices in response to an isotropic cytokine distribution and a directionally biased response to an anisotropic distribution. TGF- β 1 and - β 2 were completely devoid of motogenic activity. These results are consistent with the reported differential bioactivities of PDGF and TGF- β 3 compared to TGF- β 1 and - β 2 in animal models of wound healing

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and suggest that the sandwich assay provides a means of obtaining physiologically relevant data regarding chemo-regulated cell migration. *Cell Motil. Cytoskeleton* 63:287–300, 2006. © 2006 Wiley-Liss, Inc.

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INTRODUCTION

Dermal wound healing involves the ingress of perilesional keratinocytes, fibroblasts and vascular endothelial cells into the wound site. Locally released cytokines, including isoforms of platelet derived growth factor (PDGF) and transforming growth factor-beta (TGF- β), play an important role in the temporal and spatial regulation of these migratory events [Werner and Grose, 2003]. The transmembrane (Boyden chamber) assay is the most commonly employed protocol for assessing such chemo-regulated cell motility *in vitro* [Chen, 2004]. This assay entails the use of a multi-well chamber, each well consisting of an upper and lower medium compartment separated by a porous polycarbonate membrane coated with an adhesive matrix molecule (usually gelatin). Target cells are plated into the upper compartment and adhere to the membrane. After an incubation period (usually 3–5 h), assessment of cytokine motogenic activity is achieved by counting the number of cells which have migrated through the membrane pores and remain adherent to the lower membrane surface.

Chemo-regulated cell migration is commonly analysed in terms of chemokinesis (defined as random cell movement in response to an isotropic concentration of soluble motogen) and chemotaxis (defined as directional cell movement in response to a gradient of motogen). In the transmembrane assay, chemokinesis and chemotaxis are independently assessed by incorporating either the same (chemokinesis) or different (chemotaxis) concentrations of soluble motogen in the upper and lower medium compartments [Zigmond and Hirsch, 1973; Zigmond and Lauffenburger, 1986; Chicoine and Silbergeld, 1997; Wilkinson, 1997]. Previous studies using the transmembrane assay have reported conflicting results regarding the motogenic characteristics of PDGF and TGF- β 1: i.e. whereas certain studies demonstrated both chemokinetic and chemotactic responses by a number of target cells [Seppä et al., 1982; Postlethwaite et al., 1987; Pierce et al., 1989; Orr et al., 1990; Koyama et al., 1992; Facchiano et al., 2000], others concluded that only a chemotactic response was elicited [Koyama et al., 1990; Bischoff, 1997; Andresen and Ehlers, 1998]. In the case of TGF- β 1, certain studies failed to detect significant motogenic activity [Matsuda et al., 1992; Osornio-Vargas et al., 1993].

Interpretation of these experimental results and their extrapolation to the considerably more complex *in vivo*

milieu has been complicated by the demonstration that the activity of wound healing cytokines (as well as other soluble motogens) is not invariant, but is a dynamic characteristic critically modulated by a number of tissue-level parameters, including the nature of the extracellular matrix [Nathan and Sporn, 1991; Schor, 1994; Ellis et al., 1999]. In order to model such tissue-level control mechanisms more accurately, we have developed a quantitative migration assay utilising a “dermal-like” 3-dimensional matrix of native type I collagen fibres [Schor, 1980, 1994]. In this assay, cells are plated onto the gel surface in the presence of different concentrations of the putative motogen and the number of cells present within the 3-dimensional collagen matrix subsequently appraised after a 3–4 day incubation period. Use of the collagen gel assay and its discordance with results obtained in the conventional transmembrane assays have underscored the role played by the matrix in defining cellular response to wound healing cytokines [Schor, 1994], as well as led to the identification of effector molecules displaying hitherto unrecognised matrix-dependent motogenic activities [Schor et al., 1996, 1999, 2003].

Although providing stromal cells with a more physiologically relevant substratum than that commonly employed in the transmembrane assay, the collagen gel assay is not readily amenable to assessing cellular motogenic response to different spatial distributions of motogen. In order to address this shortcoming, the objectives of the present study have been to (i) develop an assay capable of quantifying cellular motogenic response to both an isotropic and anisotropic spatial distribution of motogen within a 3-dimensional macromolecular matrix, and (ii) compare the motogenic response of dermal fibroblasts to wound healing cytokines (PDGF-AB and TGF- β 1, - β 2 and - β 3) in the newly developed assay and the conventional transmembrane assay.

MATERIALS AND METHODS

Reagents

Human recombinant PDGF-AB, TGF- β 2 and TGF- β 3 were purchased from R&D Systems (Abington, UK) and TGF- β 1 from Sigma Chemicals (Poole, UK). Eagle's Minimal Essential Medium (MEM), donor calf serum, sodium pyruvate, glutamine, non-essential amino acids and antibiotics were also obtained from Sigma

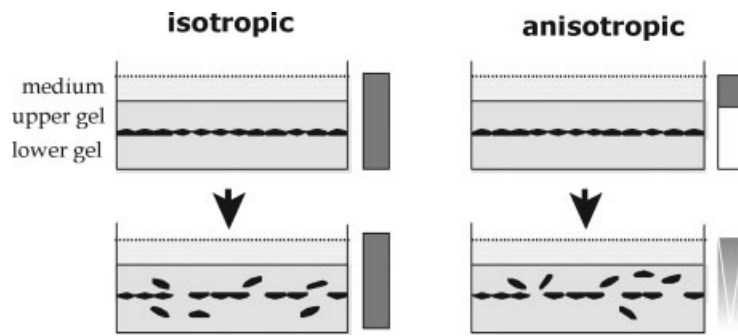


Fig. 1. Sandwich assay protocol. A monolayer of target cells is “sandwiched” between an upper and lower gel compartment (experimental details given in the *Materials and Methods* section). Assay cultures are typically incubated for four days. To evaluate cell migration in response to an isotropic spatial distribution of test motogen, this is initially incorporated at the same concentration in the upper and lower

gels, as well as in the medium overlay. Cell migration in response to an anisotropic spatial distribution of test agent is assessed by initially incorporating it only in the medium overlay; under these starting conditions, diffusion of the test compound into the underlying collagen gel compartments establishes a concentration gradient (as indicated in side bars).

Chemicals (Poole, UK). All tissue culture plasticware were obtained from SLS (Nottingham, UK). Type I collagen was extracted from rat tail tendons in 3% acetic acid, dialysed for 2 days against distilled water, diluted to 2 mg/ml and used to make 2 ml collagen gels in 35-mm plastic tissue culture dishes, as previously described [Schor, 1980].

Cells and Tissue Culture Conditions

The FSF44 fibroblast line was established in our laboratory by explant culture from foreskin obtained from a healthy 1-year-old donor. Stock cultures were maintained in Eagle’s MEM supplemented with 15% (v/v) donor calf serum, 1 mM sodium pyruvate, 2 mM glutamine, non-essential amino acids at 37°C in a moist atmosphere containing 5% CO₂. Cells were grown on 90-mm plastic tissue culture Petri dishes and passaged at a split ratio of 1:5 upon reaching confluence, usually 7–10 days after plating. All experiments were performed with cells at passages 14–20. Cells used in this study were free of mycoplasma contamination, as indicated by staining with Hoechst 33256.

Transmembrane Migration Assay

Polyvinylpropylene-free polycarbonate Nucleopore membranes (8.0 μm pore, Costar, UK) were coated with either native or denatured type I collagen by overnight immersion in a 100 μg/ml aqueous collagen solution at 37°C, followed by 3× washes with PBS, washed 1× with distilled water and then air dried [Schor et al., 1996, 1999]. The collagen-coated membranes were fitted into a 48-well microchemotaxis chamber (Neuroprobe, MD). Both upper and lower wells contained the indicated cytokine in serum free MEM (SF-MEM) plus 2 μg/ml bovine serum albumin (BSA) in assays designed to measure chemokinesis; cytokines were only added to the lower

well in experiments designed to measure chemotaxis. A total of 2.5×10^4 cells suspended in 50 μl SF-MEM + 2 μg/ml BSA were then plated into the upper wells. The chambers were incubated for 5 h at 37°C in a humidified CO₂ incubator and the membranes then removed, fixed in methanol and stained with Mayer’s Haematoxylin. Cells remaining on the top of the membranes were removed, the membranes mounted onto glass slides and examined under bright field illumination at a magnification of 250×. The number of migrated cells adherent the lower surface of the membrane were counted in 3 fields per well and 6 wells per variable (i.e. 18 fields per variable). Data are expressed as mean cell number and standard deviation per field.

Sandwich Assay for Distinguishing Between Chemokinesis and Chemotaxis in a 3-Dimensional Collagen Matrix

The “sandwich” assay is designed to quantify the effects of soluble agents on the migration of cells cultured within a physiologically relevant 3-dimensional matrix. It is set up by (i) pipetting a 2 ml collagen gelling solution into a 30-mm tissue culture dish and allowing it to set at 37°C for 1 h [Schor, 1980], (ii) plating 2×10^5 fibroblasts in 1 ml MEM onto the gel surface, (iii) removing supernatant with non-attached cells 1 h later, (iv) overlaying the adherent cell layer with a second 2 ml collagen gel to form a cell “sandwich”, and (v) adding a 1 ml medium overlay 1 h later (Fig. 1). Donor calf serum (1% v/v) is present in both lower and upper collagen gels and medium overlay. After a standard 4 day incubation period, the disposition of cells present within the upper and lower gel compartments (i.e. those cells which had migrated from their initial interface position) was ascertained with an Olympus IX70 microscope (×20 objective) fitted with a computer-controlled Prior

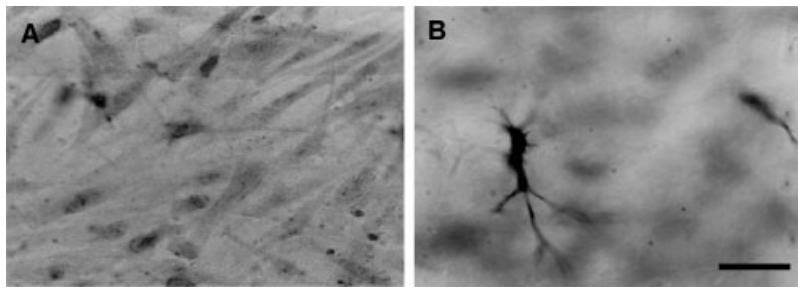


Fig. 2. Fibroblast morphology in the sandwich assay. Fibroblasts remaining at the interface position form a monolayer of flattened cells (A). In contrast, cells which have migrated into either the upper or lower gel compartments are typically refractile and stellate in appearance (B). bar = 50 μ m.

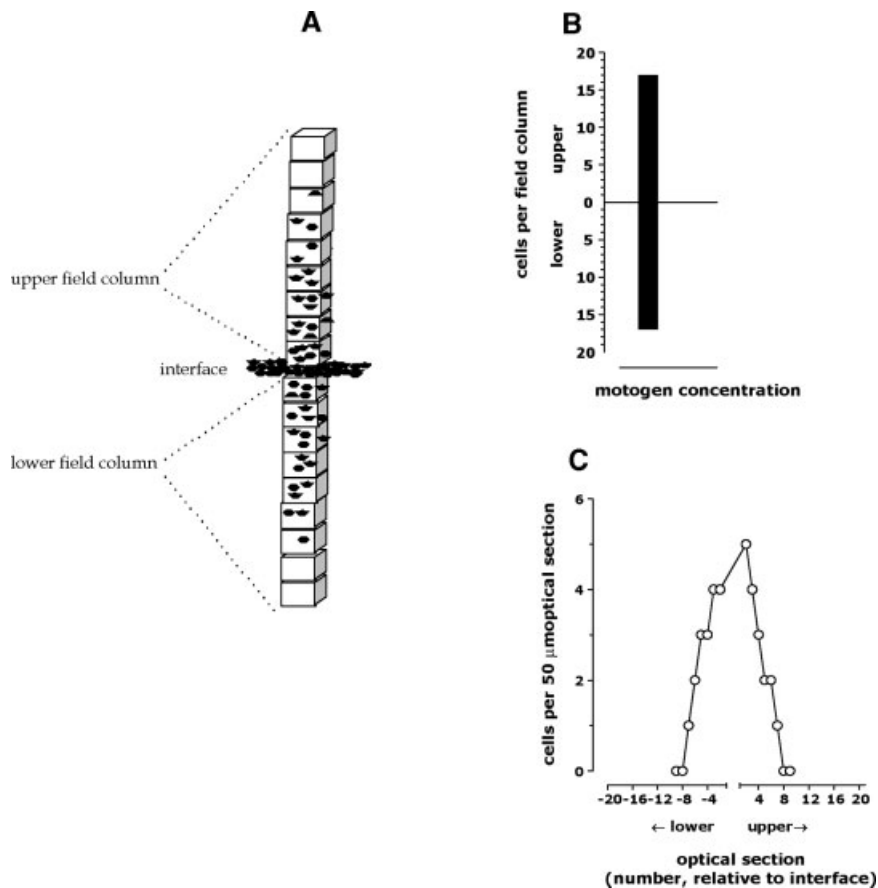


Fig. 3. Sandwich assay parameters. (A). A z-stage motor fitted to the microscope is used to move the point of focus up and down from the interface position in precise 50- μ m steps. A “field column” is defined as the vertical array of optical cuboids circumscribed by a rectangular photographic graticule and the traversed 50 μ m interval along the z-axis. In the standard sandwich assay, the field-column consists of 16 cuboids in both the upper and lower gel compartments. (B). Cells are

counted in each cuboid. Data collected from 20 randomly selected interface fields are summarised as total “cells per field column”, a format that facilitates direct comparison with results obtained in the conventional transmembrane assay, (C). Data may also be expressed as cell disposition along the z-axis as “cells per 50- μ m optical section”. Other parameters are described in Table I.

Z-Stage step motor (running under Metamorph v4 image analysis software) calibrated to move the stage up and down by 50 μ m intervals. For this purpose, the focus point was manually set at a randomly selected interface

position (Fig. 2A). The z-stage motor was then employed to move the point of focus downwards in sixteen 50- μ m steps, i.e. a total distance of 800 μ m. The number of cells present within the cuboid defined by a rectangular photo-

TABLE I. Different Formats for Expressing Sandwich Assay Data

Data format	Definition	Comment
i	Total cells per field column Calculated by summing the number of migrated cells in the vertical column of 16 optical sections on either side of the interface (Fig. 3B).	This method of data representation simplifies comparison with the “cells per field” format of the transmembrane assay.
ii	Cells per 50- μm optical section The spatial disposition of cells along the z-axis, above and below the starting interface position (Fig. 3C).	This is the least processed expression of the raw data.
iii	Fold stimulation relative to control Calculated by dividing the total number of migratory cells per field-column (as in i) for each experimental point by their respective control values (see text in <i>Results</i> section).	Is an indicator of the potency of the motogenic response.
iv	Percentage migration Calculated by dividing the total number of migratory cells per field column (as in i) by the total number of cells (ie. non-migratory + migratory) (see text in <i>Results</i> section).	Provides information regarding the proportion of cells which left the interface position.
v	Distance travelled by leading edge cell Optical sections containing the cell most distal from the interface per field-column (expressed as box and whisker plot; eg Fig. 11).	Provides information regarding the distance travelled by cells at the leading edge.

graphic graticule was ascertained at each 50- μm vertical step. Cell disposition along the z-axis in the upper gel was then similarly ascertained by returning to the interface position and moving the point of focus upwards in sixteen 50- μm steps. The graticule defined an area 0.28 mm² at the magnification used. Migrated cells are refractile and stellate in shape (Fig. 2B) and easily distinguished from those remaining at the interface. This operation was repeated in a total of 20 randomly selected interface fields per assay dish. A minimum of two replicate gels were assessed for each experimental point presented.

When a cell body spanned two optical sections, its location was assigned to the section containing the nucleus. Considering the $70 \pm 24 \mu\text{m}$ average length of fibroblasts within a 3-dimensional collagen matrix (unpublished data), the 50 μm distance between optical sections was selected to allow unambiguous assignment of greater than 95% of fibroblasts to a single optical section. No cells were observed beyond the 800 μm upper and lower counting limits. Vertical cell disposition data were obtained from 20 randomly-selected interface fields.

The above method of controlled Z-stage movement is used to define a field-column: i.e. the vertical array of 16 cuboidal optical sections above and below the selected interface field (Fig. 3A). Cells at the interface and within the two 50- μm optical sections immediately above and below it are considered non-migratory.

To evaluate cell migration in response to an isotropic spatial distribution of soluble agent, the assay is set up so that the test compound is initially incorporated at the same concentration in the upper and lower gels, as well as in the medium overlay (Fig. 1A); under these starting conditions, a positive motogenic response is

manifest by a symmetrical dose-dependent increase in the number of cells above and below the interface compared to control cultures. Cell migration in response to a concentration gradient of soluble motogen is assessed by initially incorporating the test compound only in the medium overlay (Fig. 1B); under these anisotropic (step gradient) starting conditions, motogen diffusion into the underlying collagen gel compartments establishes a concentration gradient. A positive motogenic response will accordingly result in an asymmetric cell disposition along the z-axis in the two gel compartments, with more cells present in the upper gel. As target cells encounter temporal and spatial changes in motogen concentration along the imposed diffusional gradient, motogen “dose” cannot be expressed by a single value. Under such conditions motogen “dose” is operationally denoted by the term “G:M (F)” in which G and M are the respective concentrations of motogen in the gel and medium compartments in the initial step gradient and F is the final (isotropic) concentration of motogen in both gel and medium compartments which would be achieved at diffusional equilibrium.

Data obtained in this assay may be expressed in a number of formats to highlight particular migratory parameters (Table I); these include (i) the total number of cells in the upper and lower gels (Fig. 3B), (ii) the spatial disposition of cells per 50- μm optical section along the z-axis (Fig. 3C), (iii) the relative magnitude of motogen-induced migration compared to the control, (iv) the percentage of total cells which migrated away from the interface, and (v) the distance travelled by the leading edge cells.

We have previously reported that human skin fibroblasts exhibit an approximate 4 day lag period before proliferation commenced when plated on or within type I

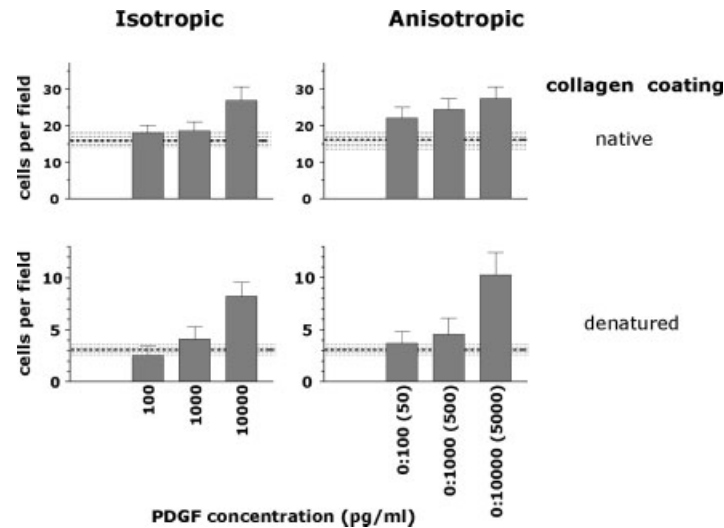


Fig. 4. The effects of PDGF-AB on fibroblast migration in the transmembrane assay. Membranes were coated with either native or denatured type I collagen (as respectively employed in the sandwich and conventional transmembrane assays). The base-line levels of cell migration across the two types of membrane (achieved in the absence of cytokine) are indicated by the dotted line (mean value) and shaded area (standard deviation). PDGF-AB was added at equal concentrations to both upper and lower medium compartments to produce an

isotropic cytokine distribution across the membrane and only in the lower medium compartment to establish an anisotropic (gradient) distribution. For consistency, cytokine "concentration" under anisotropic conditions is denoted in a manner analogous to that employed in the sandwich assay (described in *Results* and Fig. 6), i.e. initial motogen concentration in the upper assay well: initial motogen concentration in the lower assay well (isotropic motogen concentration at diffusional equilibrium).

collagen gels in the presence of 1% serum [Schor, 1980]. In agreement with these data we found that cell proliferation was not detected during the 4 day duration of the sandwich assay and that cell viability remained above 95% under all experimental conditions.

Statistical Analyses

Statistical significance between two relevant data sets was evaluated with Prism 3 software package using the Mann-Whitney two-tailed test.

RESULTS

Wound Healing Cytokines Display Distinct Motogenic Activities in the Transmembrane and Sandwich Assays

The motogenic response of human skin fibroblasts to wound healing cytokines was first assessed in the transmembrane assay using membranes coated with either denatured type I collagen (i.e. gelatin, as employed in the majority of previous transmembrane studies) or native type I collagen (as utilised in the sandwich assay). The base-line level of migration, manifest in the absence of cytokine, was significantly greater on membranes coated with native collagen (16.2 ± 2.7 cells per field) compared to denatured collagen (3.2 ± 1.3 cells per

field). PDGF-AB elicited a motogenic response on both substrata in the presence of both an isotropic and anisotropic distribution of cytokine across the membrane (Fig. 4). All three TGF- β isoforms also stimulated cell migration in this assay (Fig. 5). With the exception of TGF- β 1, the maximal relative stimulation of migration compared to control was greater on denatured collagen (2.8- to 3.8-fold) compared to native collagen (1.4- to 1.7-fold). These relatively low levels of stimulation are comparable with those previously obtained in the transmembrane assay [Pierce et al., 1989; Orr et al., 1990; Koyama et al., 1992; Bischoff, 1997; Andresen and Ehlers, 1998; Facchiano et al., 2000]. A stronger motogenic response to the tested cytokines was not obtained at concentrations either higher or lower than those presented.

Significantly different results were obtained in the sandwich assay. To facilitate comparison with the transmembrane assay, data have first been expressed as "total cells per field-column" (Table I, format i): i.e. the closest 3-dimensional equivalent of the "cells per field" format used in the transmembrane assay. After the standard 4 day incubation period, control fibroblasts displayed base-line levels of migration of only 1.0 ± 0.9 cells per field-column (Fig. 6). Under isotropic conditions PDGF-AB induced a symmetric and dose-dependent increase in upwards and downwards migration, achieving a plateau

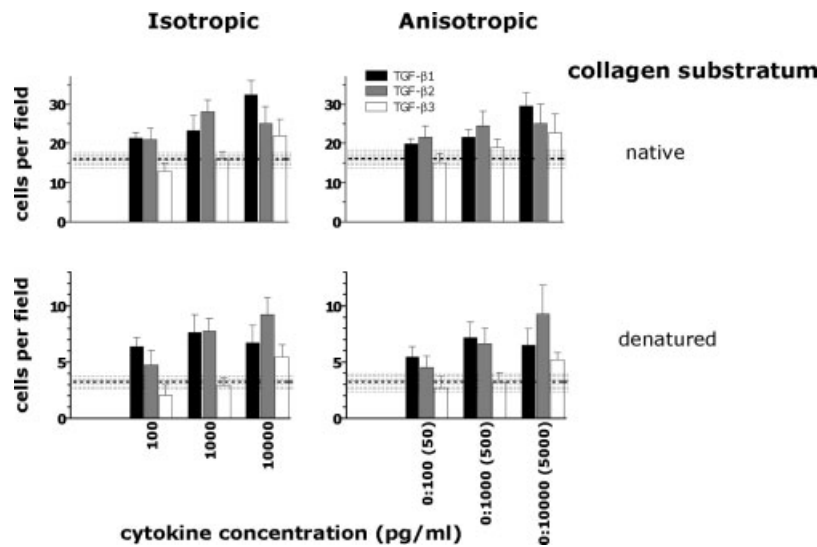


Fig. 5. The effects of TGF- β isoforms on fibroblast migration in the transmembrane assay. Assay protocols (membrane coating and cytokine distributions across the membrane) and data presentation conventions are as described in Fig. 4.

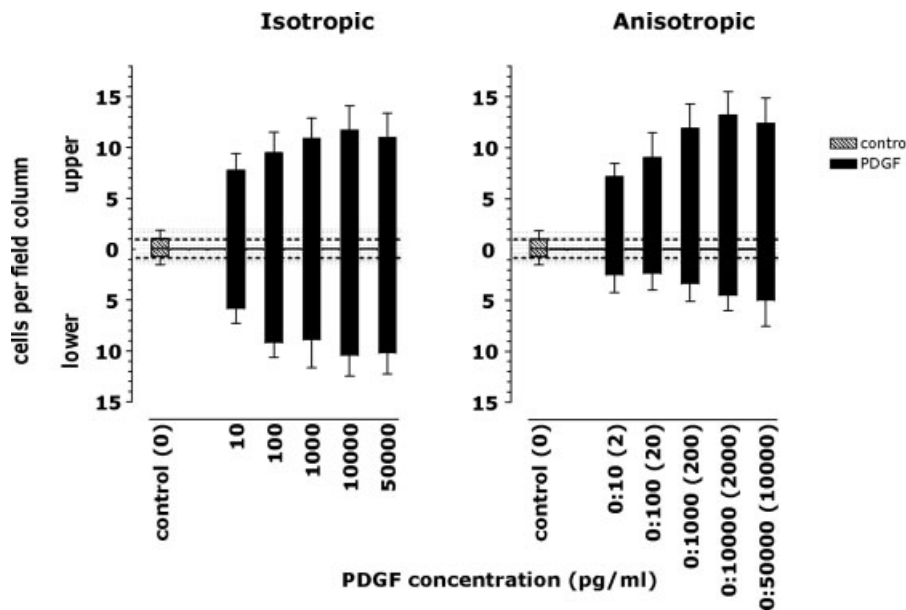


Fig. 6. Motogenic response of fibroblasts to PDGF-AB in the sandwich assay. An isotropic cytokine distribution was established by initially incorporating the indicated concentration of PDGF-AB in the upper and lower collagen gels and medium overlay. To establish an anisotropic (gradient) distribution, cytokine was initially incorporated only into the medium overlay; under such conditions motogen “dose” is operationally denoted in the abscissa by the term “G:M (F)” in which G and M are the respective concentrations of motogen in the gel and medium compartments in the initial step gradient and F is the

final (isotropic) concentration of motogen in both gel and medium compartments which would be achieved at diffusional equilibrium. The base-line levels of cell migration into the upper and lower gel compartments are indicated by the dotted lines (mean value) and shaded areas (standard deviation). Cells per field column in the upper gels under isotropic and anisotropic cytokine distributions were not statistically distinguishable; in contrast, cell numbers in the lower gels are significantly higher under isotropic conditions than achieved under anisotropic conditions ($P < 0.001$).

maximal value of 11.0 ± 2.4 and 10.6 ± 2.1 cells per field-column (in upper and lower gels, respectively) at cytokine concentrations between 100–50,000 pg/ml. An

asymmetric stimulation of cell migration was elicited by an anisotropic cytokine distribution, with maximal plateau values of 12.4 ± 2.5 cells per field-column in the

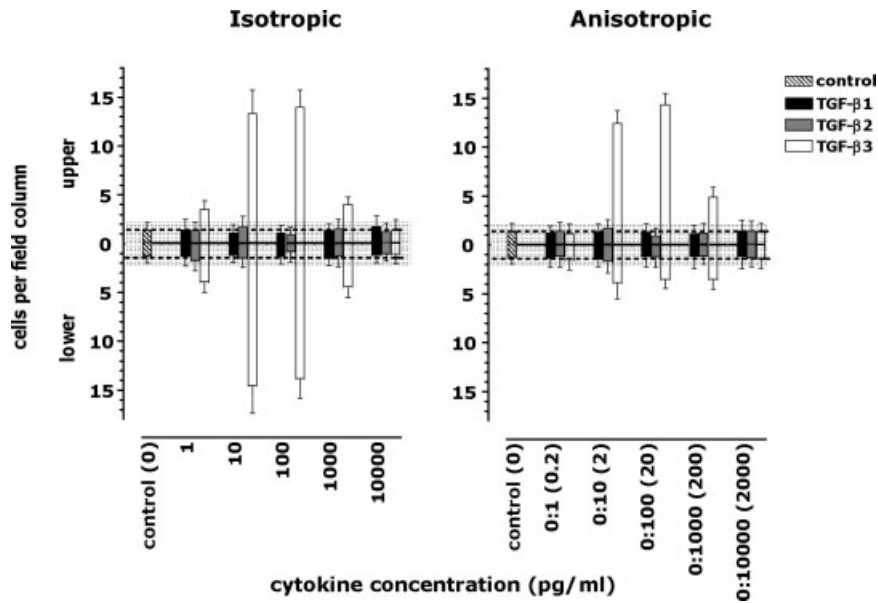


Fig. 7. Motogenic response of fibroblasts to TGF- β isoforms in the sandwich assay. Assay protocols and data presentation are as described in Fig. 6. A significant motogenic response was only obtained with TGF- β 3. Cells per field column in the upper gels under isotropic and anisotropic distributions of TGF- β 3 were not statistically distinguishable, whereas cell number in the lower gels were significantly greater under isotropic conditions ($P < 0.001$).

upper gel compared to only 5.0 ± 1.5 in the lower gel. The relative stimulation of migration compared to controls (Table I, format iii) was significantly higher in the sandwich assay (10.8–12.0-fold) than transmembrane assay (1.4- to 3.8-fold).

Corresponding data pertaining to the effect of TGF- β isoforms are presented in Fig. 7. In marked contrast to results obtained in the transmembrane assay, TGF- β 1 and TGF- β 2 were completely devoid of motogenic activity in the presence of either isotropic or anisotropic cytokine distributions, with mean values in the range of 1.1 to 2.2 cells per field-column. TGF- β 3, on the other hand, elicited a significant bell-shaped motogenic response. A symmetrical stimulation of cell migration was obtained under isotropic concentrations, with peak plateau values of 14.1 ± 2.8 cells per field-column. Therefore, a relative stimulation (Table I, format iii) of 8.8-fold was achieved at concentrations between 10–100 pg/ml. An asymmetrical cell disposition was obtained under anisotropic assay conditions, with a maximal plateau value of 13.4 ± 2.1 cells per field-column in the upper gel (8.4-fold stimulation) compared to 4.1 ± 2.4 cells per field-column in the lower gel (2.6-fold stimulation).

As all cells (both migratory and stationary) are counted in the sandwich assay, it is possible to express the number of migrated cells as a percentage of total cell number (Table I, format iv). Under control conditions the percentage of migrated cells above and below the

starting interface position were indistinguishable and fell within the range of 1.6–3.9%. The percentage of migrated cells increased symmetrically in the presence of isotropic concentrations of PDGF-AB, with maximal plateau values of $14.2 \pm 2.3\%$ (upwards) and $12.1 \pm 1.7\%$ (downwards), respectively. Under anisotropic conditions, there was a maximum of $14.1 \pm 2.2\%$ cell migration into the upper gel and $4.4 \pm 1.4\%$ into the lower gel. Comparable data for TGF- β isoforms indicated that the percentage of cells migrating from the interface in response to TGF- β 1 and - β 2 was indistinguishable from control values and fell within the range of 2.2–3.7%. In the presence of an isotropic concentration of TGF- β 3, maximal cell migration rose to 15.2% in both upward and downward directions; under anisotropic conditions, these values were 19.3% and 4.9%, respectively.

Effect of Wound Healing Cytokines on the 3-Dimensional Disposition of Fibroblasts

The spatial disposition of cells along the z-axis (Table I, format ii) in the presence of three concentrations of PDGF-AB are presented in Fig. 8. Those cells remaining at the interface and in the immediately adjacent upper and lower 50 μ m sections are classified as non-migratory and consequently not included. The low level of base-line migration in control cultures is manifest by a symmetric cell disposition above and below the interface (hatched bars). An isotropic concentration of

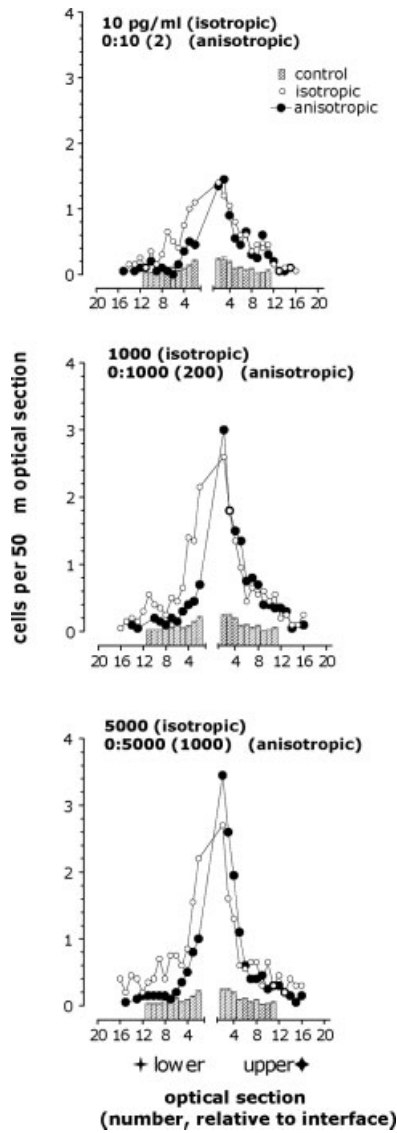


Fig. 8. Disposition of cells in response to different spatial distributions of PDGF-AB. The disposition of cells along the z-axis, expressed as “cells per 50- μ m optical section”, in the upper and lower gel compartments (abscissa) in response to isotropic and anisotropic distributions of PDGF-AB. Cell disposition in the upper gels under all tested isotropic and anisotropic cytokine concentrations was statistically indistinguishable, whereas cell disposition in the lower gels was significantly different under these two assay conditions (P values ranging from $P = 0.05$ to 0.005).

PDGF-AB (open circles) induced a dose-dependent symmetric increase in both (i) the number of cells at a given distance from the interface, and (ii) the maximal distance travelled. An asymmetric cell disposition was produced under anisotropic conditions (filled circles), with more cells present in the upper gel compartment. Comparable data obtained with TGF- β 1, - β 2 and - β 3 are presented in Fig. 9. TGF- β 1 and - β 2 had no effect on cell disposition compared to controls. In contrast, an isotropic concentra-

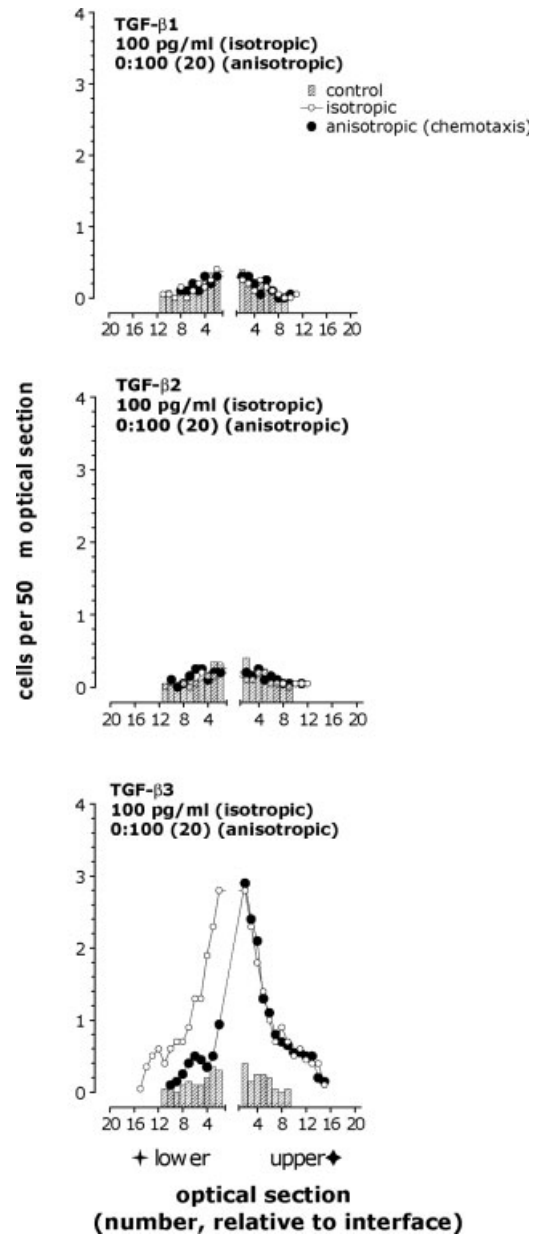


Fig. 9. Disposition of cells in response to different spatial distributions of TGF- β isoforms. Assay protocols and data presentation are as described in Fig. 6. A significant motogenic response was only obtained with TGF- β 3. In the presence of this cytokine cell disposition in the upper gel compartments was indistinguishable under isotropic and anisotropic conditions, whereas cell disposition in the lower gel compartments was statistically different ($P = 0.01$).

tion of TGF- β 3 induced a symmetrical increase in the number of migrated cells along the z-axis. An asymmetric cell disposition was obtained in the presence of an anisotropic distribution of TGF- β 3. A computer generated graphical representation of cell disposition along the z-axis in control and PDGF-treated cultures is presented in Fig. 10.

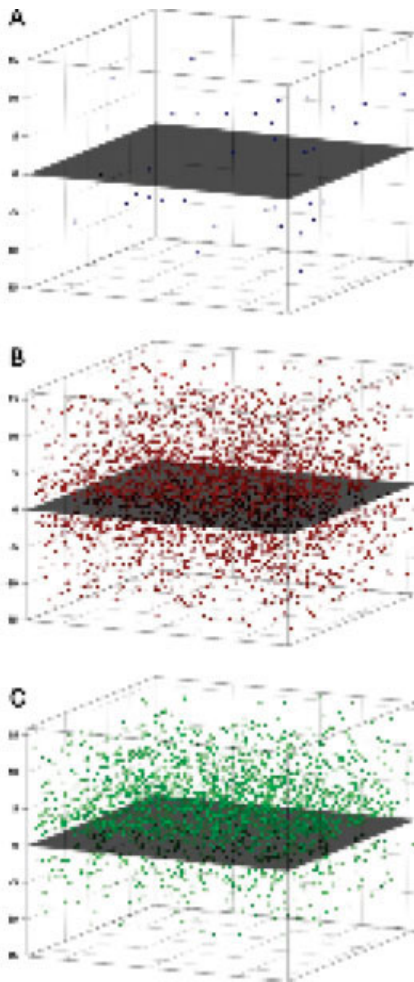


Fig. 10. Computer-generated graphic of cell disposition in response to different spatial distributions of PDGF-AB. Graphic representation of cell disposition in (A) control cultures, (B) the presence of an isotropic spatial distribution of cytokine, 1000 pg/ml, and (C) the presence of an anisotropic cytokine distribution, 0:1000 (200) pg/ml.

The effects of the wound healing cytokines on the maximal distance travelled by the target fibroblasts (i.e. leading edge) has been expressed as box and whisker plots (Table I, format v). In view of the small number of cells involved, the maximal distance travelled was grouped into $5 \times 150 \mu\text{m}$ consecutive vertical spans from the interface (i.e. 50–200, 200–350, 350–500, 500–650, 650–800 μm). In control cultures, the median distance travelled lay between 50–200 μm up and down from the interface position (Fig. 11). Isotropic concentrations of PDGF-AB elicited a symmetrical dose-dependent stimulation, in which the median distance travelled by the leading edge cell was 650–800 μm at cytokine concentrations of 1–50 ng/ml. In the presence of an anisotropic spatial distribution of PDGF-AB, distance travelled by the leading edge cell in the upward direction was indistinguishable from that elicited by isotropic

cytokine concentrations, whereas maximal stimulation of downward migration lay between 350–500 μm from the interface. As previously indicated, TGF- β 1 and - β 2 had no effect on maximal distance travelled compared to controls, whereas TGF- β 3 exhibited a significant stimulation (Fig. 12). Maximal distance travelled in the upward direction was again indistinguishable under both isotropic and anisotropic distributions of TGF- β 3, whereas downward migration was significantly higher under isotropic conditions.

DISCUSSION

Cell migration occurs within a diverse range of tissue environments *in vivo*, including the 2-dimensional surfaces upon which epithelial cells move during development and wound healing, and the 3-dimensional matrices encountered by fibroblasts and other stromal cell populations. In view of the role played by the tissue matrix in modulating cell motility [Schor, 1994], *in vitro* assays of cell migration should ideally provide a culture milieu that approximates the *in vivo* environment of the particular target cell under study. Several assays have been developed for assessing cell migration on 2-dimensional surfaces, including the monolayer “scratch”, under agarose and phagokinetic track assays [Pellegrino et al., 2003; Valster et al., 2005]. The transmembrane assay and its more recent variations have been particularly utilised to study chemo-regulated cell migration [Chen, 2004]. We initially developed the collagen gel assay to obtain quantitative data regarding fibroblast migration with a tissue-like 3-dimensional matrix [Schor, 1980]. A number of other assays have recently been described for quantifying cell migration within a defined and potentially complex 3-dimensional environment [Grinnell et al., 2006]. The sandwich assay, as presented in this communication, has specifically been developed to study the chemo-regulated migratory response of stromal cells in a more tissue-like environment than provided by the transmembrane assay.

Skin fibroblasts exhibited distinct migratory behaviour in the transmembrane and the newly developed sandwich assays. PDGF-AB and all three TGF- β isoforms stimulated cell migration in the transmembrane assay under both isotropic and anisotropic (gradient) cytokine concentrations. In contrast, only PDGF-AB and TGF- β 3 displayed motogenic activity in the sandwich assay. In the presence of isotropic concentrations of cytokine, this was manifest by a symmetrical stimulation in both the total number of cells above and below the starting interface position, as well as an increase in distance travelled. Cellular response to an anisotropic distribution of these cytokines resulted in an asymmetric dis-

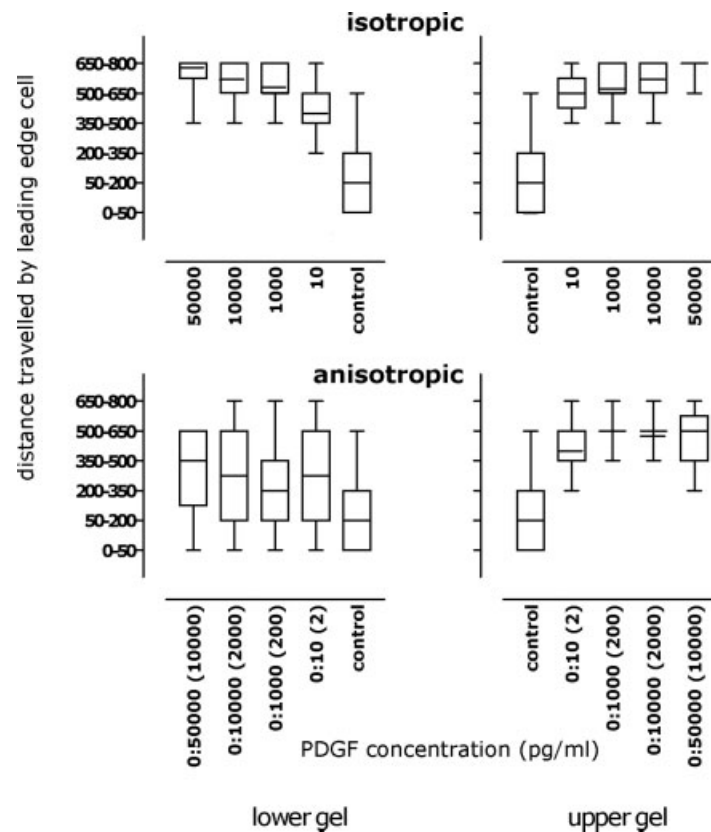


Fig. 11. Distance travelled by leading edge cell in response to PDGF-AB. Box and whisker representation of distance (μm) travelled by leading edge cell into upper and lower gel compartments in response to an isotropic and anisotropic spatial distribution of PDGF-AB: horizontal bar = median, box = interquartile range, whisker = full range. No cells were found more than 800 μm from the interface.

position of cells, with significantly more present in the upper gel compartment compared to the lower one. The motogenic response of dermal fibroblast to TGF- β 3 was clearly biphasic in nature, an observation in keeping with the biphasic migratory activity of other cytokines and soluble effector molecules [Ellis et al., 1999; Calabrese, 2001]. TGF- β 1 and - β 2 were devoid of detectable motogenic activity. The different activities of TGF- β 3 isoforms in the sandwich assay are in accord with *in vivo* results documenting (i) the diminished scarring induced by TGF- β 3 compared to the other TGF- β isoforms in a rat model of wound healing [Shah et al., 1995], and (ii) the promotion of fibroblast influx into rat incisional wounds by PDGF-BB but not TGF- β 1, in spite of their indistinguishable motogenic activity when assessed in the transmembrane assay [Pierce et al., 1989]. In contrast to the differential activities displayed by TGF- β isoforms both *in vivo* and in the sandwich assay, these cytokines have previously been reported to exert qualitatively identical motogenic activities in the transmembrane assay [Merwin et al., 1991; Parekh et al., 1994; Schor, 1994; Cordeiro et al., 2000; Olsson et al., 2000].

The congruence of results obtained in the sandwich assay with animal model data suggest that this assay may more accurately reflect cellular motogenic response *in vivo* than the commonly used transmembrane assay. This possibility is consistent with observations that fibronectin fragments containing its constituent gelatin-binding domain significantly stimulate fibroblast migration into native type I collagen substrata *in vitro* (as used in the sandwich assay) and in several animal model systems, but are devoid of motogenic activity when assessed *in vitro* on denatured collagen (as commonly employed in the conventional transmembrane assay) [Schor et al., 1996, 1999, 2003, unpublished observations]. When native collagen substrata were employed, we also reported [Ellis et al., 1992] that TGF- β 1 inhibits the motogenic activity of MSF (Migration Stimulating Factor), a novel cell activation molecule playing a hitherto unrecognised role in cancer progression and wound healing [Schor et al., 2003, 2005]. The enhanced biological relevance of results obtained in the sandwich assay carry significant implications for the establishment of assays designed to identify novel therapeutic agents. In this

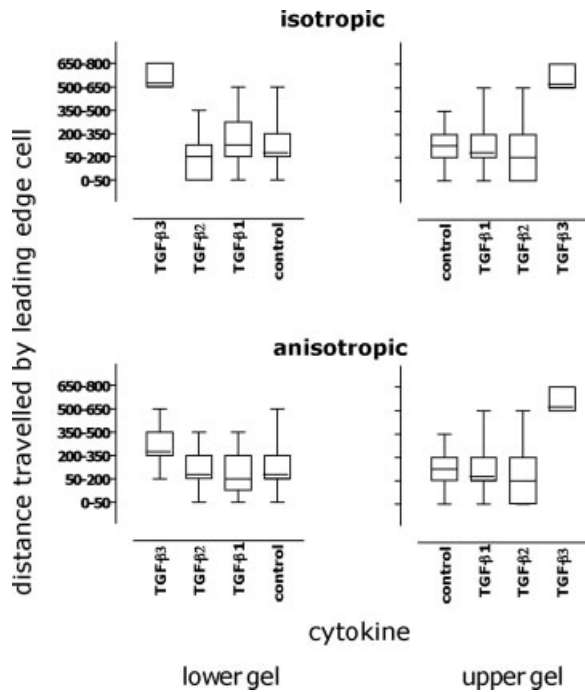


Fig. 12. Distance travelled by leading edge cell in response to TGF- β isoforms. Assay protocols and data presentation are as described in Fig. 11 and cytokine concentrations as in Fig. 9.

regard, it should be noted that recently developed high throughput screening protocols continue to be based on the transmembrane and related 2-dimensional assays [Mastyugin et al., 2004; Richards et al., 2004; Yarrow et al., 2004] which may not accurately reflect cell behaviour in vivo.

The extracellular matrix plays an important role in the control of cell migration [Schor, 1994], functioning both as (i) a modulator of cellular response to soluble motogenic factors [Schor et al., 1996; Ellis et al., 1999; Calabrese, 2001; Parameswaran et al., 2004], and (ii) as a down-stream mediator of cytokine-induced alterations in cell motility [Ellis and Schor, 1996; Ellis et al., 1997; Calabrese, 2001]. The sandwich assay has been specifically designed to provide target cells with a 3-dimensional matrix of native type I collagen fibres. The critical importance of this physiologically relevant fibrillar matrix configuration is underscored by the observation that different results were obtained in the sandwich assay and transmembrane assay using membranes coated with “native” collagen. In this context, we have previously reported that native collagen produces an amorphous mass containing few fibres when employed as a membrane coating (visualised by scanning electron microscopy) and that this differs substantially from the network of typically banded fibres comprising the collagen gel [Allen et al., 1983]. The particular physical constraints

encountered by cells moving through a 3-dimensional fibrillar environment appear to evoke distinct migratory mechanisms compared to those mediating movement upon a 2-dimensional surface [Friedl and Bröcker, 2000].

The sandwich assay is readily manipulable in terms of the number and spatial distribution of other matrix constituents which may be incorporated into the collagen gel compartments, as well as the application of physical forces (e.g. tensional stress) reported to affect cell motility and interaction with the matrix [Xu and Clark, 1996; Shreiber et al., 2001]. This fine level of experimental control provides the basis for fabricating increasingly more “tissue-like” environments for studying the hierarchy of factors that contribute to the regulation of cell migration in vivo.

The sandwich assay has been used to acquire quantitative data regarding cell migration in response to different spatial distributions of soluble effector molecules. Gradient shape has been reported to affect cell response to soluble motogens [Wang et al., 2004]. As the diffusion-driven gradient employed in the sandwich assay cannot be easily characterised and is inherently subject to temporal variation, we are currently developing techniques using various microengineering protocols [Keatch et al., 2002] to generate spatially defined and temporally stable gradients.

Chemotaxis, defined as the directional migration of cells in response to a concentration gradient of soluble motogenic factor, is posited to require the sensing of varying motogen concentration along the cell body and preferred cell orientation and migration along the gradient. A considerable amount is known regarding the signal transduction pathways and cytoskeletal motors mediating chemotaxis [Bailly et al., 2000; Haugh et al., 2000; Rönnstrand and Heldin, 2001; Kempniak et al., 2003; Parent, 2004]. Various groups have developed mathematical models to be used as a quantitative, predictive tool in the study of chemotaxis [Tranquilo et al., 1988; Sherratt, 1994; Anderson and Chaplain, 1998; Maini et al., 2004]. Models immediately applicable to the interpretation of cell migration within a 3-dimensional matrix predict a greater number of cells in the upper gel under anisotropic conditions than that achieved under isotropic conditions [Anderson and Chaplain, 1998]. It is therefore significant that data obtained in the sandwich assay indicate that no significant difference was detected under both isotropic and anisotropic cytokine distributions in the maximal plateau value of fibroblast migration into the upper gel compartment, as assessed in terms of total cell migration (Figs. 6 and 7) and cell disposition along the z-axis (Figs. 8 and 9), including distance travelled by the leading edge (Figs. 11 and 12). This unexpected finding provides a platform for future experimental and theoretical studies

designed to elucidate the link between the spatially biased disposition adopted by a cell population exposed to an anisotropic concentration of motogen and the underlying migratory characteristics of individual cells.

CONCLUSIONS

The sandwich assay generates quantitative data regarding chemo-regulated cell migration within a physiologically relevant 3-dimensional macromolecular matrix. Initial observations indicate that dermal fibroblasts display significant symmetrical and asymmetrical motogenic responses to distinct spatial distributions of PDGF-AB and TGF- β 3 (isotropic and anisotropic, respectively), whereas TGF- β 1 and - β 2 are devoid of such activity. These results stand in contrast to the similar motogenic (chemokinetic and chemotactic) activities elicited by all four cytokine in the transmembrane assay. The diverse migratory parameters quantitatively evaluated in the sandwich assay should prove useful in testing various mathematical models predicting the 3-dimensional disposition of cell populations resulting from an imposed spatial distribution of motogen. Apart from being useful in elucidating "tissue-level" migratory behaviour relevant to such pathological processes as wound healing and cancer invasion, the sandwich assay should additionally provide a rational platform for improving high throughput drug screening regimens for identifying clinically relevant motogenic and/or angiogenic agents.

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