



(86) Date de dépôt PCT/PCT Filing Date: 1998/07/23
 (87) Date publication PCT/PCT Publication Date: 1999/02/04
 (45) Date de délivrance/Issue Date: 2008/12/16
 (85) Entrée phase nationale/National Entry: 2000/01/20
 (86) N° demande PCT/PCT Application No.: US 1998/015228
 (87) N° publication PCT/PCT Publication No.: 1999/004806
 (30) Priorité/Priority: 1997/07/23 (US08/899,304)

(51) Cl.Int./Int.Cl. *A61K 38/57* (2006.01),
A61K 38/16 (2006.01), *A61P 35/00* (2006.01),
C07K 14/81 (2006.01), *C12N 5/10* (2006.01),
C12N 15/12 (2006.01)
 (72) Inventeurs/Inventors:
 BOUCK, NOEL P., US;
 DAWSON, DAVID W., US;
 GILLIS, PAUL R., US
 (73) Propriétaire/Owner:
 NORTHWESTERN UNIVERSITY, US
 (74) Agent: GOWLING LAFLEUR HENDERSON LLP

(54) Titre : PROCÉDES ET COMPOSITIONS POUR INHIBER L'ANGIOGENESE
 (54) Title: METHODS AND COMPOSITIONS FOR INHIBITING ANGIOGENESIS

(57) **Abrégé/Abstract:**

The present invention provides a method of inhibiting angiogenesis within a tissue by providing exogenous SLED to cells associated with the tissue. The presence of exogenous SLED inhibits angiogenesis within the tissue, in part by interfering with the ability of vascular endothelia to expand within the tissue. The invention also provides a method for determining the severity of a tumor by assaying for the presence of SLED within the tumor. To facilitate the inventive methods, the present invention provides pharmaceutical compositions including sources of SLED.

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁶ : A61K 38/16, 38/18, 38/17</p>	<p>A1</p>	<p>(11) International Publication Number: WO 99/04806 (43) International Publication Date: 4 February 1999 (04.02.99)</p>
<p>(21) International Application Number: PCT/US98/15228 (22) International Filing Date: 23 July 1998 (23.07.98) (30) Priority Data: 08/899,304 23 July 1997 (23.07.97) US (71) Applicant: NORTHWESTERN UNIVERSITY [US/US]; 633 Clark Street, Evanston, IL 60208 (US). (72) Inventors: BOUCK, Noel, P.; 611 N. Kenilworth Avenue, Oak Park, IL 60302 (US). DAWSON, David, W.; 3837 N. Fremont Street, Chicago, IL 60613 (US). GILLIS, Paul, R.; Apartment #3, 3737 N. Magnolia, Chicago, IL 60613 (US). (74) Agents: HEFNER, M., Daniel et al.; Leydig, Voit & Mayer, Ltd., Two Prudential Plaza, Suite 4900, 180 North Stetson, Chicago, IL 60601-6780 (US).</p>	<p>(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: METHODS AND COMPOSITIONS FOR INHIBITING ANGIOGENESIS</p> <p>(57) Abstract</p> <p>The present invention provides a method of inhibiting angiogenesis within a tissue by providing exogenous SLED to cells associated with the tissue. The presence of exogenous SLED inhibits angiogenesis within the tissue, in part by interfering with the ability of vascular endothelia to expand within the tissue. The invention also provides a method for determining the severity of a tumor by assaying for the presence of SLED within the tumor. To facilitate the inventive methods, the present invention provides pharmaceutical compositions including sources of SLED.</p>		

METHODS AND COMPOSITIONS FOR INHIBITING ANGIOGENESIS**STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER
FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT**

5 This invention was made with Government support under Grant Number CA52750 and CA64239 awarded by the National Institutes of Health. The United States Government may have certain rights in this invention.

TECHNICAL FIELD OF THE INVENTION

10 The present invention relates to methods and compositions for inhibiting angiogenesis.

BACKGROUND OF THE INVENTION

15 Angiogenesis is the fundamental process by which new blood vessels are formed. The process involves the migration of vascular endothelial cells into tissue, followed by the condensation of such endothelial cells into vessels. Angiogenesis may be induced by an angiogenic agent or be the result of a natural condition. The process is essential to a variety of normal body activities, such as reproduction, development and wound repair. Although the process is not
20 completely understood, it involves a complex interplay of molecules that stimulate and molecules that inhibit the growth and migration of endothelial cells, the primary cells of the capillary blood vessels. Under normal conditions, these molecules appear to maintain the microvasculature in a quiescent state (i.e., without capillary growth) for prolonged periods which can last for several years or
25 even decades. The turnover time for an endothelial cell is about 1,000 days. Under appropriate conditions, however (e.g., during wound repair), these same cells can undergo rapid proliferation and turnover within a much shorter period, and five days is typical under these circumstances. (Folkman and Shing, *J. Biol. Chem.*, 267(16), 10931-34 (1989); Folkman and Klagsbrun, *Science*, 235, 442-47
30 (1987)).

Although angiogenesis is a highly regulated process under normal conditions, many diseases (characterized as "angiogenic diseases") are driven by persistent unregulated angiogenesis. In such disease state, unregulated angiogenesis can either cause a particular disease directly or exacerbate an
35 existing pathological condition. For example, ocular neovascularization has been implicated as the most common cause of blindness and underlies the pathology of approximately 20 eye diseases. In certain previously existing conditions such as

arthritis, newly formed capillary blood vessels invade the joints and destroy cartilage. In diabetes, new capillaries formed in the retina invade the vitreous humor, causing bleeding and blindness.

Both the growth and metastasis of solid tumors are also angiogenesis-
5 dependent (Folkman, *J. Cancer Res.*, 46, 467-73 (1986); Folkman, *J. Nat. Cancer Inst.*, 82, 4-6 (1989); Folkman et al., "Tumor Angiogenesis," Chapter 10, pp. 206-32, in *The Molecular Basis of Cancer*, Mendelsohn et al., eds. (W. B. Saunders, 1995)). It has been shown, for example, that tumors which enlarge to greater than
10 2 mm. in diameter must obtain their own blood supply and do so by inducing the growth of new capillary blood vessels. After these new blood vessels become embedded in the tumor, they provide nutrients and growth factors essential for tumor growth as well as a means for tumor cells to enter the circulation and metastasize to distant sites, such as liver, lung or bone (Weidner, *New Eng. J. Med.*, 324(1), 1-8 (1991)). When used as drugs in tumor-bearing animals, natural
15 inhibitors of angiogenesis can prevent the growth of small tumors (O'Reilly et al., O'Reilly et al., *Cell*, 79, 315-28 (1994)). Indeed, in some protocols, the application of such inhibitors leads to tumor regression and dormancy even after cessation of treatment (O'Reilly et al., *Cell*, 88, 277-85 (1997)). Moreover, supplying inhibitors of angiogenesis to certain tumors can potentiate their
20 response to other therapeutic regimens (e.g., chemotherapy) (see, e.g., Teischer et al., *Int. J. Cancer*, 57, 920-25 (1994)).

Although several angiogenesis inhibitors are currently under development for use in treating angiogenic diseases (Gasparini, *Eur. J. Cancer*, 32A(14), 2379-85 (1996)), there are disadvantages associated with several of these proposed
25 inhibitory compounds. For example, suramin is a potent angiogenesis inhibitor, but, at doses required to reach antitumor activity, causes severe systemic toxicity in humans. Other compounds, such as retinoids, interferons and antiestrogens appear safe for human use but have only a weak anti-angiogenic effect. Still other compounds may be difficult or costly to make. In view of these problems, there
30 exists a need for methods and compositions for inhibiting angiogenesis.

BRIEF SUMMARY OF THE INVENTION

The present invention provides a method of inhibiting angiogenesis within a tissue by providing exogenous SLED (an antiangiogenic protein) to endothelial
35 cells associated with the tissue. The presence of exogenous SLED will inhibit angiogenesis within the tissue, in part by interfering with the ability of vascular endothelia to expand within the tissue. The invention also provides a method for

determining the prognosis of a tumor by assaying for the presence of SLED within the tumor. To facilitate the inventive method, the present invention provides pharmaceutical compositions including sources of SLED.

5 The methods and compositions of the present invention are clinically useful for treating a host of diseases associated with angiogenesis, and for interfering with angiogenesis associated with reproductive functions. The methods and compositions are also diagnostically useful for assessing the prognosis of tumors and other disorders associated with angiogenesis. Furthermore, the methods and compositions are useful reagents for investigation
10 of angiogenesis in the laboratory setting. These and other advantages of the present invention, as well as additional inventive features, will be apparent from the accompanying drawings and in the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

15 Figure 1 graphically illustrates the ability of SLED to inhibit the migration of endothelial cells.

Figures 2A-2D demonstrates the specificity of SLED for vascular endothelia by graphically representing the inability of various doses of SLED to inhibit the migration of cells other than vascular endothelial cells. Figure 2A
20 depicts data concerning WI-38 cells. Figure 2B depicts data concerning human foreskin fibroblasts. Figure 2C depicts data concerning vascular smooth muscle cells. Figure 2D depicts data concerning neutrophils.

Figure 3 is a dose-response curve representing the antiangiogenic activity of one type of SLED polypeptide (full length PEDF).

25 Figure 4 is a dose response curve representing the antiangiogenic activity of one type of SLED polypeptide (a fragment of PEDF).

DETAILED DESCRIPTION OF THE INVENTION

30 Within the context of the inventive method, SLED is a protein having potent antiangiogenic properties, and it includes any antiangiogenic derivative of pigment epithelium derived factor (PEDF, Steele et al., *Proc. Nat. Acad. Sci. (USA)*, 90(4), 1526-30 (1993)). One form of SLED polypeptide is set forth at SEQ ID NO:1; however, the invention is not limited to the use of this exemplary sequence. Indeed, other PEDF sequences are known in the art (see, e.g., published
35 international patent applications WO 95/33480 and WO 93/24529), and genetic sequences can vary between different species and individuals, and this natural scope of allelic variation is included within the scope of the invention.

Additionally and alternatively, a SLED polypeptide can include one or more point mutations from the exemplary sequence or naturally occurring SLED polypeptide. Thus, a SLED polypeptide is typically at least about 75 % homologous to all or a portion of SEQ ID NO:1 and preferably is at least about 80 % homologous to all or a portion of SEQ ID NO:1 (e.g., at least about 85 % homologous to SEQ ID NO:1); more preferably the SLED polypeptide is at least about 90 % homologous to all or a portion of SEQ ID NO:1 (such as at least about 95 % homologous to all or a portion of SEQ ID NO:1), and most preferably the SLED polypeptide is at least about 97 % homologous to all or a portion of SEQ ID NO:1. Indeed, the SLED polypeptide can also include other domains, such as epitope tags and His tags (e.g., the protein can be a fusion protein).

Within the context of the present invention, a SLED polypeptide can be or comprise insertion, deletion, or substitution mutants of a known PEDF sequence or derivative thereof. Preferably, any substitution is conservative in that it minimally disrupts the biochemical properties of the SLED polypeptide. Thus, where mutations are introduced to substitute amino acid residues, positively-charged residues (H, K, and R) preferably are substituted with positively-charged residues; negatively-charged residues (D and E) preferably are substituted with negatively-charged residues; neutral polar residues (C, G, N, Q, S, T, and Y) preferably are substituted with neutral polar residues; and neutral non-polar residues (A, F, I, L, M, P, V, and W) preferably are substituted with neutral non-polar residues. Moreover, the SLED polypeptide can be an active fragment of a known PEDF protein or fragment thereof. Indeed, it has been found that truncated fragments derived from SEQ ID NO:1 are active SLED polypeptides. For example, it is believed that residues 1 through 20 of SEQ ID NO:1 are cleaved during secretion and thus are dispensable for SLED activity. Moreover, other active SLED polypeptides comprise sequences derived from residues 21 through 382 of SEQ ID NO:1, such as residues 44 through 157 of SEQ ID NO:1 (e.g., residues 45 through 121 of SEQ ID NO:1). Of course, while insertion, deletion, or substitution mutations can affect glycosylation of the protein, a SLED polypeptide need not be glycosylated to possess the requisite antiangiogenic properties for use in the inventive method.

SLED polypeptides inhibit angiogenesis, in part, by attenuating the migration of endothelial cells, thus reducing the ability of endothelia to expand within the tissue. Thus, the invention provides a method of inhibiting endothelial cell migration by providing exogenous SLED to such cells. Aside from attenuating angiogenesis, the method is useful for treating disorders associated

with stimulation of endothelial cell migration such as intestinal adhesions, Crohn's disease, atherosclerosis, scleroderma and hypertrophic scars (e.g., keloids).

In accordance with the inventive method, SLED is provided to endothelial cells associated with the tissue of interest. Such cells can be cells comprising the tissue of interest, exogenous cells introduced into the tissue, or neighboring cells not within the tissue. Thus, for example, the cells can be cells of the tissue, and SLED is provided to them in situ such that the SLED contacts the cells. Alternatively, the cells can be cells introduced into the tissue, in which case the SLED can be transferred to the cells before they are so introduced into the tissue (e.g., in vitro), as well as being transferred in situ after introduction into the tissue.

The tissue with which the endothelial cells are associated is any tissue in which it is desired to inhibit the migration or expansion of endothelia, (e.g., for inhibiting angiogenesis). In one application, the tissue can be eye tissue, in which case the presence of exogenous SLED will inhibit novel angiogenesis associated with a variety of disorders of the eye. For example, the inventive method is useful for treating eye injury, hypoxia, infection, surgery, laser surgery, diabetes, retinoblastoma or other diseases or disorders of the eye. In this respect, the method is useful for preventing blindness or retarding loss of vision associated with a variety of eye diseases.

In another application, the tissue is skin tissue, in which case the presence of exogenous SLED prevents neovascularization associated with several skin diseases. For example, the inventive method is useful for treating diseases and disorders such as psoriasis, scleroderma, tumors of the skin, neovascularization as a consequence of infection (e.g., cat scratch disease, bacterial ulceration, etc.) or other skin disorders. Where SLED is provided to the skin, it can be provided to the surface of the skin or to skin tissue beneath the skin's surface. Furthermore, transfer of SLED to skin of a mammal can also stimulate the growth of hair in the skin. Without being bound by any particular theory, it is believed that SLED affects hair growth by mediating angiogenesis within the hair follicle.

In other embodiments, the tissue is a tumor (e.g., a cancerous tumor), in which case the inventive method will inhibit the growth of blood vessels within and to the tumor. Inhibiting the growth of blood vessels within tumors prevents sufficient nutrients and oxygen from being supplied to the tumor to support growth beyond a given size. Thus, the inventive method can prevent the nucleation of tumors from cancerous cells already present due to genetic predisposition (e.g., BRCA-1 mutation carriers, Li Fraumeni patients with p53 mutations, etc.) or the presence of external carcinogens (e.g., tobacco, alcohol,

industrial solvents, etc.). Aside from preventing tumorigenesis, the inventive method can retard the growth of existing tumors, thus rendering them more easily contained and excised. This application is highly advantageous for treating tumors that are difficult to operate on (e.g., brain or prostate tumors). Moreover, 5 minimizing the number of blood vessels within existing tumors lessens the probability that the tumor will metastasize. In treating tumors, the method can be used alone or in conjunction with other treatments, to control the growth of tumors. Indeed, employing the inventive method can potentiate the response of some tumors to other therapies. For example, the inventive method optionally can 10 be employed as a pretreatment for (e.g., for about a week in advance of), and continued during, a chemotherapeutic or radiation regimen.

Where the inventive method is applied to other tissues, the prevention of neovascularization effectively treats a host of disorders. Thus, for example, the inventive method can be used as part of a treatment for disorders of blood vessels 15 (e.g., hemangiomas and capillary proliferation within atherosclerotic plaques), muscle diseases (e.g., myocardial angiogenesis or angiogenesis within smooth muscles), joints (e.g., arthritis, hemophilic joints, etc.), and other disorders associated with angiogenesis (e.g., Osler-Webber Syndrome, plaque neovascularization, telangiectasia, angiofibroma, wound granularization, etc.).

20 Aside from treating disorders and symptoms associated with neovascularization, the inhibition of angiogenesis can be used to modulate or prevent the occurrence of normal physiological conditions associated with neovascularization. Thus, for example the inventive method can be used as a birth control. In accordance with the inventive method, the presence of SLED within 25 the ovaries or endometrium can attenuate neovascularization associated with ovulation, implantation of an embryo, placenta formation, etc.

Within the context of the inventive method, SLED can be supplied alone or in conjunction with other known antiangiogenic factors. For example, SLED can be used in conjunction with antibodies and peptides that block integrin 30 engagement, proteins and small molecules that inhibit metalloproteinases (e.g., marmistat), agents that block phosphorylation cascades within endothelial cells (e.g., herbamycin), dominant negative receptors for known inducers of angiogenesis, antibodies against inducers of angiogenesis or other compounds that block their activity (e.g., suramin), or other compounds (e.g., retinoids, IL-4, 35 interferons, etc.) acting by other means. Indeed, as such factors modulate angiogenesis by different mechanisms, employing SLED in combination with

PCT/US 98/15228
PEA/US 23 JUL 1999

7

other antiangiogenic agents can potentiate a more potent (and potentially synergistic) inhibition of angiogenesis within the desired tissue.

As discussed herein, SLED is a proteinaceous factor. Thus, in one protocol, the method involves providing SLED by supplying a SLED polypeptide to the cells (e.g., within a suitable composition). Any suitable method can be employed to obtain a SLED polypeptide for use in the present invention. Many suitable SLED polypeptides can be purified from tissues which naturally produce SLED or from media conditioned by a variety of SLED-producing cells (e.g., retinoblastoma cell line WER127). For example, it is known that SLED is produced by all types of muscle, megakaryocytes of the spleen, fibroblasts, kidney tubules, cerebellar Purkinje cells, pilosebaceous glands of hair follicles, and retinal cells. A particularly good source of naturally occurring SLED is vitreous and aqueous humors extracted from the eye. One protocol for purifying SLED from protein extracts of these (or other sources) is by concentration/dialysis using a 30 kDa ultrafiltration membrane followed by protein precipitation in a range of about 65% to about 95% ammonium sulfate, followed by a lentil lectin sepharose column at 0.5 M methyl- α -D-mannopyranoside, followed by gradient/isocratic elution at 0.5 M NaCl from a PHARMACIA HiTrap^{*} heparin column. Other protocols for purifying SLED polypeptides are known in the art (see, e.g., published international patent applications WO 95/33480 and WO 93/24529). A native SLED polypeptide is identified via SDS-PAGE as a protein of about 45 kDa. Other SLED polypeptides can be synthesized using standard direct peptide synthesizing techniques (e.g., as summarized in Bodanszky, *Principles of Peptide Synthesis* (Springer-Verlag, Heidelberg: 1984)), such as via solid-phase synthesis (see, e.g., Merrifield, *J. Am. Chem. Soc.*, 85, 2149-54 (1963); Barany et al., *Int. J. Peptide Protein Res.*, 30, 705-739 (1987); and U.S. Patent 5,424,398). Of course, as genes for SLED polypeptides are known (see, e.g., published international patent applications WO 95/33480 and WO 93/24529); see also GenBank accession no. U29953), or can be deduced from the polypeptide sequences discussed herein, a SLED polypeptide can be produced by standard recombinant methods.

In other protocols, SLED polypeptide can be provided to the tissue of interest by transferring an expression cassette including a nucleic acid disclosing SLED to cells associated with the tissue of interest. The cells produce and secrete the SLED polypeptide such that it is suitably provided to endothelial cells within the tissue to inhibit their migration and, thus, to attenuate angiogenesis within the tissue of interest. Coding sequences for SLED polypeptides are known (see, e.g.,

* Trade-mark

published international patent applications WO 95/33480 and WO 93/24529); see also GenBanc accession no. U29953), and others can be deduced from the polypeptide sequences discussed herein. Thus, SLED expression cassettes typically employ coding sequences homologous to these known sequences, e.g.,
5 they will hybridize to at least a fragment of the known sequences under at least mild stringency conditions, more preferably under moderate stringency conditions, most preferably under high stringency conditions (employing the definitions of mild, moderate, and high stringency as set forth in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2d edition, Cold Spring Harbor Press (1989)).

10 In addition to the SLED coding sequence, an expression cassette includes a promoter, and, in the context of the present invention, the promoter must be able to drive the expression of the SLED gene within the cells. Many viral promoters are appropriate for use in such an expression cassette (e.g., retroviral ITRs, LTRs, immediate early viral promoters (IEp) (such as herpesvirus IEp (e.g., ICP4-IEp
15 and ICP0-IEp) and cytomegalovirus (CMV) IEp), and other viral promoters (e.g., late viral promoters, latency-active promoters (LAPs), Rous Sarcoma Virus (RSV) promoters, and Murine Leukemia Virus (MLV) promoters)). Other suitable promoters are eukaryotic promoters, such as enhancers (e.g., the rabbit β -globin regulatory elements), constitutively active promoters (e.g., the β -actin promoter,
20 etc.), signal specific promoters (e.g., inducible and/or repressible promoters, such as a promoter responsive to TNF or RU486, the metallothionine promoter, etc.), and tumor-specific promoters.

Within the expression cassette, the SLED gene and the promoter are operably linked such that the promoter is able to drive the expression of the SLED
25 gene. As long as this operable linkage is maintained, the expression cassette can include more than one gene, such as multiple genes separated by ribosome entry sites. Furthermore, the expression cassette can optionally include other elements, such as polyadenylation sequences, transcriptional regulatory elements (e.g., enhancers, silencers, etc.), or other sequences.

30 The expression cassette must be introduced into the cells in a manner suitable for them to express the SLED gene contained therein. Any suitable vector can be so employed, many of which are known in the art. Examples of such vectors include naked DNA vectors (such as oligonucleotides or plasmids), viral vectors such as adeno-associated viral vectors (Berns et al., *Ann. N.Y. Acad. Sci.*,
35 772, 95-104 (1995)), adenoviral vectors (Bain et al., *Gene Therapy*, 1, S68 (1994)), herpesvirus vectors (Fink et al., *Ann. Rev. Neurosci.*, 19, 265-87 (1996)), packaged amplicons (Federoff et al., *Proc. Nat. Acad. Sci. USA*, 89, 1636-40

(1992)), pappiloma virus vectors, picornavirus vectors, polyoma virus vectors, retroviral vectors, SV40 viral vectors, vaccinia virus vectors, and other vectors. In addition to the expression cassette of interest, the vector can also include other genetic elements, such as, for example, genes encoding a selectable marker (e.g.,
5 β -gal or a marker conferring resistance to a toxin), a pharmacologically active protein, a transcription factor, or other biologically active substance.

Once a given type of vector is selected, its genome must be manipulated for use as a background vector, after which it must be engineered to incorporate exogenous polynucleotides. Methods for manipulating the genomes of vectors are
10 well known in the art (see, e.g., Sambrook et al., *supra*) and include direct cloning, site specific recombination using recombinases, homologous recombination, and other suitable methods of constructing a recombinant vector. In this manner, the expression cassette can be inserted into any desirable position of the vector.

The vector harboring the SLED expression cassette is introduced into the
15 cells by any means appropriate for the vector employed. Many such methods are well-known in the art (Sambrook et al., *supra*; see also Watson et al., *Recombinant DNA*, Chapter 12, 2d edition, Scientific American Books (1992)). Thus, plasmids are transferred by methods such as calcium phosphate precipitation, electroporation, liposome-mediated transfection, gene gun, microinjection, viral
20 capsid-mediated transfer, polybrene-mediated transfer, protoplast fusion, etc. Viral vectors are best transferred into the cells by infecting them; however, the mode of infection can vary depending on the virus.

Cells into which the SLED gene has been transferred can be used in the inventive method as transient transformants. Alternatively, where the cells are
25 cells *in vitro*, they can be subjected to several rounds of clonal selection (if the vector also contains a gene encoding a selectable marker, such as a gene conferring resistance to a toxin) to select for stable transformants.

Within the cells, the SLED gene is expressed such that the cells express and secrete the SLED polypeptide. Successful expression of the gene can be
30 assessed via standard molecular biological techniques (e.g., Northern hybridization, Western blotting, immunoprecipitation, enzyme immunoassay, etc.). Reagents for detecting the expression of SLED genes and the secretion of SLED from transfected cells are known in the art (see, e.g., published international patent applications WO 95/33480 and WO 93/24529); Steele et al., *supra*).

35 Depending on the location of the tissue of interest, SLED can be supplied in any manner suitable to provide it to endothelial cells within the tissue of interest. Thus, for example, a composition containing a source of SLED (i.e., a

SLED polypeptide or a SLED expression cassette, as described herein) can be introduced into the systemic circulation, which will distribute the source of SLED to the tissue of interest. Alternatively, a composition containing a source of SLED can be applied topically to the tissue of interest (e.g., injected as a bolus within a tumor or intercutaneous or subcutaneous site, applied to all or a portion of the surface of the skin, dropped onto the surface of the eye, etc.).

Where the source of SLED is a SLED polypeptide (e.g., within a suitable composition), it is provided in a concentration and for a time sufficient to inhibit angiogenesis within the tissue. Where SLED is produced naturally, it can be present in concentrations as high as about 250 nM. Because SLED is non-toxic, it can be supplied to tissues in a far more concentrated form. However, given SLED's potency, it can be employed in the inventive method at far reduced concentrations, such as about 50 nM or less (e.g., about 10 nM or less). Indeed, in some protocols, about 2 nM SLED or less effectively inhibits angiogenesis and endothelial cell migration. Depending on the formulation of a composition comprising the protein, it is supplied over a time course sufficient to retard angiogenesis within the desired tissue. In some protocols (e.g., where the SLED is supplied to the surface of skin or to the eye), repeated application enhances the antiangiogenic effect and may be required in some applications. Where the source of SLED is a SLED expression cassette, the cells expressing the cassette produce an effective amount of the protein (i.e., sufficient to inhibit angiogenesis in the tissue).

To facilitate the inventive method, the invention provides a pharmacological composition comprising a source of SLED and a suitable diluent. In addition to the source of SLED, the composition includes a diluent, which includes one or more pharmacologically-acceptable carriers. Pharmaceutical compositions for use in accordance with the present invention can be formulated in a conventional manner using one or more pharmacologically or physiologically acceptable carriers comprising excipients, as well as optional auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen. Thus, for systemic injection, the source of SLED can be formulated in aqueous solutions, preferably in physiologically compatible buffers. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. For oral administration, the source of SLED can be combined with carriers suitable for inclusion into tablets, pills, dragees, capsules, liquids, gels,

5 syrups, slurries, diposomes, suspensions and the like. For administration by inhalation, the source of SLED is conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant. The source of SLED can be formulated for parenteral administration
10 by injection, e.g., by bolus injection or continuous infusion. Such compositions can take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. For application to the skin, the source of SLED can be formulated into a suitable gel, magma, creme, ointment, or other carrier. For
15 application to the eyes, the source of SLED can be formulated in aqueous solutions, preferably in physiologically compatible buffers. The source of SLED can also be formulated into other pharmaceutical compositions such as those known in the art.

20 Because it is known that SLED is absent from some tumors, the invention also provides a method for determining the prognosis of a tumor by assaying for the presence of SLED within the tumor. The method involves obtaining tissue or fluid from the tumor and detecting the presence or absence of SLED within the tissue or fluid. Greater the SLED concentration within the tumor correlates with a lesser likelihood that the tumor is undergoing angiogenesis. Thus, higher SLED
25 concentration within the tumor is indicative of a relatively early stage of tumerogenesis and is, thus, an optimistic indication. Conversely, the absence of SLED within a given tumor is indicative of a more advanced stage of tumerogenesis. The method can employ an assay for the presence of PEDF gene expression (e.g., via rtPCR, Northern hybridization, in situ hybridization etc.).
30 Alternatively, the method can employ an assay for the presence of secreted SLED (e.g., immunological assays, SLED purification and PAGE analysis, etc.). Reagents for detecting the presence of SLED within such tumors are known in the art (see, e.g., published international patent applications WO 95/33480 and WO 93/24529).

35 While it is believed that one of skill in the art is fully able to practice the invention after reading the foregoing detailed description, the following examples further illustrate some of its features. As these examples are included for purely illustrative purposes, they should not be construed to limit the scope of the invention in any respect.

The procedures employed in these examples, such as cell culture, manipulation of protein and DNA, etc. are well known in the art (see generally Sambrook et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor

Laboratory, Cold Spring Harbor, NY (1989)). Accordingly, in the interest of brevity, the experimental protocols are not discussed in detail.

EXAMPLE 1

5 This example demonstrates that SLED prevents endothelial cell migration.

The migration of different vascular endothelial cell types was determined by adding SLED to cultured endothelial cells. Specifically, endothelial cells isolated from bovine adrenal capillaries, human umbilical chords, and human dermal microvascular tissue.

10 The cells were plated on gelatinized Nucleopore membranes (5 μm pores for bovine capillary cells and 8 μm pores for other cells) in an inverted modified Boyden chamber. After two hours, the chamber was reinverted and test substances added to the top wells of each. Specifically, populations were exposed to either culture medium alone (control), 10 ng/ml bFGF, 2 nM SLED (full length
15 PEDF), or both 10 ng/ml bFGF (Fibroblast Growth Factor) and 10 nM SLED. The cells were then permitted to migrate for 3-4 hours. Following this, the membranes were fixed and stained, and the number of cells that had migrated were counted.

The results of the assay are presented in Figure 1 as a percentage of
20 maximal migration (error bars represent standard error measurement, $n=4$). As is depicted, all three types of vascular endothelial cells exhibited nearly 100% migration in the presence of bFGF. However, in the presence of SLED, considerably less migration was observed. These results demonstrate that SLED inhibits endothelial cell migration. These results are surprising, given that the
25 PEDF protein is known to induce neural differentiation of cultured retinoblastoma tumor cells, to be a neurotrophic factor for cerebellar granular cells and a cytostatic factor for glial cells (Taniwaki et al., *J. Neurochem.*, 68, 26-32 (1997); Sugita et al., *J. Neurosci. Res.*, 49, 710-18 (1997); Tombran-Tink et al., *Exp. Eye Res.*, 53, 411-14 (1991); Becerra, "Structure-Function Studies on PEDF," Chapter
30 21, in *Chemistry and Biology of Serpins*, Church et al., eds. (Plenum Press, 1997)).

EXAMPLE 2

This example demonstrates that the prevention of cell migration by SLED is specific for endothelial cells.

35 The ability of SLED to prevent migration of fibroblasts or smooth muscle was tested using cells obtained from human diploid fibroblast cell line WI-38,

human foreskin fibroblasts, vascular smooth muscle, and normal human neutrophils.

The assay was performed as indicated in Example 1, except that the dose of SLED varied from 0.01 nM to about 50 nM and that the migration assay was performed without inverting the chambers. Moreover, the inducer of migration varied with the cell type (IL-8 was used at 1 µg/ml and PDGF was used at 250 pg/ml).

The results of this experiment are presented in Figures 2A-2D. As indicated in these figures, SLED did not inhibit migration of any of the cell lines. This result indicates that the antimigratory activity of SLED is specific for vascular endothelial cells.

EXAMPLE 3

This example demonstrates that SLED is among the most potent inhibitors of endothelial cell migration.

Using a protocol similar to that outlined in Example 1, bovine adrenal capillary endothelial cells were exposed to bFGF, SLED, and several known antiangiogenic factors. The amount of a given factor necessary to achieve 50% of migration was determined and is reported here as ED₅₀. A smaller ED₅₀ measurement indicates a more potent antiangiogenic factor. The results of this experiment, presented in Table 1, indicate that SLED is a highly potent antiangiogenic factor.

Table 1

Agent	ED₅₀ (nM)
SLED	0.1-0.5
Thrombospondin	0.5
Endostatin	3.0
Angiostatin	3.5
Retinoic Acid	15
Tissue Inhibitor of Metalloproteinase-1	3500
Captopril	10,000

EXAMPLE 4

This example demonstrates that SLED inhibits the angiogenic activity of known angiogenic agents.

Using a protocol similar to that outlined in Example 1, bovine adrenal capillary endothelial cells were exposed to five known angiogenic agents alone or in combination with 0.1 $\mu\text{g/ml}$ SLED. In particular, aFGF was employed at a concentration of 50 ng/ml, bFGF was employed at a concentration of 10 ng/ml, 5 IL-8 was employed at a concentration of 40 ng/ml, PDGF was employed at a concentration of 250 pg/ml, and VEGF was employed at a concentration of 100 pg/ml.

The results of the assay are presented in Figure 3. As is depicted, the migration of the cells was considerably inhibited by SLED, regardless of the 10 angiogenic agent. These results demonstrate that SLED-mediated inhibition of vascular endothelial migration is not specific for bFGF induction, but that SLED acts generally to inhibit migration of these cells.

EXAMPLE 5

15 This example demonstrates that SLED inhibits neovascularization *in vivo*.

Pellets containing various proteins were implanted in the avascular corneas of rats. Pellets either contained or lacked bFGF, and the pellets also contained either SLED or bovine serum albumin (BSA) as a control. After seven days, the corneas of the rats were examined to note whether angiogenesis had occurred.

20 The results of this assay are presented in Table 2. As indicated, no vascularization was observed from injecting pellets lacking bFGF. However, vascularization was observed in all eyes implanted with bFGF and BSA. Co-injection of bFGF and SLED, however, resulted in no neovascularization in any cornea. These results indicate that SLED is a potent inhibitor of angiogenesis in 25 *vivo*.

Table 2*

Treatment	Without bFGF	With bFGF
SLED (8nM)	0/3	0/3
30 BSA	0/2	4/4

*results expressed as number of corneas with angiogenesis/number of test animals.

EXAMPLE 6

35 This example demonstrates that SLED polypeptides other than the full PEDF protein are active antiangiogenic agents.

Trypsin digestion of the complete PEDF protein cleaves the protein at amino acid 352, removing the approximately 3-5 kDa carboxy-terminal portion of the protein (Becerra et al., *J. Biol. Chem.*, 270, 25992-99 (1995)). This procedure was employed to generate the fragments, and the truncated N-PEDF fragment
5 was purified from trypsin by heparin affinity chromatography.

Using a protocol similar to that outlined above, various concentrations of either full length PEDF or the truncated peptide were assayed for their respective abilities to affect endothelial cell migration. Data generated for the truncated peptide are indicated in Figure 4. Comparison of these data with the activity of
10 the full length PEDF (see Figure 3) reveals both proteins to be similarly potent at inhibiting endothelial cell migration. These results indicate that peptides other than full length PEDF are active SLED polypeptides.

EXAMPLE 7

15 This example demonstrates that exogenous SLED applied to the skin promotes the growth of hair therein.

In this example, the ability of SLED to induce hair growth is investigated using an established mouse model. In particular, in the C57BL/6 mouse strain, a change in skin pigmentation correlates with the physiology of the skin being in
20 anagen (growing), catagen (transitional), or telogen (resting) phase (see, e.g., Jiang et al., *J. Invest. Dermatol.*, 104, 523-25 (1995); Slominski et al., *J. Invest. Dermatol.*, 102, 862-69 (1994); Paus et al., *Lab. Invest.*, 71, 134-40 (1994); and Paus et al., *Lab. Invest.*, 60, 365-69 (1989)). Six or seven week old C57BL/6 mice have pink skin, indicating telogen phase. Depilation of these mice triggers hair
25 growth and skin darkening.

To test for the effect of SLED on hair growth, six or seven-week-old C57BL/6 mice are inspected for the absence of skin pigmentation to verify that their skin is in telogen (resting) stage. Following this, they are anesthetized and their hair is removed by clipping (rather than shaving) so as not to trigger anagen.
30 A plasmid having a SLED expression cassette is precipitated onto gold particles. Similarly, a control plasmid having a β -galactosidase expression cassette is also precipitated onto gold particles. To avoid significant trauma to the skin (which could trigger anagen), the plasmids are transferred to the prepared skin of the mice at 250 psi using a gene gun (see, e.g., Rakmilevich et al., *Proc. Nat. Acad. Sci.*
35 (USA), 93, 6291-96 (1996)).

At weekly intervals following gene transfer, animals are sacrificed and the presence of circulating levels of administered gene product are assessed by Western blotting. Additionally, skin from the prepared and transfected skin is assayed for the presence of gene product within the skin by
5 immunohistochemistry. The mice are also visually observed weekly for the presence of coloration within the treated areas of skin.

Results indicate that transfer of the β -galactosidase or SLED expression cassette leads to detectable product in the animals. However, skin transfected with the β -gal expression cassette does not progress to anagen any more rapidly than
10 untreated skin. Conversely, skin treated with the SLED expression cassette becomes pigmented more rapidly than untreated skin. The results indicate that transfer of exogenous SLED to the skin promotes the growth of hair.

15

While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations of the preferred embodiments may be used and that it is intended that the invention may be practiced otherwise than as specifically described herein.
20 Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.

-17-

SEQUENCE LISTING

GENERAL INFORMATION

APPLICANT: Northwestern University

TITLE OF INVENTION: Methods and Compositions for Inhibiting Angiogenesis

FILE REFERENCE: 200290.0034/23CN

CURRENT APPLICATION DATA

APPLICATION NUMBER: CN 2,298,018

FILING DATE: 1998-07-23

PRIOR APPLICATION DATA

APPLICATION NUMBER: PCT/US98/15228

FILING DATE: 1998-07-23

APPLICATION NUMBER: US 08/899,304

FILING DATE: 1997-07-23

PATENT AGENT INFORMATION

NAME: Helene D'Iorio

REGISTRATION NUMBER: 4133

REFERENCE/DOCKET NUMBER: 08-885929CA

TELECOMMUNICATION INFORMATION:

TELEPHONE: (613) 786-0166

TELEFAX: (613) 563-9869

NUMBER OF SEQUENCE ID NOS: 1

COMPUTER-READABLE FORM

MEDIUM TYPE: Diskette

COMPUTER: IBM Compatible

OPERATING SYSTEM: DOS

SOFTWARE: PatentIn Ver. 2.1

INFORMATION FOR SEQ ID NO.: 1

SEQUENCE CHARACTERISTICS

LENGTH: 362

TYPE: PRT

ORGANISM: Homo sapiens

SEQUENCE DESCRIPTION: SEQ ID NO.: 1

Met Gln Ala Leu Val Leu Leu Leu Cys Ile Gly Ala Leu Leu Gly His
 1 5 10 15

Ser Ser Cys Gln Asn Pro Ala Ser Pro Pro Glu Glu Gly Ser Pro Asp
 20 25 30

Pro Asp Ser Thr Gly Ala Leu Val Glu Glu Glu Asp Pro Phe Phe Lys
 35 40 45

-18-

Val Pro Val Asn Lys Leu Ala Ala Ala Val Ser Asn Phe Gly Tyr Asp
 50 55 60

Leu Tyr Arg Val Arg Ser Ser Met Ser Pro Thr Thr Asn Val Leu Leu
 65 70 75 80

Ser Pro Leu Ser Val Ala Thr Ala Leu Ser Ala Leu Ser Leu Gly Ala
 85 90 95

Glu Gln Arg Thr Glu Ser Ile Ile His Arg Ala Leu Tyr Tyr Asp Leu
 100 105 110

Ile Ser Ser Pro Asp Ile His Gly Thr Tyr Lys Glu Leu Leu Asp Thr
 115 120 125

Val Thr Ala Pro Gln Lys Asn Leu Lys Ser Ala Ser Arg Ile Val Phe
 130 135 140

Glu Lys Lys Leu Arg Ile Lys Ser Ser Phe Val Ala Pro Leu Glu Lys
 145 150 155 160

Ser Tyr Gly Thr Arg Pro Arg Val Leu Thr Gly Asn Pro Arg Leu Asp
 165 170 175

Leu Gln Glu Ile Asn Asn Trp Val Gln Ala Gln Met Lys Gly Lys Leu
 180 185 190

Ala Arg Ser Thr Lys Glu Ile Pro Asp Glu Ile Ser Ile Leu Leu Leu
 195 200 205

Gly Val Ala His Phe Lys Gly Gln Trp Val Thr Lys Phe Asp Ser Arg
 210 215 220

Lys Thr Ser Leu Glu Asp Phe Tyr Leu Asp Glu Glu Arg Thr Val Arg
 225 230 235 240

Val Pro Met Met Ser Asp Pro Lys Ala Val Leu Arg Tyr Gly Leu Asp
 245 250 255

Ser Asp Leu Ser Cys Lys Ile Ala Gln Leu Pro Leu Thr Gly Ser Met
 260 265 270

Ser Ile Ile Phe Phe Leu Pro Leu Lys Val Thr Gln Asn Leu Thr Leu
 275 280 285

Ile Glu Glu Ser Leu Thr Ser Glu Phe Ile His Asp Ile Asp Arg Glu
 290 295 300

Leu Lys Thr Val Gln Ala Val Leu Thr Val Pro Lys Leu Lys Leu Ser
 305 310 315 320

Tyr Glu Gly Glu Val Thr Lys Ser Leu Gln Glu Met Lys Leu Gln Ser
 325 330 335

Leu Phe Asp Ser Pro Asp Phe Ser Lys Ile Thr Gly Lys Pro Ile Lys
 340 345 350

Leu Thr Gln Gly Gly Thr Pro Gly Trp Leu
355 360

1. Use of an effective amount of exogenous PEDF to inhibit angiogenesis within a tissue.
2. Use of an effective amount of exogenous PEDF in the manufacture of a medicament to inhibit angiogenesis within a tissue.
3. Use of an effective amount of exogenous PEDF to inhibit endothelial cell migration.
4. Use of an effective amount of exogenous PEDF in the manufacture of a medicament to inhibit endothelial cell migration.
5. The use of claim 1 wherein said tissue is a tumor tissue.
6. Use of an effective amount of exogenous PEDF to inhibit angiogenesis in a tumor.
7. A pharmaceutical composition comprising an anti-angiogenic effective amount of PEDF and a pharmaceutically acceptable carrier for inhibiting angiogenesis.
8. Use of transfected endothelial cells comprising an isolated nucleic acid encoding PEDF for preparing a medicament for inhibiting angiogenesis within a tissue, wherein said cells express and secrete PEDF polypeptide.
9. Use of transfected endothelial cells comprising an isolated nucleic acid encoding PEDF for inhibiting angiogenesis within a tissue, wherein said cells express and secrete PEDF polypeptide.
10. Use of a population of cells transfected with a vector comprising an isolated nucleic acid encoding PEDF for preparing a medicament for inhibiting angiogenesis within a tissue, wherein said cells express and secrete PEDF polypeptide, and wherein said population of cells is not a population of endothelial cells.
11. Use of a population of cells comprising a nucleic acid encoding PEDF for inhibiting angiogenesis within a tissue, wherein said cells express and secrete PEDF polypeptide.

12. The use of any one of claims 8 to 11, wherein said nucleic acid is as deposited in GenBank Accession number U29953.
13. The use of any one of claims 8 to 11, wherein said nucleic acid is as deposited in GenBank Accession number U29953 and encodes a protein exhibiting substantially the same antiangiogenic activity as that set forth in SEQ ID NO: 1.
14. The use of claim 10 or 11, wherein said PEDF is expressed from non-integrated or stably integrated DNA.
15. The use of claim 10 or 11, wherein said use is systemic.
16. The use of claim 10 or 11, wherein said use is topical.
17. The use of any of claims 1, 2, 3 or 5 which further comprises use of another antiangiogenic factor in conjunction with PEDF.

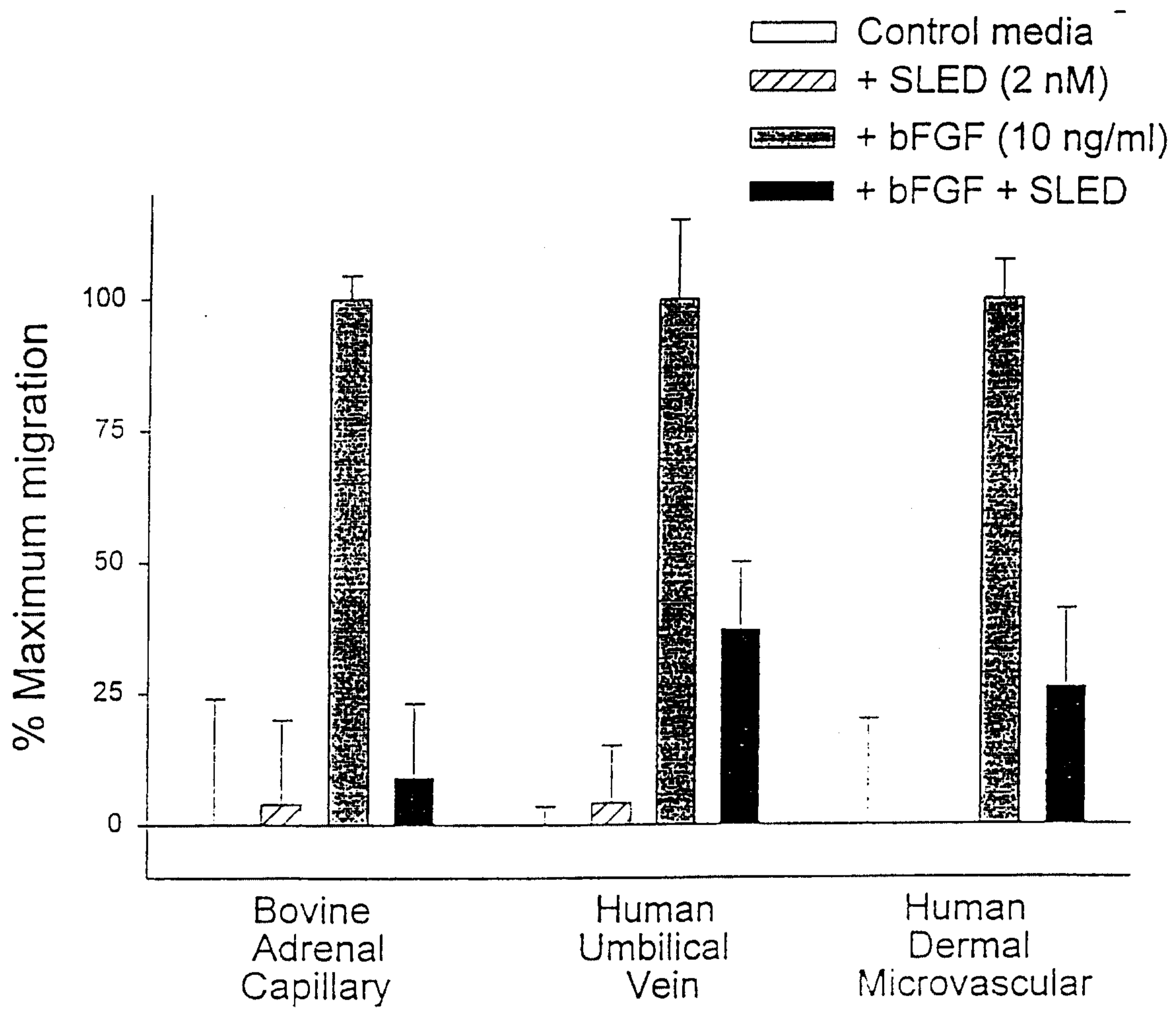


Figure 1

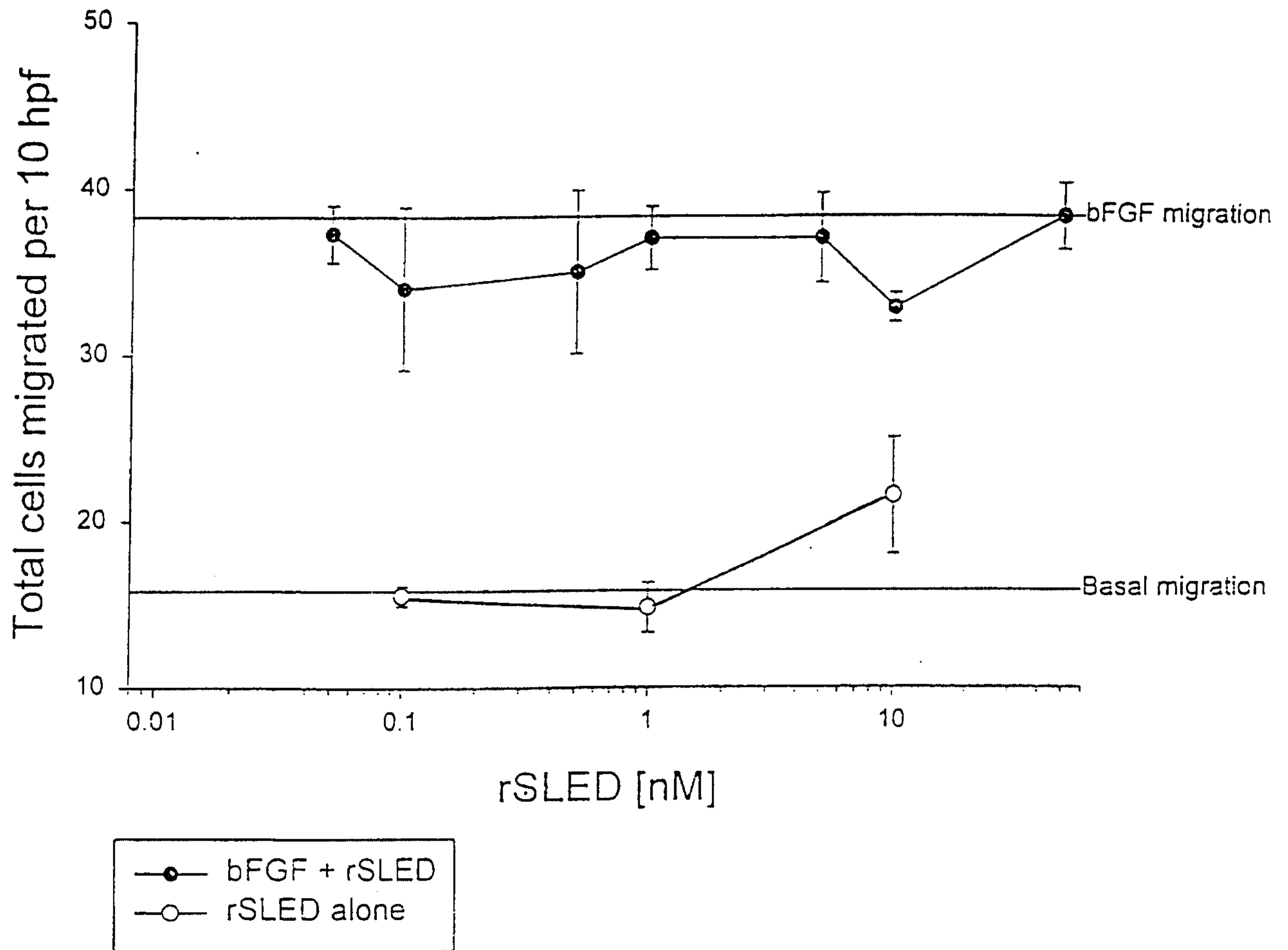


Figure 2A

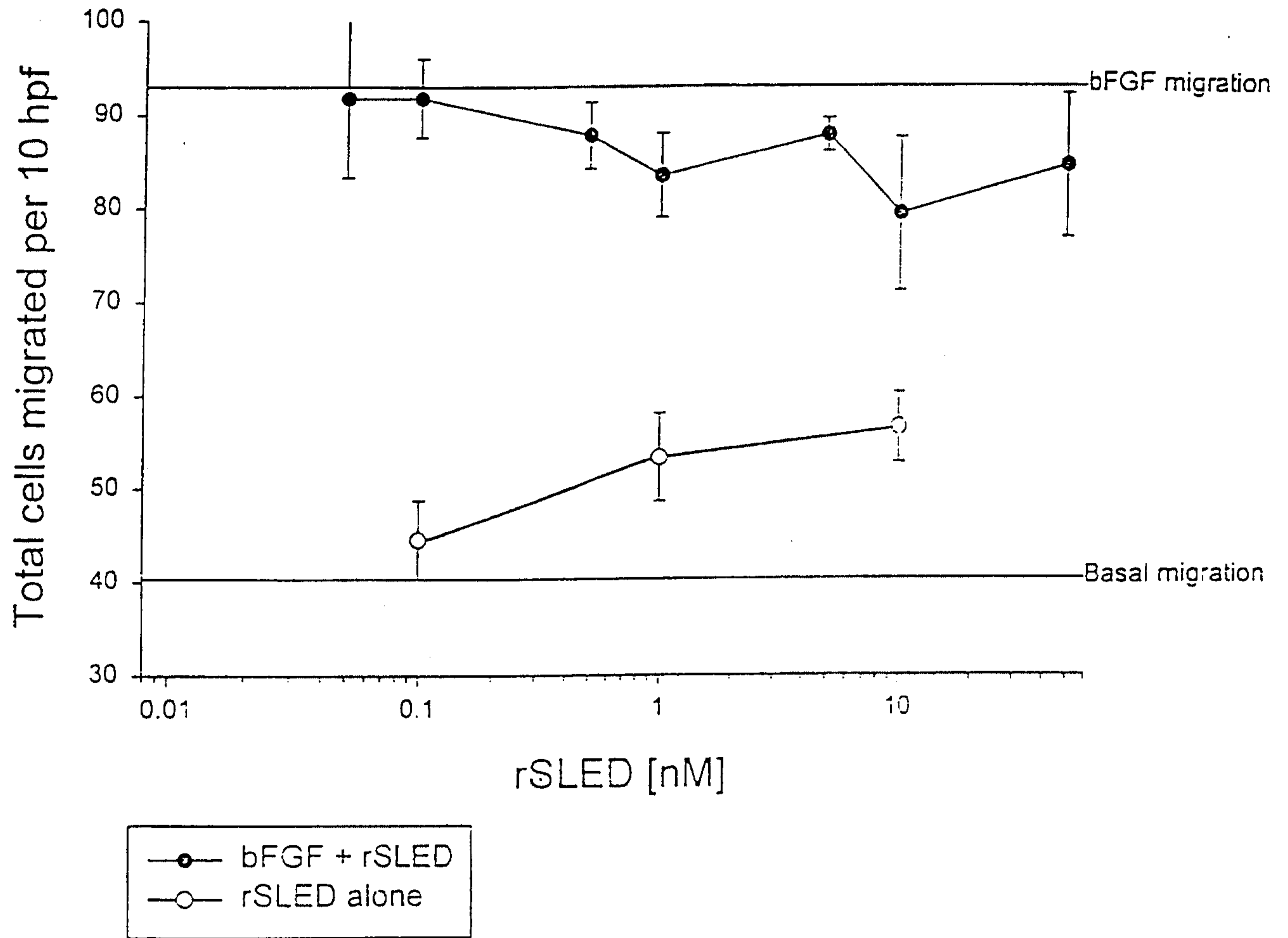


Figure 2B

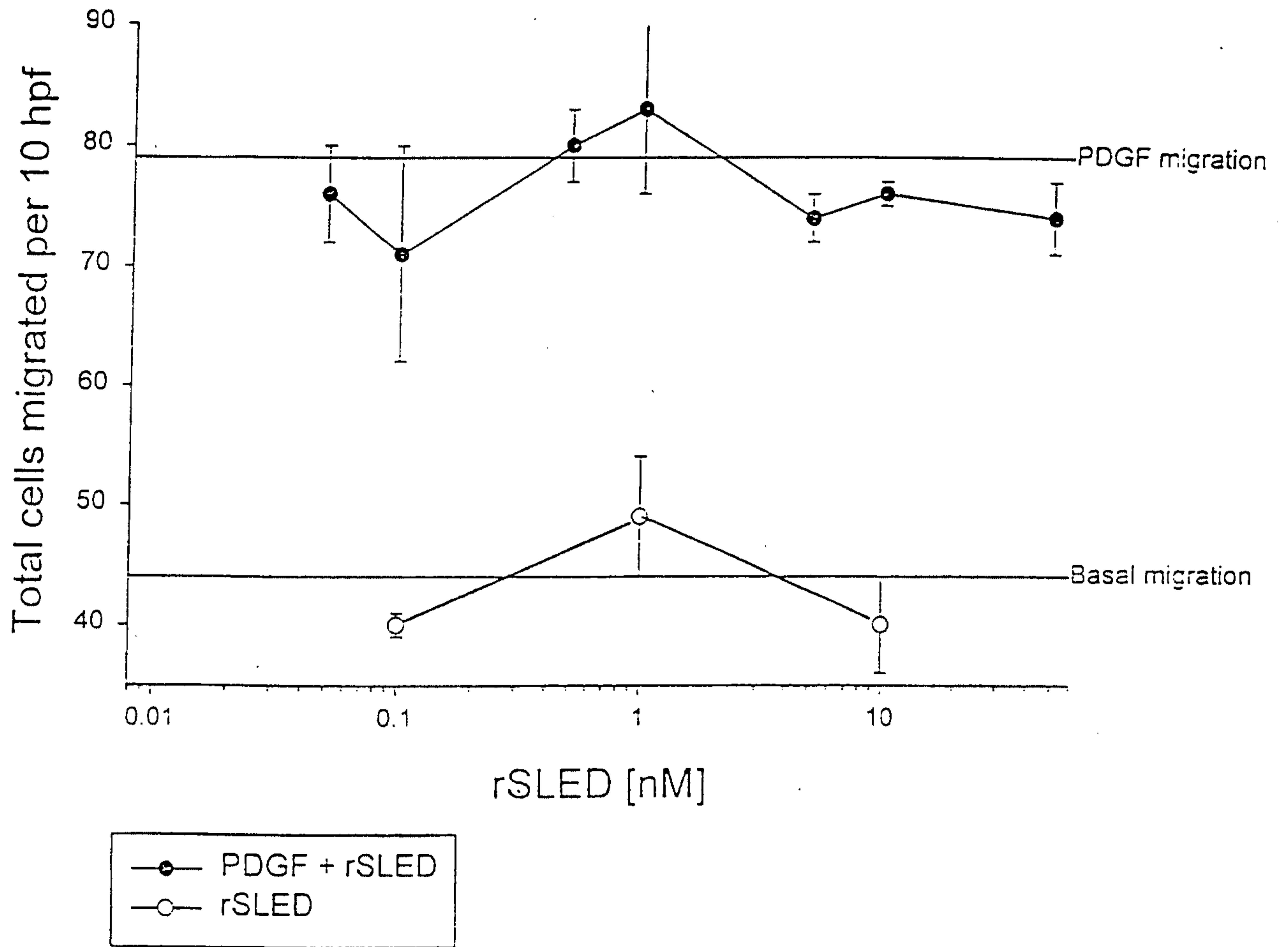


Figure 2C

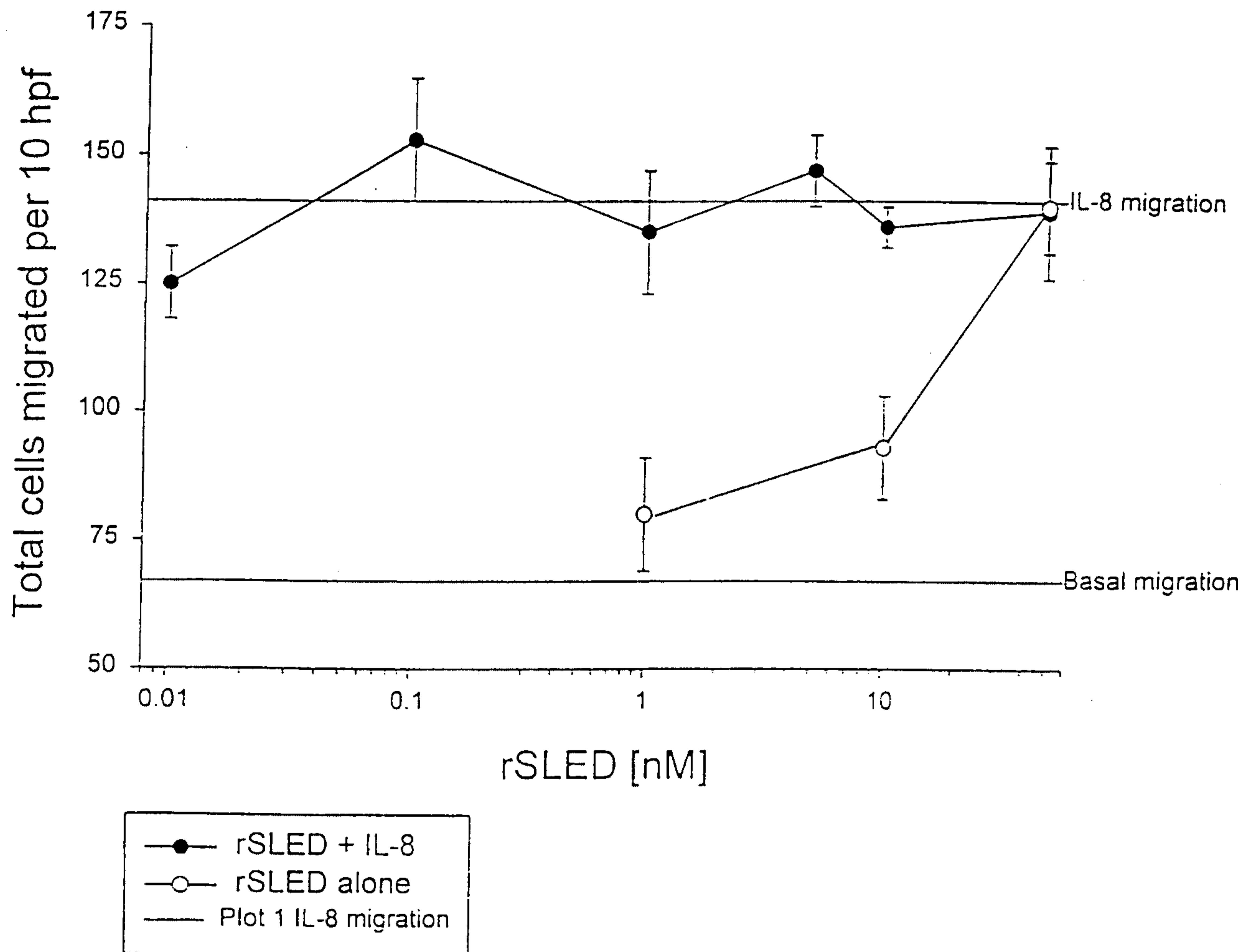


Figure 2D

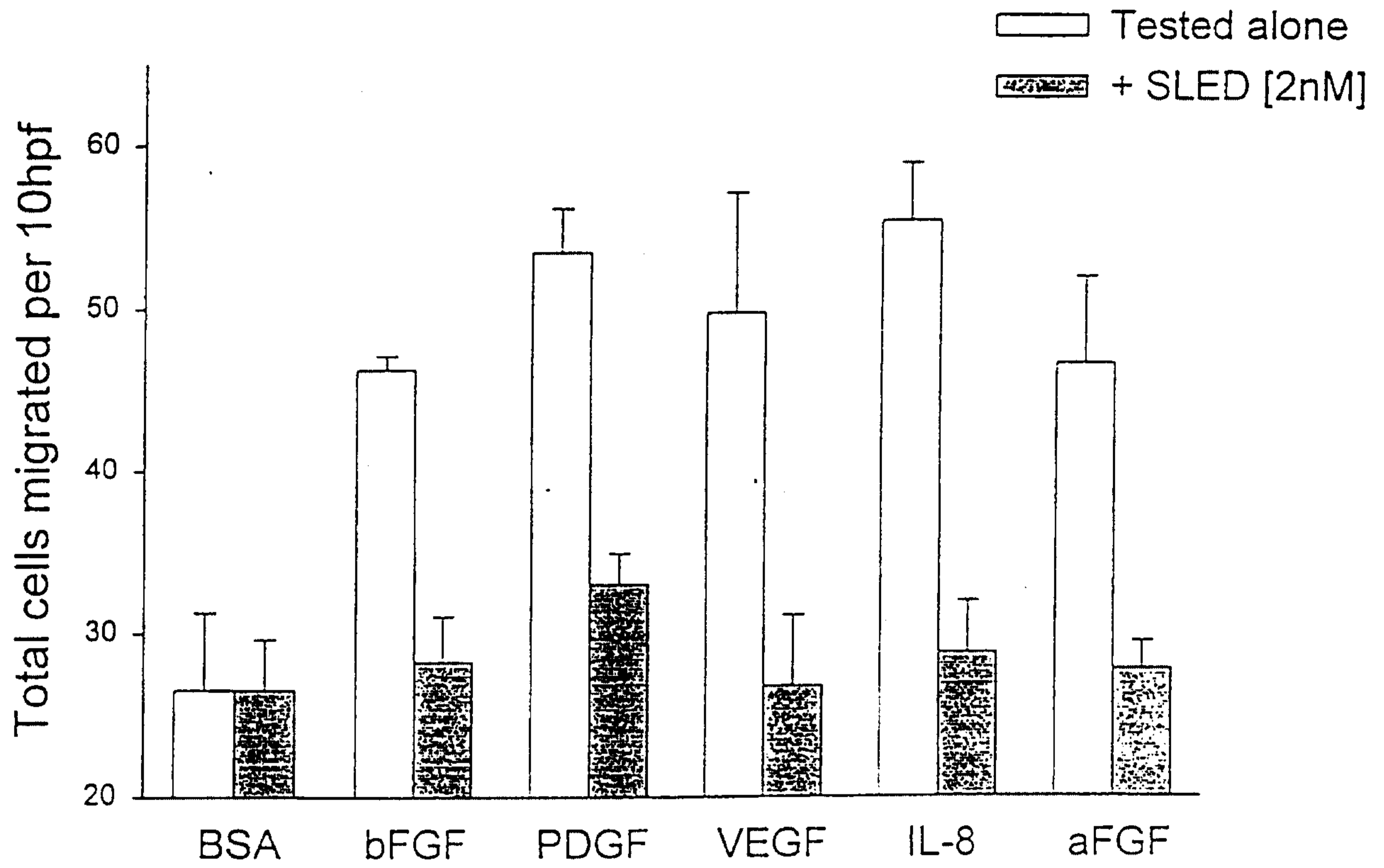


Figure 3

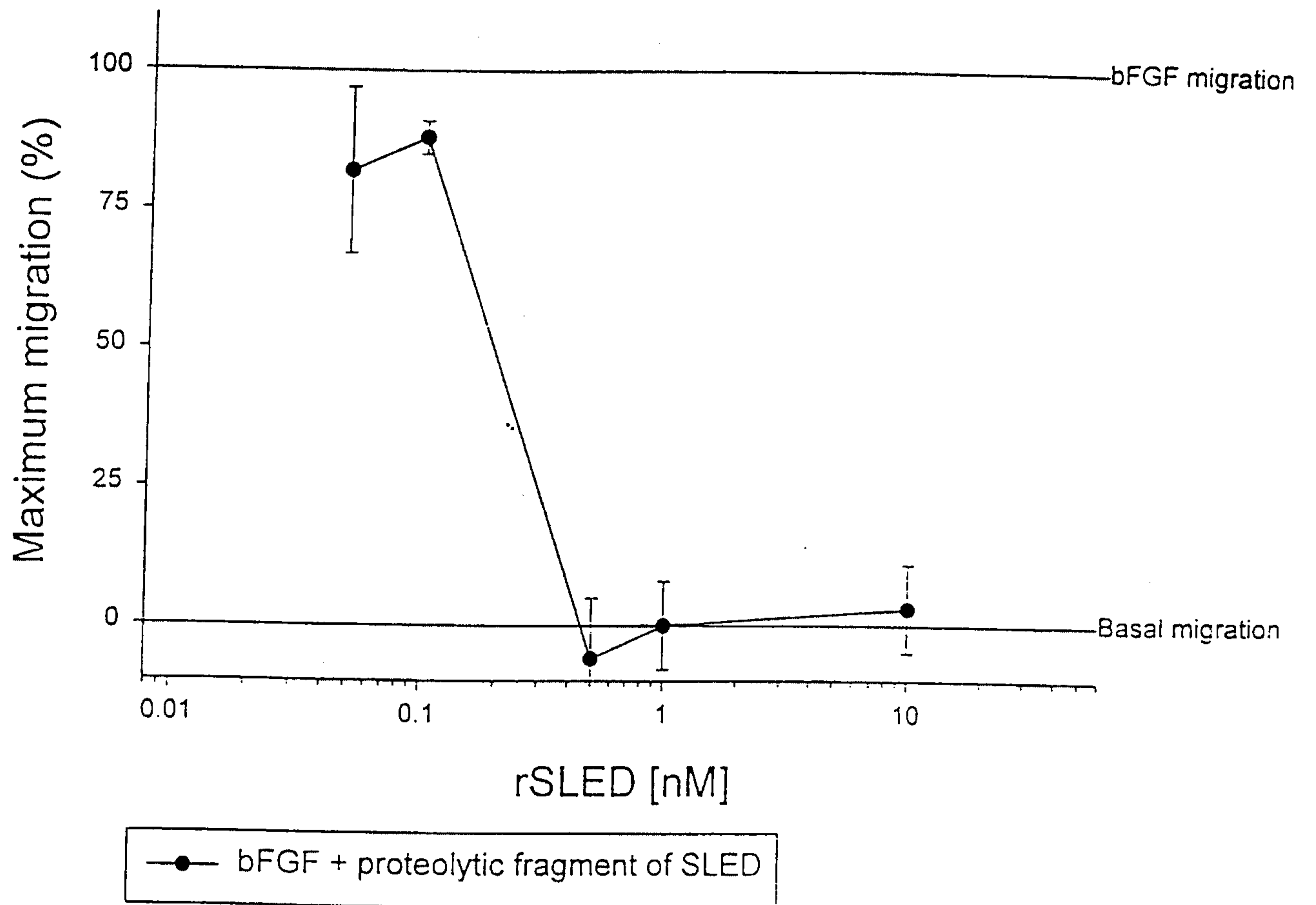


Figure 4