

rhBMP-2, rhVEGF₁₆₅, rhPTN and thrombin-related peptide, TP508 induce chemotaxis of human osteoblasts and microvascular endothelial cells

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Received 17 November 2004; accepted 22 December 2004

Abstract

Osteogenesis and angiogenesis are inter-linked and tightly regulated processes involved in growth, repair, and bone remodeling. Bone morphogenic protein 2 (BMP-2), vascular endothelial growth factor (VEGF), pleiotrophin (PTN) and thrombin-related peptide, TP508 have all been found to have the ability to promote bone fracture healing by enhancing both the osteogenesis and angiogenesis processes. One of the underlying mechanisms proposed is that mediators for osteogenesis may also be involved in mediating angiogenesis and vice versa. The aim of this study was to examine the chemotactic effects of rhBMP-2, rhVEGF₁₆₅, rhPTN and TP508 on human osteoblasts and endothelial cells. Using a direct-viewing chemotaxis assay system, we report for the first time, the direct quantitative observation of chemotaxis of both human osteoblastic cells and microvascular endothelial cells towards sources of rhBMP-2, rhVEGF₁₆₅, rhPTN and TP508. This study confirmed that rhBMP-2, rhVEGF₁₆₅, rhPTN and TP508 have chemotactic effects on both human osteoblastic and endothelial cells, indicating that these factors are directly involved in promoting angiogenesis and osteogenesis by recruiting osteoblasts and endothelial cells via chemotaxis.

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Keywords: Chemotaxis; Osteoblast; Endothelial cells; rhVEGF₁₆₅; rhBMP-2; rhPTN; TP508

Introduction

It is well known that bone morphogenic protein 2 (BMP-2) promotes osteogenic cell proliferation and differentiation in vitro and in vivo [24]. There are BMP receptors in osteoblasts and endothelial cells [13,24]. Vascular endothelial growth factor (VEGF) secreted by endothelial and osteoblastic cells, has an essential role in angiogenesis and maintaining osteoblast function [4,9,13]. The presence of VEGF receptors on osteoblastic cells has been demonstrated [3,31]. Pleiotrophin

(PTN) was first identified as a neurogenic growth factor, and later found to have potent effects on regulation of osteoblast recruitment [12,20,27,28,36]. The receptor for PTN is N-syndecan, which is widely expressed in both osteoblastic and endothelial cells [12,18]. The thrombin-related peptide, TP508, is a synthetic 23 amino acid peptide, which represents the receptor-binding domain of thrombin [2]. TP508 mimics thrombin by interacting with receptors on cells involved in tissue repair and has been shown to enhance revascularization of injured tissue, and promote soft tissue wound healing, cartilage repair, and fracture repair [2,14,17,22,23,25,26]. One of the underlying mechanisms of TP508 in promoting tissue repair is that TP508 may be chemotactic to osteoblasts and endothelial cells [17,25,26].

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Angiogenesis is essential for the increased delivery of oxygen and nutrients required for the reparative processes of bone healing. The processes of osteogenesis and angiogenesis are intimately linked during development and repair [32], mediators for osteogenesis may also be involved in mediating angiogenesis and vice versa. The aim of this study was to examine the chemotactic effects of rhBMP-2, rhVEGF₁₆₅, rhPTN and TP508 on human osteoblasts and endothelial cells.

Materials and methods

Human osteoblastic and endothelial cell culture

Human bone-derived osteoblastic cells (HOBs) were cultured from trabecular bone explants obtained at the time of orthopedic procedures performed on patients who had no evidence of metabolic bone disease. The bone fragments were washed repeatedly with culture medium to remove adherent marrow and blood cells and to expose the trabecular surface of the bone. Small bone chips ($\approx 5 \times 5 \times 5$ mm) were then placed in T-75 flasks, each containing 15 ml DMEM (Sigma, Pole, UK) supplemented with 15% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (50 μ g/ml) and amphotericin B (2.5 μ g/ml), and cultured at 37 °C in a 5% CO₂ atmosphere. The first medium change was made at day 8 and thereafter the medium was changed every five days until the flask became confluent around weeks 3–4. The cells were then passaged or used for the study. All cells used in this study were passage 2–4. The phenotype of the HOBs was confirmed by immunocytochemistry, they were stained positively for alkaline phosphatase, type I collagen, osteocalcin, BMP-2, BMP receptors I and II.

Human microvascular endothelial cells (HMECs) were from an immortalized cell line derived from human dermal tissues [21,35]. Experiments comparing the phenotypic characteristics of HMECs with human dermal microvascular endothelial cells or human umbilical vein endothelial cells revealed that HMECs have features of both small and large vessel endothelial cells [35]. HMECs were initially transfected with simian virus 40A large T antigen and have been passaged more than 100 times without signs of senescence. HMECs express von Willebrand factor (vWF), take up acetylated low-density lipoproteins, and rapidly form tubes when cultured on matrigel [21]. HMECs were cultured in MCDB 131 medium (Invitrogen Ltd., UK) supplemented with 15% FCS, 20 mM L-glutamine, 77.5 U/ml streptomycin and 25 U/ml penicillin at 37 °C in 5% CO₂. The HMECs were plated in T75 flask at a density of 10⁵ cells/ml, and they usually became confluent after 7–14 days of culture. The phenotype of the HMECs was confirmed by immunocytochemistry, they were stained positively for CD31, collagen Type IV, VEGF receptor I (Flt-1) and II (Flk-1) (Santa Cruz Biotechnology, Inc., CA, USA) and vWF (Sigma, Poole, UK).

Assembly of Dunn chemotaxis chamber

The Dunn chemotaxis chamber permits the direction of migration of individual cells to be measured in relation to the direction of the gradient, and the time course of the response to be followed [1,6,37]. The chamber consists of two concentric wells ground into one face of a glass microscope slide and separated by an annular platform which has been optically polished to lie precisely 20 μ m below the upper face of the chamber. This distance defines the depth of the diffusion gap when the wells are covered with a cover slip (Fig. 1). When the inner circular well of the chamber is filled with control medium and the outer annular well with medium containing the chemoattractant, a directed linear diffusion gradient becomes established in the diffusion gap within 10–30 min, which usually has a half-life of 10–30 h [37].

HOB and HMEC cells (10⁵ cell/ml) were seeded onto 22 \times 22 mm acid-washed clean glass coverslips, and allowed to settle for 24 h prior to use. To set up chemoattractant gradients, both concentric wells of the Dunn chamber slide were first filled with plain medium (DMEM

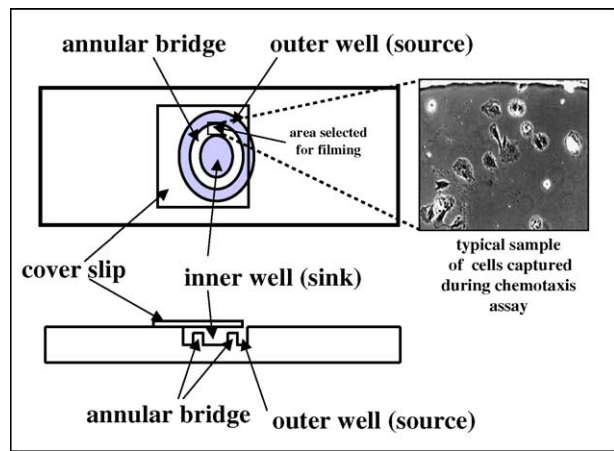


Fig. 1. Diagram shows a Dunn chamber slide. When the inner circular well of the chamber is filled with control/plain medium and the outer well with medium containing the chemoattractant, a linear diffusion gradient becomes established in the diffusion gap over the annular bridge. The recording field was chosen with the upper margin pointing to the direction of increasing concentration of the gradient.

and MCDB 131), a glass cover slip seeded with HOBs or HMECs was then inverted onto the chambers in an offset position leaving a narrow slit at one edge for refilling the outer well. After firmly seating the cover slip by applying slight pressure to its margin, the glass coverslip was sealed in place using hot dental wax around all the edges except for the filling slit. The medium in the outer well was then drained from the slit by a piece of clean soft tissue paper, before being replaced with medium containing the following factors: 100 ng/ml of either rhVEGF₁₆₅, rhBMP2, rhPTN (PeproTech EC Ltd., London, UK), or 1, 10 and 100 μ g/ml TP508 (Chrysalin®, Chrysalis BioTechnology, Inc., Galveston, TX, USA). The slit was then finally sealed off with the hot dental wax. For negative control experiments, the outer well was filled with plain medium containing nothing or heat-inactivated (80 °C in water bath for 20 min) of one of the above-mentioned factors.

Recording, tracking and statistical evaluation of cell migration

The assembled Dunn chamber slide was placed on the microscope stage, which was heated to 37 °C. Recording of cell migration in the diffusion gap of the Dunn chamber slide began within 15 min of assembling the chamber via a Nikon inverted microscope equipped with a 10 \times phase contrast objective and coupled to a digital CCD camera. One part of the annular platform was selected (usually with 10–15 cells). The chamber slide was positioned by locating the outer edge of the annular platform to coincide with the upper margin of the recording field so that the direction of increasing concentration of the gradient was vertically upwards (Fig. 1). Using a shutter control device at a time-lapse interval of 5 min over the duration of 9 h, serial images of the cells in the selected field were then digitally recorded and saved. The captured sequences were displayed rapidly as a movie and interactive tracking of cells with a superimposed mouse pointer resulted in the generation of cell trajectories, each consisting of a sequence of (x,t,y,t) position coordinates obtained from the 109 consecutive images of each cell using AQM 2001 software (Kinetic Imaging Ltd., Manchester, UK). Calculations for the speed of cell migration were derived from this data. The mean speed and 99% confidence limits were then calculated for each time-lapse interval, and an overall figure derived for the 9 h period.

A purposely written notebook in Mathematica 3.0 was used to test the directionality of the cell migration. Briefly, each cell trajectory was converted to a single angle representing the direction from the starting point of the trajectory to the point at which it first crossed a virtual horizon. The distance from the starting point to the horizon was chosen to be 30 μ m for both cell types. Trajectories that never reached this

horizon were eliminated. These data were summarized in a circular histogram showing the number of cell migration directions lying within each 18° interval. The Rayleigh test for unimodal clustering of directions was applied to the data and a probability (p) value of less than 0.01 was considered to have statistical significance. In the cases of significant directional migration, the mean direction was represented as an arrow and the 99% confidence interval as a grey sector on the circular histogram. The sample size N represents the number of cells measured, and the minimal number of cells required for each statistical analysis is 30, and the data may be pooled from different experiments.

Results

Chemotaxis of HOBs and HMECs to rhBMP-2, rhVEGF₁₆₅, rhPTN and TP508

The directional clustering shown in the circular histograms (Fig. 2) were significant in HOBs exposed to rhBMP-2 (100 ng/ml, Fig. 2A), rhVEGF (100 ng/ml, Fig. 2C), rhPTN (100 ng/ml, Fig. 2E) and TP508 (1, 10 and 100 µg/ml, Fig. 2G). HMECs also showed chemotaxis to rhBMP-2 (100 ng/ml, Fig. 2B), rhVEGF (100 ng/ml, Fig. 2D), rhPTN (100 ng/ml, Fig. 2F) and TP508 (1, 10 and 100 µg/ml, Fig. 2H). Migration was randomly distributed in cells exposed to plain media (Fig. 2I and J) or to heat-inactivated proteins (Fig. 2K and L) in both HOBs and HMECs.

Comparison of cell migration speeds

The mean speed of cell migration of HOBs and HMECs in response to various growth factors and peptide concentrations were shown in Fig. 3. HOBs appeared to move faster than HMECs when treated with rhBMP-2 (100 ng/ml), rhVEGF (100 ng/ml) and rhPTN (100 ng/ml). The fastest HOBs movement was seen when the cells were stimulated with 100 ng/ml rhPTN (34.38 ± 2.80 µm/h, $p < 0.01$ vs. control, t -test), followed by 100 ng/ml rhBMP-2 (24.42 ± 3.12 µm/h, $p < 0.05$ vs. control, t -test). The speeds of cell migration in the rest of the treatment groups were similar (between 10 and 20 µm/h) with no statistical difference when compared with control (without treatment). In the HMECs, the fastest movement was observed when the cells were stimulated by 100 µg/ml TP508 (19.44 ± 1.0 µm/h, $p < 0.01$ vs. control, t -test), followed by 10 µg/ml TP508 (15.36 ± 1.06 µm/h, $p < 0.01$ vs. control, t -test) and there was no statistical difference in cell migration speed in the rest of the groups, where the speeds were all between 10 and 15 µm/h.

Discussion

The Dunn chamber chemotaxis analysis system allows the behaviour of small numbers of cells to be directly monitored in a stable linear gradient of a che-

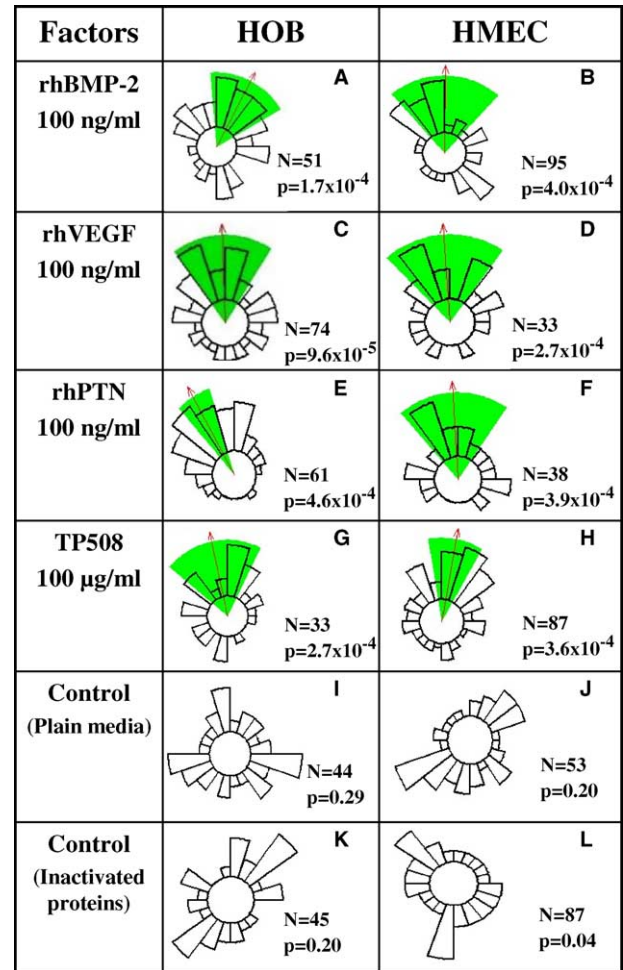


Fig. 2. Circular histograms show cell displacements pooled from the HOBs and HMECs chemotaxis analysis. All circular histograms show the proportion of cells that migrated within each 18° interval, with the source of chemoattractant at the top. The arrow and the grey segment show the mean significant migration direction and its 99% confidence interval, respectively. The Rayleigh statistical test showed that HOBs migrated significantly towards to rhBMP-2 (A); rhVEGF₁₆₅ (C); rhPTN (E) and HMECs migrated significantly towards to rhBMP-2 (B); rhVEGF₁₆₅ (D); rhPTN (F). When the control plain media or heat-inactivated proteins were added in the outer well, no chemotactic response was seen in either HOBs (I) and (K) or HMECs (J) and (L), showing that cells moved randomly. In each histogram, the number of cells (N) and Rayleigh test p value is indicated (p).

moattractant which provides unique measurements of the direction of migration of individual cells in relation to a gradient along with the time course of the response. This is a useful tool to examine chemotactic effects of various growth factors and cytokines on any given cell type [1,6,37]. The specificity of chemotaxis to a given factor in the present study was confirmed with methodology controls: when plain media or heat-inactivated proteins were added to the outer (source) well, cells moved randomly with no significant directional chemotaxis. We have demonstrated that there were direct and significant chemotactic effects of rhBMP-2, rhPTN,

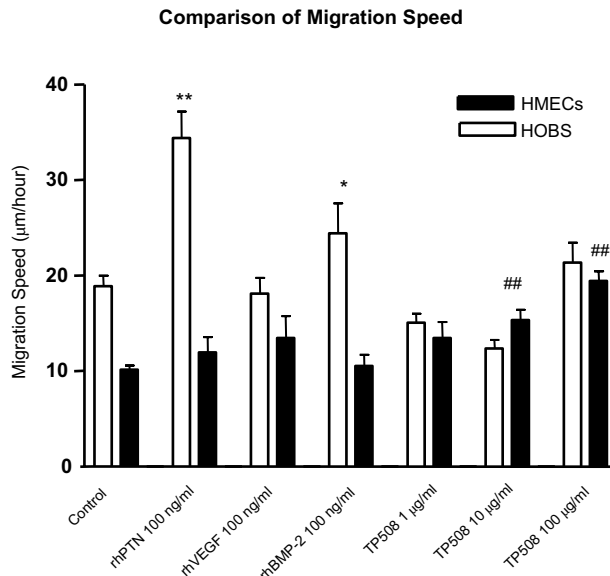


Fig. 3. Diagram shows the mean speeds of cell migration ($\mu\text{m}/\text{h}$) of HOBs and HMECs in response to various factors, error bars show standard error. The fastest HOBs migration was seen towards 100 ng/ml rhPTN, second by 100 ng/ml rhBMP-2 ($*p < 0.05$ and $**p < 0.01$ vs. control), whereas in HMECs, the fastest migration was seen towards 100 $\mu\text{g}/\text{ml}$ TP508 and seconded by 10 $\mu\text{g}/\text{ml}$ TP508 ($###p < 0.01$ vs. control). There was no statistical difference in cell migration speed in the rest of the treatment groups when compare with controls (with no treatment, *t*-test).

TP508 and rhVEGF₁₆₅ on both the human osteoblastic and microvascular endothelial cells. In agreement with the previous reports where chemotaxis was concluded from indirect means, such as in vivo vascular in-growth assay or qualitative cell migration assay [2,3,14,15], the present study provided direct qualitative and quantitative evidence that BMP-2, VEGF, PTN and thrombin-related peptide (TP508) are all potent chemoattractants to osteoblastic and endothelial cells, confirming that most of the known osteogenic and angiogenic factors may have both osteogenic and angiogenic properties. The dose of various factors tested for chemotaxis in this study was selected based on previous studies and they may not be the optimal doses for inducing maximal chemotaxis, but the tested doses did induce positive chemotaxis of both HOBs and HMECs and serve the purpose of this current study.

BMP-induced bone formation in vivo is a complex multistage process and is likely to involve the activities of multiple locally produced growth factors and systemically available hormones. During bone formation and fracture healing there is a cross-talk between endothelial cells and osteoblasts. Osteoblastic or osteoprogenitor cells respond to treatment with the BMPs by increasing cell proliferation and differentiation [24]. In this study as well as in many previous studies, BMP-2 had chemotactic effects on mesenchymal cells and osteoblastic cells [8], suggesting the enhancement of bone formation by

rhBMP-2 may be related to an increase in recruitment of bone-forming cells to the injured sites. BMP receptors have been identified in arteries and vascular smooth muscle cells, and in cellular migration studies, incubation with BMP-2 produced efficacious, concentration and time-dependent chemotaxis of human vascular smooth muscle cells [34]. The chemotactic response of HMECs to rhBMP-2 seen in this study suggests that BMP-2 plays a role in enhancement of angiogenesis. Reports have suggested that BMPs stimulate osteoblastogenesis and angiogenesis through the production of VEGF-A, in the presence of VEGF-A antibody, BMP-stimulated angiogenesis were arrested [4]. Mayr-Wohlfart et al. has confirmed that VEGF-A, but not VEGF-E, induces chemotaxis of primary human osteoblasts [15].

The VEGF and VEGF receptor system plays a central regulatory role in physiological and pathological angiogenesis and osteogenesis [7,29,32]. During embryogenesis, the VEGF/VEGF receptor system is critically involved in the formation of the vascular system by regulating both the growth and the survival of blood vessels. A variety of pathological conditions such as rheumatoid arthritis and osteoarthritis, are associated with the up-regulation of VEGF and the VEGF receptors [10,33]. Osteoblasts produce VEGF and other factors that can induce VEGF receptor up-regulation in bone and endothelial cells and thus regulate angiogenesis and osteogenesis [16]. Early in osteoblast differentiation the expression of the VEGF gene is low whereas during mineralization osteoblastic cells express high levels of VEGF and VEGF receptors, which correlates with the progress of bone remodeling [3]. The involvement of different VEGF receptors at different stages of bone formation and remodeling remains to be further defined. Differences in various VEGF receptor expression in osteoblasts and endothelial cells during bone repair and remodeling may provide insights in understanding skeletal conditions such as fracture non-union and osteoporosis.

PTN is an extracellular matrix-associated protein, present in matrices, which acts as targets for the deposition of new bone [5,11,12]. During embryonic development, the PTN gene is widely expressed in many tissues [11]. In post-natal life, PTN expression is mainly seen in the nerve (brain) and bone tissues [19,30]. PTN has diverse functions in stimulating neurogenesis, tumour cells migration and angiogenesis [5]. The receptor for PTN is N-syndecan, which is widely expressed in both osteoblastic and endothelial cells [12,18]. Recently it has been suggested that PTN has multiple roles in bone formation that are dependent on its concentration, the time of development and the interaction with other factors [12]. rhPTN at 50 mg/ml was found to be chemotactic for human osteoprogenitors, and as low as 10 pg/ml rhPTN has been shown to stimulate total colony formation, alkaline phosphatase-positive colony formation

and alkaline phosphatase-specific activity in a time-dependent manner [36]. In the present study, we have demonstrated that rhPTN at 100 ng/ml not only has chemotactic effects on HOBs but also on HMECs, suggesting its involvement in regulating osteogenesis and angiogenesis. The HOB cell migration speed towards 100 ng/ml rhPTN was faster than the other treatments, confirming that PTN may have a more specific role in osteoblast recruitment through chemotaxis, as previous studies suggested [12,28].

TP508 may represent a biologically active thrombin peptide released during degradation of the fibrin clot, and function as an upstream effector that triggers and regulates the expression of other growth factors and enzymes during soft tissue and bone repair [17,25,26]. Part of the TP508 mechanism may be related to its enhancement of neovascularization of the injured tissue [2,17,26]. In a previous study, we have found an increase in blood vessel number and maturation in the distraction regenerates with TP508 treatments [14]. TP508 has been shown to be angiogenic when assayed on chicken chorioallantoic membranes, and the peptide was chemotactic to human endothelial cells [17]. In concurrence with the previous findings the present study confirmed that TP508 was chemotactic to HOBs and HMECs at 1–100 µg/ml concentrations. In the HMECs, the fastest movement was seen when the cells migrated towards 100 µg/ml TP508, and both HOBs and HMECs showed a dose-dependent increase in the cell migration speed in the TP508 treated groups. Taking the data together, TP508 may be involved in regulating angiogenesis and osteogenesis, promoting fracture repair through enhancing osteoblast and endothelial cell recruitment.

In conclusion, this study confirmed conclusively that rhBMP-2, rhVEGF₁₆₅, rhPTN and TP508 all have chemotactic effects on both human osteoblastic and endothelial cells, indicating that these growth factors and peptide may be directly involved in promoting angiogenesis and osteogenesis by recruiting osteoblasts and endothelial cells via chemotaxis.

Acknowledgments

This study was funded by a project grant from The British Orthopaedic Association Wishbone Trust (project grant 309) to Gang Li. The authors wish to acknowledge OrthoLogic Corporation, USA and the R&D office, Department of Health and Social Services, Northern Ireland for partial support of this work.

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