Quantitative analysis of the responses of murine bone marrow mesenchymal stem cells to EGF, PDGF-BB and fibronectin by factorial design methodology

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Abstract A 2-level full factorial design was firstly employed to explore the responses of murine bone marrow mesenchymal stem cells stimulated by various combinations of EGF, PDGF-BB, and fibronectin. EGF and PDGF-BB individually, rather than fibronectin, had significant effects on cell proliferation. The synergism between PDGF-BB and fibronectin, as well as the antagonism between EGF and PDGF-BB were also detected. Moreover, cells changed from a flattened and spread to a smaller and elongated shape with addition of factors. The factors also moved cells out of G0/G1 phase, increasing the fractions of cells in S and G2/M phases.

Keywords Bone marrow mesenchymal stem cells · Epidermal growth factor · Factorial design · Fibronectin · Platelet derived growth factor-BB

Introduction

In vivo, bone marrow mesenchymal stem cells (BMSCs) reside in a complex microenvironment characterized by soluble growth factors and insoluble

extracellular matrix (ECM) components. In-depth understanding how growth factors and ECM proteins regulate cell behavioral responses is essential to both fundamental scientific exploring and technological applications of BMSCs. Regulation of cell responses is further complicated by unexpected interactions of various factors. To date, most experiments have studied the responses of BMSCs to individual factors, and few studies have rigorously analyzed and optimized the effects of multiple factors as well as the multifactorial interactions between these factors. Factorial design, as a long-standing methodology in engineering studies, primarily aims to understand the sources of variation in responses and to assess the effects of factors relative to unexplained variation (Chun et al. 2003). Accordingly, applying the approach to BMSCs is an effective way to deduce major and interactive effects of different factors.

In present study, three factors were selected for testing based on their documented effects on BMSCs: epidermal growth factor (EGF) (Gronthos and Simmons 1995; Kratchmarova et al. 2005), platelet derived growth factor-BB (PDGF-BB) (Gronthos and Simmons 1995; Kratchmarova et al. 2005) and fibronectin (FN) (Gronthos et al. 2001). EGF and PDGF-BB are potent mitogen and chemoattractant of connective tissue cells and exert their effects on target cells via binding to structurally related cell surface tyrosine kinase receptors (Schwartz and Baron 1999). FN, one of the major ECM components, has been proven to be distributed throughout various tissues to regulate cell behaviors by binding

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to cell-surface matrix receptors, primarily integrin $\alpha_5\beta_1$ (Danen and Yamada 2001). We applied a 2-level full factorial design to identify and analyze the behavioral responses of BMSCs regulated by EGF, PDGF-BB, and FN, and to detect synergism and antagonisms among these factors in such complex systems.

Materials and methods

Isolation and expansion of BMSCs

BMSCs were isolated from the bone marrow of 6-week-old male Sprague Dawley rats (150–170 g) according to a protocol modified from Meirelles Lda and Nardi (2003). Briefly, following sacrifice of the rats, femurs and tibias were aseptically excised, cleaned and washed in PBS containing penicillin/streptomycin (100 U/mL and 100 mg/mL) (Sigma). The epiphyses then were removed and the marrow was harvested by inserting a syringe needle (20-gauge) into one end of the bone and flushing with 5 mL control medium [CM, α -MEM (GIBCO) containing 10% fetal bovine serum (FBS) (PAA Laboratories GmbH, Austria)]. After being drawn and expelled from the same syringe three times to disperse the marrow, the cells were washed twice in CM, plated in gelatin-coated plates at a density of 10^6 cells/cm², and cultured at 37 °C in a humidified atmosphere containing 95% air and 5% CO₂. After 72 h, unattached cells were removed by two washes with CM. Subsequent medium changes were performed every 3 days. When cultures became near-confluent, cells were detached with 0.05% trypsin–0.53 mM EDTA (GIBCO).

Characterization of BMSCs

BMSCs were characterized with respect to their capability of osteogenic and adipogenic differentiation. For osteogenic differentiation, cells were seeded at 3×10^3 cells/cm² and maintained for 21 days in osteogenic medium that consisted of CM supplemented with 100 nM dexamethasone (Sigma), 10 mM β -glycerophosphate (Sigma), and 0.05 mM ascorbic acid-2-phosphate (Sigma) (Meirelles Lda and Nardi 2003). Osteogenic differentiation was histologically

confirmed using alkaline phosphatase (ALP) staining and Alizarin Red S staining (Meirelles Lda and Nardi 2003). Cells were stained for ALP using C AKP stain kit (Jianchen; Nanjing, China) according to the manufacturer's instructions. Briefly, cells were fixed with ice-cold 70% ethanol at 4 °C for 30 min and rinsed in distilled water. Cells were incubated in substrate for 15 min at 37 °C and stained with hematoxylin for 10 min at room temperature. For Alizarin Red S staining, cells were fixed with ice-cold 70% ethanol at 4 °C for 1 h. After being rinsed with distilled water, the cells were stained with 40 mM Alizarin Red S (SCRC, Shanghai, China), pH 4.2, for 10 min at room temperature.

For adipogenic differentiation, cells were seeded at 3×10^4 cells/cm² and maintained for 28 days in adipogenic medium that consisted of CM supplemented with 10 μ g/mL insulin (Sigma), 100 μ M indomethacin (Sigma), 1 μ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma) (Meirelles Lda and Nardi 2003). Adipogenic differentiation was evaluated by Oil Red-O staining as an indicator of intracellular lipid accumulation (Meirelles Lda and Nardi 2003). Briefly, cells were fixed with 4% formaldehyde at room temperature for 1 h and rinsed with 70% ethanol and distilled water. Cells were stained with 3 mg/mL Oil Red-O (SCRC, Shanghai, China) in isopropyl alcohol (SCRC, Shanghai, China) for 30 min at room temperature.

Experimental design and statistical analysis

A two-level full factorial experimental design was applied to investigate the influence of EGF (R&D Systems), PDGF-BB (Chemicon) and FN (Chemicon), as well as their interactions on cell proliferation, cell morphology and cell cycle profile of BMSCs. Table 1 shows the design matrix for the two-level full factorial design experiments. The factorial design was composed of 8 experimental units plus 4 replicated centerpoints, to obtain an independent estimate of the intrinsic variability (pure error) in the data. The concentrations chosen based on reported literature values were not optimized during this study. Table 2 summarizes the high (coded +1) and low (coded –1) levels of each factor. Experimental results obtained for all of these factorial combinations were used to estimate effect coefficients of the following regression equation as follows:

Table 1 Design matrix for the two-level full factorial design experiments

EGF	PDGF-BB	FN
−1	−1	−1
1	−1	−1
−1	1	−1
1	1	−1
−1	−1	1
1	−1	1
−1	1	1
1	1	1
0	0	0
0	0	0
0	0	0
0	0	0

Table 2 The levels of each factor

	−1	0	1
EGF (ng/mL)	0	5	10
PDGF-BB (ng/mL)	0	5	10
FN (μg/mL)	0	5	10

$$Y = K + \beta_1(x_1) + \beta_2(x_2) + \beta_3(x_3) + \beta_{1,2}(x_1 \cdot x_2) + \beta_{1,3}(x_1 \cdot x_3) + \beta_{2,3}(x_2 \cdot x_3)$$

where Y represents the cell proliferation in terms of OD value revealed by MTT assay; x_i indicates the scaled levels of each factor (−1 or 1); β_i and $\beta_{i,j}$ denote the main effect term coefficients and the second-order interaction term coefficients, respectively; K is the intercept. Statistical analysis of the factorial experiment results was performed using MINITAB statistical software (Minitab, State College, PA). Main effects, two-factor interactions, and three-factor interaction, along with the statistical significance of each of these properties, were calculated according to standard factorial analysis formulae.

Cell seeding

BMSCs (Passage 2) were seeded at 3×10^3 cells/cm² on uncoated or FN-coated culture plates and allowed to attach for 8 h in CM, after which CM were replaced by new CM or medium supplemented with EGF

and/or PDGF-BB. Tissue culture plates (96 well plates and 100 mm cell culture dish) were coated with FN by adding the solution of substrate (200 μL/cm²) and incubating overnight at 4 °C. Plates were washed twice with PBS before cell seeding.

Cell proliferation assay

Cell proliferation was quantitatively determined in 96-well plates using MTT assay in three sets of experiments (Krampera et al. 2005). Briefly, culture medium was replaced with 100 μL medium without FBS, and 10 μL MTT (Sigma) solution in PBS (5 mg/mL) was added to each well. After 4-h incubation at 37 °C, the medium was removed, and extraction solution (0.04 N HCl in absolute isopropanol) was added to allow the converted dye release. Absorbance of converted dye was measured at a wavelength of 570 nm with an ELISA reader (Bio-Tek Instruments, USA). To avoid that contact inhibition would influence the results, cell were cultured for 5 days to reach 60–80% confluence.

Cell areas and widths measurements

Cell morphology was observed for 6 days and photographs were taken in typical areas of 100 mm culture plates using phase contrast microscopy every day. The areas and the maximal widths perpendicular to the long axes of individual cells in typical fields were quantitatively measured by Image-Pro Plus image analysis software (Media Cybernetics, Silver Spring, MD). Mitotic cells were ignored.

Cell cycle profile assay

BMSCs cultured in 100 mm culture plates for 5 days were detached and washed twice in PBS. The collected cells were then thoroughly resuspended in 70% ice-cold ethanol fixative and kept on ice for 18 h. After cells were washed with PBS once again, staining solution containing propidium iodide (20 μg/mL) (Sigma), RNase A (200 μg/mL) (Sigma) and Triton X-100 (0.1%) (Fluka) was added. Cells then were incubated at room temperature for 30 min. Cell fluorescence was measured by a FACSCalibur flow cytometer (Becton Dickinson, USA). The linear DNA content data were processed by Modfit software

package (Verity Software, Topsham, ME) for cell cycle analysis (Krishan 1975).

Results and discussion

Characterization of murine BMSCs

Approximately 5×10^7 bone marrow cells were initially plated in one 100 mm cell culture dish. At 1 week after plating, 50~100 cell colonies with various sizes were formed. Primary cells usually reached confluence after 10~13 days. However, 3 days was sufficient for cells in subsequent culture to become confluence. BMSCs exhibited bipolar to polygonal fibroblast-like morphology in gelatin coated tissue culture dishes. The expanded BMSCs (Passage 2) were characterized by their ability to

selectively undergo osteogenic and adipogenic differentiation, as assessed histologically (Fig. 1). BMSCs cultured in osteogenic medium for 3 weeks underwent a dramatic change in cell morphology from spindle to cuboidal shape. A significantly greater ALP activity was observed by ALP staining (Fig. 1a). Mineralized matrix deposition was demonstrated by red-positive nodules above the cell monolayer (Fig. 1c). In contrast, BMSCs cultured in CM retained their fibroblast-like shape, and showed little purple stain as well as the absence of mineralized matrix deposition (Fig. 1b, d). BMSCs cultured in adipogenic medium for 4 weeks accumulated lipid droplets as evidenced by red stain of Oil Red O (Fig. 1e), while BMSCs cultured in CM maintained their fibroblast-like shape and did not show any small vacuole (Fig. 1f). The capacity for expansion in an undifferentiated state and the maintained

Fig. 1 Differentiation of BMSCs. Cells (Passage 2) were maintained in differentiation medium (a, c, e) or in CM (b, d, f) for 21 days (a, b, c, d) or 28 days (e, f) and then analyzed by ALP staining (a, b), Alizarin Red S staining (c, d), and Oil Red O staining (e, f). Shown are phase-contrast pictures at $10 \times$ magnification. Scale bar = 1 mm

ability for osteogenic and adipogenic differentiation establish the feasibility of using BMSCs for clinical applications.

Analysis of the effects of EGF, PDGF-BB, and FN on BMSCs proliferation from two-level factorial design experiments

Analysis of the “main effect” of each factor individually and “two-factor effects” on cell proliferation was done by standard statistical methods for analyzing factorial experiments (Fig. 2). Statistical analysis yielded coefficients β and their respective P value for the main effects as well as the second-order interaction terms of Eq. (1) (Table 3). Although all of the effects were positive, the significances were not identical.

As expect, EGF had the strongest effect on cell proliferation ($p < 0.01$) and PDGF-BB also had a significant effect ($p < 0.01$). However, the combined EGF and PDGF-BB had an antagonistic effect ($0.01 < p < 0.05$). Namely, EGF initiated many of the same signal transduction events as PDGF-BB, but the cross-talk between the two receptors is not reciprocal. This may be caused by the process called transmodulation by which, in cells expressing

Fig. 2 Factorial analysis of the effects of the three factors as well as their combinations on BMSCs proliferation. Cells (Passage 2) were plated at a density of 3×10^3 cells/cm² and grown for 5 days in 8 combinations of three factors and then measured by MTT assay. Centerpoints were not considered in the evaluation of the effects. The abscissa indicates the standardized effects of each factor, either individually (main effect) or in combinations (two-factor effects), according to standard factorial analysis formulae

Table 3 Effect coefficients of the regression equation^a

Term ^b	Coefficient	<i>p</i> value
Constant	0.13919	0.000
EGF	0.01031	0.000
PDGF	0.00862	0.002
FN	0.00125	0.619
EGF*PDGF	0.00587	0.026
EGF*FN	0.00738	0.006
PDGF*FN	0.01019	0.000
EGF*PDGF*FN	0.00494	0.057

^a $Y = K + \beta_1(x_1) + \beta_2(x_2) + \beta_3(x_3) + \beta_{1,2}(x_1 \cdot x_2) + \beta_{1,3}(x_1 \cdot x_3) + \beta_{2,3}(x_2 \cdot x_3)$, where no 3-factor interaction was elucidated

^b Term denotes K , and β_i and $\beta_{i,j}$

receptors of both PDGF-BB (PDGFR) and EGF (EGFR), PDGF-BB can reduce the binding of EGF to its receptor (Liu and Anderson 1999; Schwartz and Baron 1999). Interestingly, while EGF and PDGF-BB both shifted the effect of FN towards promoting cell proliferation, FN did not have a significant effect on BMSCs proliferation on its own ($p > 0.05$). It is likely that integrin-mediated adhesion to FN alone is insufficient to drive BMSCs proliferation in the absence of exogenous growth factors. Although integrins, EGFR and PDGFR can activate extracellular signal-regulated kinase (ERK) independently, the emerging picture is that ERK activation must exceed a threshold to drive cell proliferation and exceeding this threshold requires input from both integrins and growth factor receptors (Danen and Yamada 2001; Schwartz and Baron 1999). Yet more intriguingly, PDGF-BB and FN had a synergetic effect as expect, whereas EGF and FN did not have. This is possibly due to the phosphatidylinositol 3-kinase (PI3K) pathway, a critical control point that determines the differences of EGF and PDGF. Kratchmarova et al. (2005) found that accumulation of components of the PI3K pathway was detected only cells were treated with PDGF but not with EGF.

Effect of EGF, PDGF-BB, and FN on morphology of BMSCs

BMSCs were visually evaluated to demonstrate changes of the cell morphology with time and with medium supplemented with multifarious combinations of factors based on 2-level full factorial design.

Representative pictures of BMSCs cultured in CM alone and CM with different combinations of the three factors at 1–6 days are showed in Figs. 3, 4, respectively. The morphology of BMSCs subtly changed with incubation time in all 8 media. BMSCs underwent a time-dependent transition from thin spindle-shaped cells initially to larger flattened cells at much later times in culture. Similarly, factors significantly affected the cell morphology. Generally, BMSCs cultured in CM were flattened and spread, and tended to form separate and small colonies at first several days, restricting cells growing in limited areas during subsequent period of culture. In contrast, BMSCs cultured in CM with factors were smaller with a more elongated spindle-like shape and formed “mesh-like” cellular junctions in the whole plate, enhancing the bound of cell growth.

Furthermore, cell areas and widths of the 8 largest cells in typical fields from the 8 cultures were compared with an image analysis program (Figs. 5, 6). Although there were no significant differences among cells cultured in different media with various combinations of factors, the areas and the maximal

widths of the 8 largest cells cultured in CM were approximately 2-fold greater than cells in the presence of factors ($p < 0.05$).

Cultures of BMSCs are morphologically heterogeneous. Colter et al. (2001) have demonstrated that they contain extremely small cells that are rapidly self-renewing (RS cells). Our results further confirmed that the small and thin cells had relatively high potential of proliferation. Cell adhesion to FN via integrin activates focal adhesion kinase, leading to reorganization of the actin cytoskeleton, and subsequently to changes in cell shape. In turn, cytoskeletal-associated proteins, including integrins and growth-factors receptors, couple within focal adhesions, activating the ERK or Rho GTPase signaling, which is related to cell proliferation (McBeath et al. 2004; Schwartz and Baron 1999).

Effect of EGF, PDGF-BB, and FN on cell cycle profile of BMSCs

The cycle distribution profile for BMSCs cultured in 8 media was shown in Fig. 7a relatively large subset

Fig. 3 Morphology of BMSCs culture in CM (a–f). Cells (Passage 2) were plated at a density of 3×10^3 cells/cm² and grown for 6 days in CM. Pictures were taken in typical fields every day (Day 1, a; Day 2, b; Day 3, c; Day 4, d; Day 5, e; and Day 6, f). Shown are phase-contrast pictures at 10 $\times$ magnification. Scale bar = 1 mm

Fig. 4 Morphology of BMSCs cultured in CM with EGF, PDGF-BB, and FN (a–f). Cells (Passage 2) were plated at a density of 3×10^3 cells/cm² and grown for 6 days in CM with the three factors. Pictures were taken in typical fields every day (Day 1, a; Day 2, b; Day 3, c; Day 4, d; Day 5, e; and Day 6, f). Shown are phase-contrast pictures at $10 \times$ magnification. Scale bar = 1 mm

Fig. 5 The 8 largest cells based on area in typical fields from cultures in CM (left) and CM with three factors (right). Every cell area was measured using ImagePro Plus software on the

basis of representative pictures of BMSCs incubated for 2 days. Then, the 8 largest cells were ordered by length

Fig. 6 Cell areas and cell widths of the 8 largest cells in typical fields from cultures of BMSCs. The areas and the maximal widths perpendicular to the long axes of the 8 largest cells from the 8 cultures was measured using ImagePro Plus

software on the basis of representative pictures of BMSCs incubated for 2 days. Data were expressed as mean $\pm$ standard deviation ($n = 8$) and analyzed using the student's t -test to compare the pairs of groups with each other. * $p < 0.05$

Fig. 7 The cell cycle profile of BMSCs from the 8 cultures. The percentage of cells in each phase of the cell cycle was determined by flow cytometry for BMSCs cultured in 8 different media for 5 days

(65.82% ~ 75.4%) of BMSCs remained in growth-arrest state (G0/G1 phase) in any medium. There was only a very small proportion (1.71% ~ 10.24%) of the cell population undergoing mitosis, and significant fractions (16.33% ~ 28.29%) of the cells were detected in S phase. In many tissues, the subset of cells that replicates infrequently and is quiescent exhibits a self-renewal potential. Although in the present work this has not been clarified, our data suggest that the surprisingly large ex vivo expansive potential of BMSCs resides on the self-renewal properties of the subset of quiescent cells.

We performed a direct comparison of the cell cycle stages of BMSCs cultured in different media. It was observed that the three factors influenced both the proportion of BMSCs in each phase of the cell cycle and the fraction of actively proliferating cells. One of the most striking differences was that the three factors moved cells out of G0/G1 phase, increasing the fractions of cells in S and G2/M phases. Regulation of the cell cycle by both growth factors and ECM proteins is restricted to the G1 phase. Integrin-mediated cell adhesion to FN cooperates with EGF or PDGF-BB to enhance and sustain ERK activity, which in turn supports the transcriptional activation of cyclin D1 in the G1 phase (Danen and Yamada 2001). Extensive crosstalk takes place between integrin and growth factor receptor signaling pathways, and mitogenic signaling is weak and

transient in the absence of integrin-mediated cell adhesion. Consequently, the cells cultured with the combination of EGF and FN, as well as the combination of PDGF-BB and FN had relatively less cells in G0/G1 phase than cells with EGF, PDGF-BB and FN alone.

Moreover, the results of cell cycle are accordance with cell proliferation data, indicating that the expansion of BMSCs following stimulation with EGF, PDGF-BB and FN may be primarily mediated by the recruitment of nonmitotic cells into mitosis.

In summary, this work demonstrates that EGF, PDGF-BB, FN and their various combinations have a significant and complex influence on cell proliferation, cell morphology and size as well as cell cycle profile of BMSCs. The result of this study, apart from providing more information on the properties of BMSCs, may further contribute to the potential use of BMSCs as a target for clinical application. However, how these multiple signals trigger intracellular signaling events to govern these behavioral responses is still needed to be investigated.

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