Original Paper

Inflammatory Microenvironment Changes the Secretory Profile of Mesenchymal Stem Cells to Recruit Mesenchymal Stem Cells

Junchao Xing^{a,b,c,d} Tianyong Hou^{a,b,c,d} Huiyong Jin^{a,b,c,d} Fei Luo^{a,b,c,d}
Zhengqi Chang^{a,b,c,d} Zhiqiang Li^{a,b,c,d} Zhao Xie^{a,b,c,d} Jianzhong Xu^{a,b,c,d}

^aNational and Local United Engineering laboratory of Tissue Engineering, Department of Orthopaedics, Southwest Hospital, the Third Military Medical University, ^bCenter of Tissue Engineering Research and Application, the Third Military Medical University, ^cLaboratory of Tissue Engineering in Chongqing City, ^dCenter of Regenerative and Reconstructive Engineering Technology in Chongqing City, Chongqing, China

Key Words

Mesenchymal stem cells • Cell migration • Inflammatory microenvironment • Chemokine secretion

Abstract

Background/Aims: Human bone-marrow mesenchymal stem cells (hBMSCs) are widely transplanted into inflammatory microenvironment to accelerate tissue regeneration. Transplanted hBMSCs recruit host hBMSCs through a poorly understood mechanism. This study was aimed to determine whether and how inflammatory microenvironment influenced the host-hBMSCs-recruiting capability of transplanted hBMSCs. **Methods:** Pro-inflammatory factors, including IL-1 β , IL-6 and TNF- α , were utilized to mimic inflammatory microenvironment. hBMSCs were cultured and conditioned media (CM) were collected. The effects of inflammatory microenvironment on the host-hBMSCs-recruiting capability of cultured hBMSCs were revealed by transwell migration assays. Employing semi-quantitative and quantitative cytokine antibody assays, we examined the secretory profile of cultured hBMSCs. **Results:** CM from cultured hBMSCs exerted excellent host-hBMSCs-recruiting capability, which was significantly promoted by exposure to inflammatory microenvironment. Within inflammatory microenvironment, hBMSCs secreted more chemokines related to cell migration. Finally, 21 cytokines were verified as potential factors accounting for the enhanced host-hBMSCs-recruiting capability of cultured hBMSCs exposed to inflammatory microenvironment. **Conclusion:** These results strongly suggested that in clinic, inflammatory microenvironment might promote the host-hBMSCs-recruiting capacity of transplanted hBMSCs by increasing chemokines secretion. Modulation of such characteristics of hBMSCs might provide novel therapeutic ideas in the context of hBMSCs.

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Tianyong Hou
and Jianzhong Xu

NO. 29 Gaotanyan Street, Shapingba District, Chongqing City, 400038 (China)
Tel. +86-023-68754164, Fax 86-023-68754164
E-Mail tianyonghou@126.com and E-Mail prof_jianzhongxu@163.com

Introduction

Mesenchymal stem cells (MSCs)-based tissue engineering strategies hold great potential for various kinds of musculoskeletal diseases, especially those accompanied by large bone defects as a result of severe trauma, inflammation and tumor resection. Thereinto, MSCs are frequently transplanted with bioscaffolds into the defects to accelerate bone regeneration. Accumulating evidence indicate that successful application of MSCs transplantation, not confined to the field of bone tissue engineering, largely depends on the mobilization and homing of the endogenous stem cells, a result of indirect paracrine effects by proteins secreted from transplanted MSCs, rather than their direct transdifferentiation [1-4]. This notion is further reinforced by our previous findings that bone repair mediated by transplanted MSCs was largely attributed to the expansion and differentiation of endogenous MSCs [5]. Moreover, emerging new findings suggested that transplanted donor MSCs could recruit host stem/progenitor cells to the transplantation site for tissue repair [6, 7]. These observations have kindled much interest in mechanisms governing host MSCs migration induced by transplanted MSCs.

Currently, there is a general consensus that the homing of endogenous stem cells to the injury site is a prerequisite for tissue regeneration and determines the efficacy of MSCs transplantation [8]. In the early phase of wound healing, that is, inflammatory phase, an intricate cascade of growth factors and inflammatory mediators was released by cellular participants in a temporally and spatially controlled manner [9]. As a result, indigenous MSCs were attracted to the injured tissues and then exerted reparative property [10-13]. During growth, MSCs produce multifarious chemokines and cytokines spontaneously, some of which are important in dictating migration of stem cells, such as CC chemokine ligands (CCL2, CCL4, CCL5, CCL20) and CXC ligands (CXCL8, CXCL12) [14, 15]. After transplantation, the secretory property of MSCs may be mainly influenced by surrounding local inflammatory microenvironments. However, the molecular identities of secreted proteome (secretome) of transplanted MSCs are unclear, and moreover, how and to what extent inflammatory microenvironment affects transplanted-MSCs-induced tropism of endogenous MSCs are yet to be clarified. Establishing this information will provide new insights into the cellular mechanisms underlying the tissue regeneration mediated by transplanted MSCs and the modulation of endogenous stem/progenitor cells homing for *in situ* tissue regeneration.

IL-1 β , IL-6 and TNF- α are three major pro-inflammatory factors (PF) induced by inflamed tissues, and their concentrations rise and peak in the inflammatory phase of transplantation and tissue regeneration [16]. They play important roles in inflammatory diseases by inducing various inflammatory cytokines and chemokines [16, 17]. Moreover, IL-1 β , IL-6 and TNF- α induce migration of MSCs by binding to the respective receptors and activating the corresponding downstream signals [18-21]. Thus, IL-1 β , IL-6 and TNF- α may regulate the secretome of MSCs and are ideally suited for imitating the *in vivo* inflammatory microenvironment.

Therefore, this study was aimed to explore the secretome of human bone-marrow mesenchymal stem cells (hBMSCs) in inflammatory microenvironment and seek the factors involved with hBMSCs migration. PF including IL-1 β , IL-6 and TNF- α were added into the culture of hBMSCs and the resulting conditioned media (CM) were collected. We then evaluated the effects of inflammatory microenvironment on the host-hBMSCs-recruiting capacity and secretome of cultured hBMSCs by Transwell Migration Assay and Human Cytokine Microarray.

Materials and Methods

Cell isolation and culture

All protocols involving human subjects were approved by Ethics Committee, Southwest Hospital, Third Military Medical University, with all subjects providing informed consents. Briefly, nucleated cells

were isolated from iliac crest bone marrow aspirates of healthy donors (15 donors in total, aged 23 to 45, 6 male and 9 female) by Ficoll (Sigma-Aldrich, USA) gradient centrifugation. For each donor, cells were kept separate and resuspended in basic culture medium containing Dulbecco's modified Eagle's medium/F12 (DMEM/F12; 1:1; Hyclone) containing 10% fetal bovine serum (FBS; Gibco, USA), and 100 units/ml penicillin/streptomycin (Gibco, USA). Cells were placed in 75-cm² flasks (Corning Costar Corp, USA) and incubated with basic culture medium at 37°C in 95% humidified air and 5% CO₂. Medium was replaced every 3 days and cultures with 80%-90% confluence were detached with 0.25% trypsin-EDTA (Hyclone, USA). Before use, hBMSCs of passage 4 (P4) were characterized by morphology, immunophenotypic analysis, and osteogenic and adipogenic differentiation. For immunophenotypic analysis, cells were harvested and washed with fluorescence-activated cell sorting (FACS) buffer (2% FBS and 0.1 % NaN₃ (KeLong Chemical, Chengdu, China) in phosphate buffered saline (PBS; Zhongshan Golden Bridge Biotechnology, Beijing, China)). Then, cells were incubated with specific fluorescence-conjugated antibodies (1:100 dilution) for 1 h and analyzed by flow cytometry with a FACSCalibur flow cytometer (BD Biosciences, USA). Human monoclonal antibodies were used as follows: anti-CD29-PE, anti-CD44-PE, anti-73-APC, anti-90-APC, anti-CD105-PE, anti-CD31-PE, anti-CD34-APC, and anti-45-APC, and the recommended isotype control for each fluorochrome (all from BD biosciences, USA). For analysis of multilineage differentiation capacity, cells were detached from cultures reaching 80-90% confluency, washed and seeded at 5×10⁴/well in a 24-well culture plate (Corning Costar Corp, USA). Osteogenesis was induced by culture in DMEM/F12 with 50μM ascorbic acid, 10 mM β-glycerophosphate, and 0.2 μM dexamethasone (all from Sigma-Aldrich, USA). On day 14, the calcium deposits were shown using the Leukocyte Alkaline Phosphatase Kit (Sigma-Aldrich, USA) according to the manufacturer's recommendations. To induce adipogenic differentiation, cultures were stimulated for 21 days in DMEM/F12 supplemented with 1% FBS, 1 μM dexamethasone, 50 μM 3-isobutyl-1-methyl-xanthine (IBMX), 100 ng/ml insulin (all from Sigma-Aldrich, USA). The medium was changed twice a week. After Oil Red O (Sigma-Aldrich, USA) staining, lipid droplets were visualized with light microscope. Finally, cells had a spindle-shaped morphology, were homogeneously CD29⁺, CD44⁺, CD73⁺, CD90⁺, CD105⁺, CD31⁺, CD34⁺, CD45⁺ (Fig. 1A), and were able to differentiate into osteoblasts (Fig. 1B) and lipoblasts (Fig. 1C).

Preparation of conditioned media

Confluent hBMSCs (equivalent to transplanted hBMSCs) from 15 donors were separately washed twice with serum-free DMEM/F12 and incubated with serum-free medium alone or supplemented with 4 ng/ml IL-1β, 10 ng/ml IL-6, and 20 ng/ml TNF-α (all from Peprotech Asia, USA). After 48 hours, the supernatants were collected, centrifuged, aliquoted, and stored at -80°C. Serum-free medium with the presence or absence of IL-1β, IL-6, and TNF-α served as control. The grouping of CM was detailed in Table 1.

Migration assay

Migration assays were performed in transwell inserts (8 μm pores; Corning Costar Corp, USA). For each cell batch, 700 μl of CM from each group was added into the bottom compartment. Non-treated hBMSCs (2×10⁴, equivalent to endogenous hBMSCs) in 200 μl serum-free medium were seeded into the top chamber and allowed to migrate at 37°C for 36 hours. Then, cells on the upper side of the filter (non-migrating cells) were removed with a cotton wool swab (Norgen Biotek, Canada). Migrated cells on the lower face were washed with PBS and fixed with 4% paraformaldehyde (Boster Biological Technology, Wuhan, China). The membrane was then placed onto glass slides with the bottom side upwards and migrated cells were stained with 4', 6 diamidino-2 phenylindole (DAPI; Invitrogen, USA), and observed under a microscope. For each group, the number of migrated cells was counted on 10 random high power fields (200× magnification) and averaged. Migration assay was separately conducted on 15 batches of hBMSCs obtained from 15 donors.

Cytokine antibody assay

To screen chemokines associated with hBMSCs migration, high-throughput RayBio Human Cytokine Antibody Assays (RayBiotech, Norcross, USA) were employed as per manufacturer's instructions. The assay was repeated in quadruplicate, on 4 batches of CM randomly selected from 15 batches. Using this assay, 262 different human cytokines can be screened simultaneously. Briefly, the membranes were blocked with blocking buffer for 1 hour and then incubated with CM sample (100 μl from each group) for 2 hours at room temperature. After extensive wash with wash buffer I (5 times, 5 min each) and wash buffer II (twice, 5 min each), membranes were incubated with a cocktail of biotin-conjugated antibodies at room temperature.

Fig. 1. Characterization of hBMSCs. (A) Histogram analysis of cell surface markers showed that hBMSCs were positive for CD29, CD44, CD73, CD90, CD105, and negative for CD31, CD43, and CD45. After induction of osteogenic and adipogenic differentiation, calcium deposits (B) and lipid droplets (C) were observed, suggesting that cultured hBMSCs were able to differentiate into osteoblasts and lipoblasts. hBMSCs, human bone-marrow mesenchymal stem cells.

After 90 min, membranes were repeatedly washed and then horseradish peroxidase-conjugated streptavidin (Cy3 equivalent) was added and incubated at room temperature for 1 h, followed by a set of washes. Finally, fluorescent signals were detected by a GenePix™ 4000B laser scanner (Axon Instruments, Sunnyvale, CA94089, USA). The relative protein levels were obtained by subtracting the background staining and normalizing to the positive controls on the same membrane.

Quantitative human cytokine array

To further quantify the levels of screened chemokines, a custom Quantibody human cytokine array was performed on the same 4 batches of samples. The array is a multiplex sandwich enzyme-linked immunosorbent assay (ELISA)-based system, which spotted the membrane with 42 cytokine or chemokine-specific antibodies per our request. The proteins of interest are shown in Table 2. The protocol was similar to that of cytokine antibody assay. Each antibody toward cytokine or chemokine, together with the positive, negative and internal controls, was arrayed in quadruplicate. Data were extracted and analyzed with RayBio QAM-TH17-1 software (RayBiotech, Inc.). When the cytokine levels were below the sensitivity, a value of 0 was used for the comparisons of the levels.

Table 1. The grouping of conditioned media. * Pro-inflammatory factors included 4 ng/ml IL-1 β , 10 ng/ml IL-6, and 20 ng/ml TNF- α . hBMSCs, human bone-marrow mesenchymal stem cells

Groups	hBMSCs	Pro-inflammatory factors*
A	Yes	Yes
B	Yes	No
C	No	No
D	No	Yes

Table 2. Proteins detected by Quantibody human cytokine array

ANG	ENA-78	IGF-II	IL-2 R α	MCP-3	MIP-3 β	RANTES
ANG-1	EOT2	IL-10	IL-6	MDC	NAP-2	SDF-1
Cathepsin S	GCP-2	IL-11	IL-8	MIP-1 α	OPG	TNF RII
CD14	GM-CSF	IL-16	LAP	MIP-1 β	OPN	TRAIL R3
CXCL16	GRO	IL-1 α	MCP-1	MIP-1 δ	PDGF-AA	u PAR
EGF	IGFBP-3	IL-1 β	MCP-2	MIP-3 α	RANK	VEGF

Fig. 2. Migration capacity of hBMSCs toward different CM. (A) showed the images of *in vitro* migration assay using transwell culture system. CM (200 μ l) and hBMSCs (2×10^4) were added into the bottom and top chamber, respectively. Migration was terminated after 36 hours. Migrated hBMSCs were stained with DAPI. Bar = 100 μ m. (B) Quantification of transwell results. The number of migrated cells was counted on 10 random high power fields for each CM. Bars are mean $\pm$ SD; n=10; *p<0.0083. hBMSCs, human bone-marrow mesenchymal stem cells. CM, conditioned media. DAPI, 4', 6 diamidino-2 phenylindole. DMEM/F12, Dulbecco's modified Eagle's medium/F12. PF, pro-inflammatory factors.

Statistical analysis

All comparable values were expressed as mean $\pm$ SD. Between any two groups, the mean numbers of migrated cells toward CM were compared using one-way ANOVA (SPSS version 13.0). Using bonferroni correction, $P < 0.0083$ ($0.05/6$) was considered statistically significant. The results of cytokine antibody arrays from different CM were compared by ANOVA tests with Post Hoc Test (SPSS version 13.0). $P < 0.05$ was considered statistically significant.

Results

CM from PF-stimulated hBMSCs facilitated migration of hBMSCs

To investigate the hBMSCs-recruiting capacity of hBMSCs in the inflammatory microenvironment, migration assays were performed. As shown in Figure 2, the chemotactic activity of hBMSCs+ DMEM/F12 was markedly higher than hBMSCs- DMEM/F12 ($P < 0.0083$), while slightly higher than PF+ DMEM/F12 ($P > 0.0083$). This suggested that the bioactive factors released by non-treated hBMSCs had analogous chemotactic power to PF. Moreover,

Table 3. The fold change of human cytokines between samples. #DIV/0!: the value of control is 0. PF, pro-inflammatory factors, include 4 ng/ml IL-1 β , 10 ng/ml IL-6, and 20 ng/ml TNF- α . hBMSCs, human bone-marrow mesenchymal stem cells. DMEM/F12, Dulbecco's modified Eagle's medium/F12

Cytokine name	Fold change PF-stimulated hBMSCs versus non- treated hBMSCs	Fold change non-treated hBMSCs versus DMEM/F12	Cytokine name	Fold change PF-stimulated hBMSCs versus non- treated hBMSCs	Fold change non-treated hBMSCs versus DMEM/F12	Cytokine name	Fold change PF-stimulated hBMSCs versus non- treated hBMSCs	Fold change non-treated hBMSCs versus DMEM/F12
Angiogenin	0.889747779	4.957937582	MIP-3-alpha	0.993957347	1.112664564	Lymphotoctin	0.631396288	1.059306047
BDNF	0.904458467	0.761156891	NAP-2	1.511251805	0.015094287	MIF	0.566395035	1.633601567
BLC	0.715342714	0.913535291	NT-3	1.058963291	1.44301381	MIP-1alpha	0.583886288	0.042156496
BMP-4	0.825264182	1.315416773	PARC	1.077860127	1.007432788	MIP-1beta	0.933938325	0.178783873
BMP-6	0.918090508	0.712450333	PDGF-BB	1.266331735	1.025451525	MIP-3beta	0.718471822	1.111520373
CK beta 8-1	0.974971068	1.928618577	RANTES	1.635487318	1.119618717	MSP-alpha	0.863967507	0.938573643
CNTF	0.794643439	0.905878859	SCF	0.95971854	0.844791612	NT-4	0.805412504	0.978713963
EGF	0.808392819	1.520933608	SDF-1	1.052782156	1.293907659	Osteoprotegerin	4.025217328	15.29335487
Eotaxin	0.808467761	1.220677615	TGF-beta 1	1.151268229	0.99506587	Oncostatin M	0.666910393	0.936968942
Eotaxin-2	1.740775411	0.557144446	TNF-alpha	1.199898604	0.972579523	PIGF	0.341901704	1.172148086
Eotaxin-3	0.929202889	1.12978248	TNF-beta	1.037930956	0.921504639	sgp130	0.824991376	1.139108678
FGF-6	0.200523504	1.042632582	Acrp30	0.702020963	1.533860771	sTNF RII	0.513428148	1.887619116
FGF-7	1.374645784	0.581291424	AgRP	0.823724458	0.972409847	sTNF-RI	0.695014063	0.858524009
Flt-3 Ligand	0.802973248	0.983829719	Angiopoietin-2	0.729638115	1.032435147	TECK	0.69214484	0.878908901
Fractalkine	0.958072749	0.898988682	Amphiregulin	0.659518064	1.313657454	TIMP-1	0.635060763	22.78806399
GCP-2	57.69076413	#DIV/0!	Axl	0.465371274	1.122011362	TIMP-2	0.698226636	23.48340668
GDNF	0.663295209	1.004251093	bFGF	0.414165373	0.995452735	Thrombopoietin	0.586042764	1.05922697
GM-CSF	1.586538209	0.638477147	b-NGF	0.684566799	1.037072947	TRAIL R3	0.501979657	1.389567918
I-309	0.548993311	2.049280867	BTC	0.575345398	0.82795766	TRAIL R4	0.701972925	0.900707713
IFN-gamma	1.031546017	1.016718396	CCL-28	0.5394139	1.221745706	uPAR	0.755323768	1.043584781
IGFBP-1	1.448151959	2.628670032	CTACK	0.670110267	1.067962003	VEGF	0.838931625	1.30075603
IGFBP-2	1.149439575	38.13843236	Dtk	0.737139397	1.01619849	VEGF-D	0.707191467	0.771221603
IGF-I	1.464649326	1.061876271	EGF-R	0.624475322	0.667340091	Activin A	0.585903658	1.512167322
IL-10	0.899647374	1.048102546	ENA-78	2.092098706	0.808196644	ALCAM	0.680879691	0.730914267
IL-13	1.664292718	1.08484795	Fas/TNFRSF6	0.842268758	1.04039735	B7-1(CD80)	0.486161595	0.525564399
IL-15	1.004109858	1.060629504	FGF-4	0.876215881	0.973825291	BMP-5	1.0836273	0.518948807
IL-16	0.629166925	0.896875073	FGF-9	0.63070869	1.049350371	BMP-7	0.48112035	0.794873022
IL-1alpha	0.899362855	1.085878195	GCSF	0.59444537	1.009481191	Cardiotrophin-1	0.632863925	0.720691461
IL-1beta	0.737584273	1.026818534	GITR	0.012448013	0.945934707	CD14	0.313269861	0.614069687
IL-1ra	0.558788747	1.130744307	GRO	32.85759626	0.917788156	CXCL-16 DR6 (TNFRSF21)	2.090980185	1.324202816
IL-2	1.040046888	1.008333415	HCC-4	0.874583964	1.055798644	Endoglin	0.747027164	0.953210301
IL-3	0.367959245	0.898659159	HGF	0.475072046	1.127111414	ErbB3	0.648441592	1.118563846
IL-4	1.614572963	0.972551248	ICAM-1	0.811149535	0.958661122	E-Selectin	0.746879772	1.127185175
IL-5	1.091852805	0.94305161	IGFBP-3	1.794803172	0.986009985	Fas Ligand	0.433372087	1.378502925
IL-6	29.26752243	1.266425233	IGFBP-6	0.738098529	1.186117567	ICAM-2	0.521098605	1.159341476
IL-7	1.246123343	0.938424384	IGF-I SR	0.561306111	0.978412377	IGF-II	1.247919984	1.235030765
Leptin	1.946985043	0.942626594	IL-1 R4/ST2	0.57279091	1.545534087	IL-1 R II	0.680982521	0.773099279
LIGHT	0.800848201	1.035803497	IL-1 RI	0.776620672	1.073798552	IL-10 Rbeta	1.206928311	0.38699947
MCP-1	11.50214058	0.752973	IL-11	0.880680283	0.960425923	IL-13 Ralpha2	0.622323875	0.714083369
MCP-2	30.02210012	0.935779428	IL-12 p40	0.427943483	1.050386109	IL-18 BPalpa	0.549792775	0.453782966
MCP-3	4.853721864	1.049501181	IL-12 p70	0.54323907	1.063832996	IL-2 Ralpha	#DIV/0!	0
MCP-4	1.615995807	1.680460662	IL-17	#DIV/0!	#DIV/0!	IL-2 Rbeta	0.348203319	0.70866519
M-CSF	1.229757558	2.354878804	IL-2 Ralpha	0.794347255	0.98043179	IL-2 Rgamma	0.155599091	1.367406326
MDC	1.645842015	0.850304731	IL-6 R	0.726914885	1.123405165	IL-21R	0.765273517	0.965227995
MIG	0.98631027	1.133900505	IL-8	0.971004697	1.166891817	IL-5 Ralpha	0.601296992	1.039794371
MIP-1-delta	11.02025193	#DIV/0!	I-TAC	0.654058563	1.058672011			

continued

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Cytokine name	Fold change PF-stimulated hBMSCs versus non-treated hBMSCs	Fold change non-treated hBMSCs versus DMEM/F12	Cytokine name	Fold change PF-stimulated hBMSCs versus non-treated hBMSCs	Fold change non-treated hBMSCs versus DMEM/F12	Cytokine name	Fold change PF-stimulated hBMSCs versus non-treated hBMSCs	Fold change non-treated hBMSCs versus DMEM/F12
IL-9	0.583002994	1.293515615	Luteinizing hormone	0.66550569	0.805959633	Cathepsin S	1.585653391	1.230665704
IP-10	0.663547222	1.078963	LIMPII	0.755250157	0.985658627	CCL14a	0.911029948	2.624213633
LAP	0.467579495	1.422041323	LYVE-1	1.288127911	0.86445011	CCL21	1.266066433	0.894784548
Leptin R	0.74649695	0.814497732	Marapsin	0.941651087	1.239442593	CD23	1.02372928	1.159702113
LIF	0.564190306	0.971019602	MICA	0.883858995	0.599550177	CD40 Ligand	0.865854035	0.981910821
L-Selectin	0.674612873	0.752368059	MICB	0.933372835	0.952254675	CEA	0.831824628	0.964092822
M-CSF R	0.738655308	0.728490799	MMP-2	1.005746665	0.740642525	CEACAM-1	0.990579951	1.183104174
MMP-1	1.527944695	0.568866045	MMP-7	1.046869243	3.322310756	Cripto-1	0.941891558	1.299384256
MMP-13	0.69370569	0.459102033	MMP-8	0.912159816	1.237931078	CRP	0.999561922	1.19669486
MPIF-1	0.328881054	0.532540769	MMP-10	0.794950259	0.908251571	DAN	1.47639869	2.504170326
NGF R	0.414444779	1.514896312	NCAM-1	0.827307741	0.595159172	DKK-1	1.187386682	1.413837792
PDGF AA	0.682920472	0.878983086	Nidogen-1	0.777277737	1.40015797	DKK-4	1.305625749	1.137421051
PDGF-AB	0.737815001	1.231036023	NrCAM	0.732178039	1.269672901	DPPIV	0.855377425	2.662576548
PDGF Ralpha	1.24772856	2.094149626	NRG1-beta1	0.414476244	0.903743588	E-Cadherin	1.043648232	0.877605871
PDGF Rbeta	0.622926138	1.22498058	Osteopontin	0.977483932	0.919782223	EDA-A2	1.457171595	3.152281199
PECAM-1	0.271066168	1.11318888	PAI-1	1.071933648	1.335345637	EpCAM	0.909981583	3.08876758
Prolactin	0.675661601	1.026199587	Platelet factor 4	1.088369294	1.041528551	ErbB2	1.765120525	2.061972387
SCF R	0.748637136	0.613658771	PSA-total	1.064775703	0.698427877	Erythropoietin R	1.38934688	1.137480276
SDF-1beta	0.625923238	0.457360372	RAGE	0.799187312	0.9142585	FSH	0.459633554	#DIV/0!
Siglec-5	1.654131577	0.149208161	RANK	0.96666594	3.523880296	HB-EGF	1.18791524	1.016429682
TGF-alpha	0.472823454	0.952926069	Resistin	0.7441992	0.774535836	hCGa, intact	1.007988248	1.074445918
TGF beta2	0.395344872	0.54059149	SAA	1.0483143	1.974528077	HVEM	0.993453577	1.231649235
Tie-1	0.484172931	0.63996041	Siglec-9	0.849035015	0.868077467	IL-13 R alpha1	1.161259609	1.43433544
Tie-2	0.257493028	1.149726687	TACE	0.191839476	1.152729344	IL-17B	0.908452202	40.77407203
TIMP-4	0.667438114	1.482364085	TIM-1	0.601678357	0.748200102	IL-17C	1.088914743	1.227454764
VE-Cadherin	0.539558296	0.783078291	TRAIL R2	1.544307782	0.176343458	IL-17F	1.191642549	0.92987024
VEGF R2	0.693335029	1.14258002	Trappin-2	1.427511395	1.156309249	IL-17R	0.79896034	0.940454603
VEGF R3	0.428364565	1.285062802	TREM-1	0.707634294	1.441985651	Procalcitonin	1.078606741	1.296472879
Adiposin	1.0167339	3.569529502	TSH	0.846806576	1.366278447	PSA-free	1.006366618	1.14188258
BCAM	0.471544361	0.455275046	TSLP	0.927565749	2.659401565	Shh N	1.259822603	1.013259418
CD30	1.018756566	1.90929455	VCAM-1	0.906489009	1.068641358	Thyroglobulin	0.971384028	1.053278484
CD40	0.616774229	0.760168968	VEGF-C	0.856055886	0.800759613	Ubiquitin+1	1.362519593	1.016953326
FcR RIIB/C	0.925308382	0.850352884	XEDAR	1.010275099	0.916337066			
Ferritin	1.019243136	0.975670774	4-1BB	1.334893195	1.086537993			
FLRG	0.527558559	1.510472933	ACE-2 Alpha-fetoprotein	0.730699497	2.078678182			
Follistatin	0.881887071	0.925736191	Angiopoietin-1	1.050478739	0.835183198			
Furin	0.657290712	1.101755409	Angiostatin	1.066978405	1.048073033			
Galectin-7	0.652300334	0.934287606	ANGPTL4	1.122046618	0.909700412			
GDF-15	1.075727971	0.83494794	Bate2 M	1.197981073	1.36640384			
Growth Hormon	0.792880054	0.743246021	beta IG-H3	1.011914814	135.9135734			
IL-10 R alpha	1.934381384	1.775826287	CA125	0.854961569	1.261420093			
IL-22	1.418791867	0.875127392	CA15-3	0.667694381	1.350667252			
IL-28A	1.802504352	1.156569855	CA19-9	0.749097434	1.244357236			
IL29	0.745802608	0.586403837	Carbonic Anhydrase IX	0.706136698	1.104255076			
IL-31	1.00525282	0.739772141		0.888624872	1.25293461			
Insulin	0.904100304	0.807890863		1.072478294	1.04302096			

CM from PF-stimulated hBMSCs induced the greatest chemotaxis as compared with any other group ($P < 0.0083$), indicating that inflammatory microenvironment enhanced the secretion of bioactive components from hBMSCs. Taken together, these results are consistent with

Fig. 3. Spectral description of 262 cytokines using RayBio human antibody assay (AAH-CYT-G4000) of CM from PF-stimulated hBMSCs (Column A), non-treated hBMSCs (Column B), and DMEM/F12 (Column C). The assay was repeated in quadruplicate. The relative levels of each protein in three groups of CM were normalized into a range from -1 to 1, and then plotted by Matlab (version 7.0). The expression levels of 11 cytokines were significantly elevated in the CM of PF-stimulated hBMSCs, as compared with those in the CM of both untreated hBMSCs and DMEM/F12 alone. These differentially expressed cytokines were marked with red points in front of their names and included C-X-C motif ligand 16 (CXCL-16), Osteoprotegerin, growth-related oncogene (GRO), epithelial cell-derived neutrophil-activating peptide 78 (ENA-78), macrophage inflammatory protein-1-delta (MIP-1-delta), monocyte chemoattractant protein 1 (MCP-1), MCP-2, MCP-3, Interleukin 6 (IL-6), granulocyte chemotactic peptide-2 (GCP-2), and Interleukin 2 receptor alpha (IL-2R alpha). CM, conditioned media. PF, pro-inflammatory factors. hBMSCs, human bone-marrow mesenchymal stem cells. DMEM/F12, Dulbecco's modified Eagle's medium/F12.

Fig. 4. Summary of signal intensities and protein concentrations of differentially expressed cytokines that associate with the migration of hBMSCs. Quantitative human cytokine assay was repeated in quadruplicate. Logarithmic transformation was performed on actual values for noticeable cognition. A: Compared to CM from non-treated hBMSCs, there were significant increases ($p < 0.05-0.01$) in the relative levels of 21 cytokines in the CM from PF-stimulated hBMSCs. The other 21 cytokines detected (not shown in this figure) did not exhibit significant differences between these two groups. B: The concentrations of 21 differentially secreted proteins in different CM were measured, showed in logarithmic pattern. The actual mean concentrations were located above the columns. hBMSCs, human bone-marrow mesenchymal stem cells. CM, conditioned media. PF, pro-inflammatory factors. DMEM/F12, Dulbecco's modified Eagle's medium/F12.

Preliminary screening of migration-related factors

RayBio® Human Cytokine Antibody Assays were performed for preliminary cytokine screening. The expressions of 262 cytokines were simultaneously analyzed. The average fold change from CM of non-treated hBMSCs, whose mean was assigned a value of 1, for all cytokines was reported in Table 3. As shown in Figure 3, culturing hBMSCs with PF induced significant increases in secretion of 11 cytokines. The levels of 2 proteins were elevated 4.0-9.9 fold, 3 proteins were increased 10.0-29.9 fold, and 3 proteins were stimulated greater than 30.0 fold (Table 3). Take DMEM/F12 as control (with mean assigned a value of 1), only 3 of these 11 cytokines (Osteoprotegerin, MIP-1-delta, GCP-2) showed a dramatic increase in expression (> 2 -fold increase and $p < 0.05$) due to the introduction of hBMSCs (Table 3). Taken together, these data reinforced the notions that hBMSCs secreted a variety of cytokines to recruit MSCs and inflammatory microenvironment is effective in stimulating cytokine production from hBMSCs.

Validation of chemokines involved in hBMSCs migration

After ANOVA tests on the relative levels of 262 cytokines, 11 cytokines were selected as differentially expressed (Fig. 3). The concentrations of them were then quantified by a

multiplex-ELISA-based cytokine array. Despite these 11 cytokines, the levels of additional 31 cytokines were also evaluated, as most of them were known to be implicated in cell migration according to the existing literature. Only cytokines whose mean signal intensities were > 2 fold above control levels and significantly different from control group ($p < 0.05$) were included in the Figure 4. As compared with CM from non-treated hBMSCs, CM from PF-stimulated hBMSCs showed more profound increases in secretion of 21 cytokines, including 9 of the 11 cytokines preliminarily screened except CXCL16 and IL-2R α (Fig. 4A). Thereinto, the maximal concentration occurred in GRO, with an average of 14.037 ng/mL for CM from PF-stimulated hBMSCs. Besides, the concentration of IL-6 was 4.468 ng/mL in the CM from PF-stimulated hBMSCs and reduced by nearly half of the initial concentration (10 ng/mL) (Fig. 4B).

Discussion

In clinic, MSCs transplantation has been extensively performed for treating a wide array of diseases. Initially, their intrinsic multipotent differentiation properties generated the most interest, but more recently, their paracrine effect, which is manifested as the ability to define and manipulate the local microenvironment by releasing various bioactive molecules, is drawing the attention [23]. After local transplantation, donor MSCs interacted with surrounding microenvironment, resulting in recruitment of endogenous progenitor cells into the injury site to facilitate tissue regeneration [4, 6, 7]. Local inflammatory responses, incited by injury, have predominant responsibilities for host MSCs recruitment, with multiple inflammatory mediators (cytokines, chemokines and other potential chemoattractants) being involved [16]. Due to the presence of various bioactive cellular and molecular components, inflammatory microenvironment is likely to exert impacts on the secretome of transplanted MSCs and the relevant tropism of endogenous MSCs. A better understanding regarding this information is crucial for the comprehension of the cellular and molecular mechanisms governing MSCs transplantation, which remains the major bottleneck in the development of MSCs-based tissue engineering.

IL-1 β , IL-6, and TNF- α are representative molecules in the inflammatory microenvironment and play critical roles in the inflammatory diseases by triggering highly complicated biological cascades involving chemokines and cytokines [15, 16]. Besides, each of them has been identified as a powerful MSCs chemoattractant [18-20]. Herein, we utilized IL-1 β , IL-6, and TNF- α to mimic the *in vivo* inflammatory microenvironment, as well as serve as a positive control for migration assay. For wound healing, the mobilization and homing of endogenous MSCs to the injured tissue is an essential but extremely complex process, and the molecular mechanisms are almost unknown [3, 4]. In the present study, we demonstrated that cultured hBMSCs exhibited a potent capacity of attracting hBMSCs by secreting soluble chemokines, which was consistent with previous *in vivo* studies [3, 6, 7]. Such chemotactic power was comparable to that elicited by IL-1 β , IL-6, and TNF- α , further validating the paracrine effects of transplanted MSCs. Moreover, the presence of inflammatory microenvironment (IL-1 β , IL-6, and TNF- α) enhanced the host-hBMSCs-recruiting capacity of cultured hBMSCs, indicating promotional roles of inflammatory microenvironment in the paracrine effects of transplanted hBMSCs. Based on this, one can speculate that inflammatory microenvironment and resident transplanted MSCs interact with each other, bringing about synergetic effects to facilitate host MSCs homing to the transplantation site.

In order to rationally modulate and take advantage of the migration capacity of endogenous MSCs, the underlying mechanisms should be understood. Cell migration is generally attributed to chemotactic signals incited by growth factors or chemokines. Currently, the chemotactic signal pathways guiding the migration of MSCs are under lively investigation. A distinct set of soluble cytokines, including chemokines and growth factors, are emerging as dependable factors participating in the directed migration of MSCs [15]. Since the CM from PF-stimulated hBMSCs possessed a superior host-hBMSCs-recruiting

Fig. 5. Schematic diagram of the potential effects of inflammatory microenvironment (with the presence of IL-1 β , IL-6, and TNF- α) on the secretome of hBMSCs. Among the screened cytokines, the secretions of MIP-1 α , RANTES, GRO, ENA-78, GCP-2, IL-8 and IL-6 can be stimulated by both IL-1 β and TNF- α . Besides, IL-1 β can increase the secretion of MIP-3 α and TNF- α while the hypersecretion of MCP-1 is mainly attributed to the presence of TNF- α . As with other differentially expressed cytokines, the secretory changes are difficult to explain according to the existing literature. Also, the effect of IL-6 on the cytokine production from MSCs remains unknown.

power, we hypothesised that such chemotactic disparity may be a representation of soluble chemoattractants with different concentrations therein. As showed by cytokine antibody microarrays, secretion of 21 proteins from transplanted hBMSCs was significantly increased by exposure to inflammatory microenvironment and included those involved in chemotaxis, invasion, cell signaling, cytoskeleton reorganization, and degradation of extracellular matrix. This indicated that the enhanced host-hBMSCs-recruiting activity of PF-stimulated hBMSCs was a synthetic result elicited by a set of bioactive molecules. It is worth noting that among various potent chemoattractants for stem cells, SDF-1 has attracted much attention in recruiting endogenous progenitors and guiding *in situ* regeneration. By signaling through the cognate receptor CXCR4, SDF-1 plays a predominant role in the activation, mobilization, homing and retention of CD34⁺ HSCs [24-27]. Several studies have demonstrated that local injection or delivery of SDF-1 can home MSCs to multitude target organs such as heart [28], kidney [29], brain [30], and bone [31]. Such capacity is further confirmed by successful applications of SDF-1 in tissue engineering strategies [32-34]. Thus, SDF-1 appears to be a major factor regulating MSCs migration. However, in this study, the SDF-1 production by transplanted hBMSCs was not increased in the inflammatory microenvironment, and the concentration was relatively lower than that in other studies where the introduction of SDF-1 significantly enhanced MSCs migration. These findings not only dented the leading role of SDF-1, but also indicated that unlike the case for CD34⁺ HSCs, it seemed an arduous task to define a major ligand/receptor system governing the migration of MSCs. Besides, we found that some of the identified cytokines, including IL-8, RANTES, MCP-1, MCP-2, and MCP-3, were also in close relationship with leukocyte migration [10]. As the mechanism underlying MSCs migration is still in infancy, these findings provided great inspiration for the current investigations taking leukocytes, but not HSCs, as research archetypes for MSCs trafficking.

A somewhat surprising finding from the present study was that GRO possessed the greatest level in the CM derived from PF-stimulated d hBMSCs. The GRO subgroup consists of three members, CXCL1/GRO- α , CXCL2/GRO- β , and CXCL3/GRO- γ , and belongs to the IL-8 cytokine family [35]. This subfamily is a potent angiogenic chemokine group famous for stimulating chemotaxis of leukocyte, endothelial cells, as well as regulating inflammation and angiogenesis [36, 37]. Moreover, GRO have been demonstrated to be crucial in the mobilization and homing of stem cells [38-40]. During *in vitro* expansion, MSCs constitutively secrete GRO chemokines [35, 41, 42], which in turn regulate the biological behaviors of MSCs through binding to their cell-surface cognate receptor, CXCR2 [22, 36, 37]. Besides, GRO production from MSCs can be significantly increased by the stimulation of IL-1 β and TNF- α [15, 18, 41]. Taken together, it is reasonable to conclude that hypersecretion of GRO is a previously unrecognized molecular feature that specifically accompanies transplanted hBMSCs in inflammatory microenvironment and at least in part, GRO accounted for the enhanced capacity of transplanted hBMSCs in recruiting endogenous hBMSCs. Another

interesting finding was that the levels of IL-1 β and TNF- α in the CM from PF-stimulated donor hBMSCs were very low or slightly higher, whilst, the concentration of IL-6 was nearly half of the initial value and notably higher than that in the CM from non-treated hBMSCs. This indicated that hypersecretion of proteins might be mainly due to the presence of IL-1 β and TNF- α . Supporting this notion is the fact (as showed in Fig. 5) that IL-1 β can stimulate MSCs to secrete MIP-1 α , RANTES, MIP-3 α , ENA-78, GCP-2, IL-8 as well as GRO [18, 41] and, TNF- α mainly accounts for hypersecretion of MIP-1 α , RANTES, ENA-78, GCP-2, IL-8, GRO, and MCP-1 [15, 43]. However, to the best of our knowledge, the influence of IL-6 on the secretome profile of MSCs has not been reported. On the other hand, both IL-1 β and TNF- α can increase the IL-6 production from MSCs [18, 41, 43]. Considering the final concentration of IL-6, it is illogical to discount the secretagogue effect of IL-6, although further investigation is needed. Collectively, these reinforced the notion that the secretory variation of transplanted hBMSCs was a result from the synthetical actions of IL-1 β , IL-6 and TNF- α and further complicated the relationship between inflammatory microenvironment and MSCs.

The major limitation of the present study is that the microarrays do not encompass all proteins probably involved in hBMSCs migration, and additional experiments (e.g. introduction of purified cytokines and blocking antibodies) aimed at further proofing the hBMSCs-recruiting roles of screened cytokines are lacking. However, anyway, it is a comprehensive comparison for fundamental screening and further studies exploring the biological behaviors of 21 identified cytokines are ongoing. Moreover, it is difficult to conclude whether the up-secreted 21 cytokines in the CM from PF-stimulated donor hBMSCs would be pathophysiologically important *in vivo*, but if their secretion could be modulated spatially and timely in a precise pattern, the administration of them would contribute substantially to the mobilization and migration of endogenous hBMSCs. It is also worth noting that the present study is encouraging in providing candidate bioactive molecules for researches in the field of *in situ* tissue regeneration, a tissue engineering concept focusing on recruiting host mesenchymal stem cells to injury site to conduct tissue regeneration [44]. As certain human recombinant bioactive molecules (e.g. BMP-2 and BMP-7) have been successfully used in clinic [45], with great enthusiasm, we here propose more in-depth researches aiming to development novel tissue-engineering products based on the present study. Actually, we are currently attempting to investigate the cross-talks between 21 identified cytokines and the surrounding transplantation microenvironment. Meanwhile, the downstream signal events in endogenous hBMSCs following the combination of these cytokines and their receptors are being identified, aiming to ravel the functional mechanism of transplanted-donor hBMSCs *in vivo*.

In this study, we analyzed the effects of inflammatory microenvironment on the chemotactic ability and secretome of transplanted hBMSCs. We observed that inflammatory microenvironment stimulated cultured hBMSCs to secrete more cytokines related to cell migration, which preliminarily uncovered the veil over the functional mechanism of transplanted hBMSCs. Moreover, 21 chemoattractant molecules were determined to be potentially involved in this effect. These new findings provided a basis for future studies on the interactions between transplanted donor cells and host microenvironment in the application of MSCs-based strategies. Further understanding of the biological effects of transplanted MSCs and their cytokines associated with migration of host MSCs will help to define and manage the roles of transplanted MSCs.

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