HMGB1 promotes cellular chemokine synthesis and potentiates mesenchymal stromal cell migration via Rap1 activation

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Abstract. The migration of mesenchymal stem cells (MSCs) and osteogenic differentiation occupy an important role in fracture healing. High mobility group box 1 (HMGB1), a widely distributed inflammatory factor in fractures, has been confirmed to act as a chemoattractant to MSCs. To investigate the effect of HMGB1 on MSC migration and the underlying mechanism, the synthesis of MSC chemokines, and the consequent activation of signaling pathways following HMGB1 stimulation, were evaluated. A Quantibody® array was performed to determine which chemokines were secreted from MSCs with or without treatment with HMGB1. The results indicated differential chemokine synthesis by MSCs following treatment with HMGB1, including that of CCL4 and CCL13. In addition, the Ras-associated protein-1 (Rap1) signaling pathway was markedly activated in the HMGB1-treated groups, suggesting that HMGB1 may enhance the migrational ability of MSCs via Rap1 activation. Furthermore, HMGB1 was able to promote the secretion of various chemokines derived from MSCs, which would, in turn, increase the mobility of MSCs. Taken together, these results provide a mechanistic basis for developing novel approaches to promote fracture healing.

Introduction

A number of studies have revealed that the concentration of high mobility group box 1 protein (HMGB1), an inflammatory factor, is markedly increased in the bone fracture microenvironment (1-3). HMGB1 was also shown to promote the osteogenic differentiation of mesenchymal stem cells (MSCs) (2). Previous studies have demonstrated the directional migration of MSCs towards the bone fracture site, and have shown that the osteogenic differentiation ability of MSCs has an essential role in the wound-healing process (4-6). In addition, MSCs synthesize various cytokines, including stem cell factor, thrombopoietin and interleukin-6 (7,8), which exert an important influence on the behavior and activity of peripheral cells, and on the MSCs themselves (9,10).

Ras-associated protein-1 (Rap1), a member of the Ras superfamily of small GTPases, has the function of reversing the oncogenic potential of Ras, inducing a change in cell morphology activated by mutant K-ras, and transmitting oncogenic signals (11,12). Rap1, similarly to other GTPases, demonstrates binary switches by cycling between inactive GDP-bound and active GTP-bound conformations, and regulates multiple cellular signaling pathways after receiving a cellular stimulus (12-17). In addition, the migrational ability of MSCs was reported to increase via activation of the Rap1 signaling pathway (18). Furthermore, HMGB1 has been confirmed to activate the RAS/mitogen-activated protein kinase (MAPK) signaling pathway of MSCs (19). Given that Rap1 is a member of the Ras family, an hypothesis was developed for the present study that HMGB1 may also potentiate MSC migration via Rap1 activation, and a series of experiments have been performed to verify this hypothesis.

In the present study, a scratch assay has been used to determine the effect of HMGB1 on the mobility of MSCs. To further study the molecular mechanism of MSC activation via HMGB1, a Quantibody® array was applied to detect the cytokines synthesized by MSCs with and without treatment with HMGB1. The identification of differentiated cytokines under these two conditions was used to reveal the effect of HMGB1 on the MSCs. These results may provide a basis for developing novel approaches in bone-fracture-healing therapy.

Materials and methods

Reagents. Recombinant human HMGB1 protein (Sigma-Aldrich, St. Louis, MO, USA) was commercially purchased. A concentration of 25 ng/ml protein was used in the experiments detailed below. The Rap1 inhibitor (Cell Signaling Technology, Inc., Danvers, MA, USA), which is a transferase inhibitor, was used at a concentration of 5 μM. The reagents listed above were handled and used according to the manufacturer's protocols.

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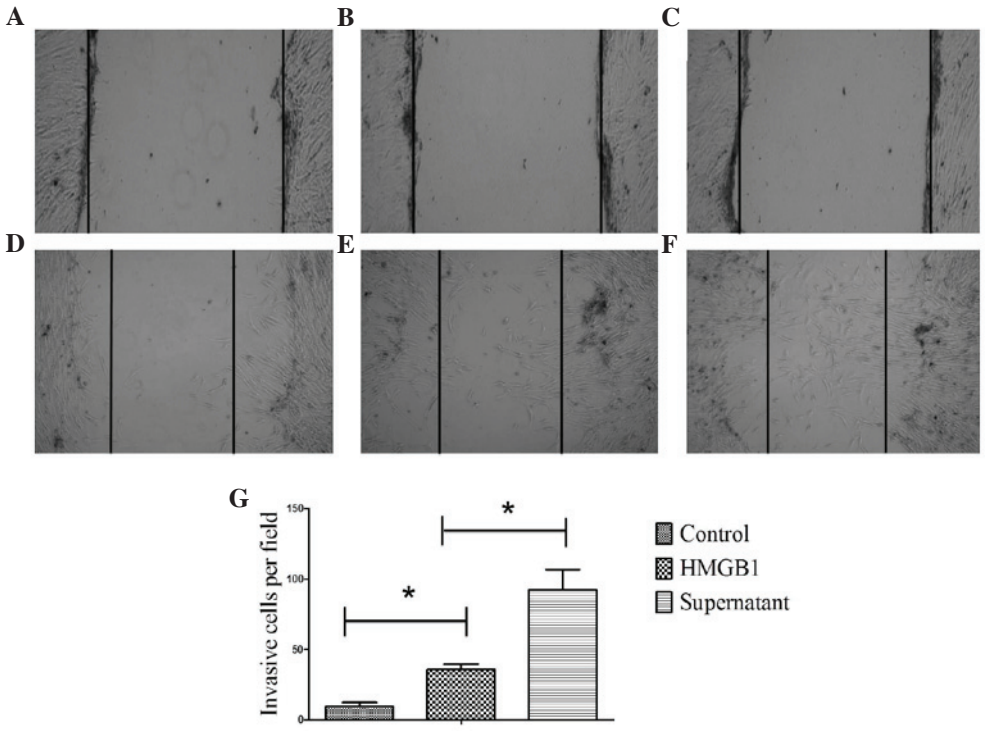


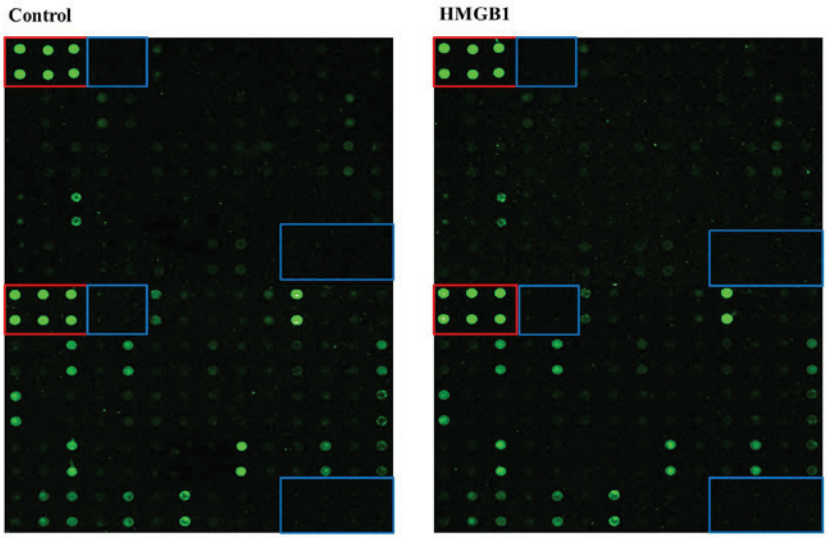
Figure 1. HMGB1 potentiates the migration of MSCs, as demonstrated by a scratch assay. The control (left column), HMGB1 (middle column) and supernatant (right column) groups are presented at (A-C) 0 h and (D-F) 48 h. The solid lines denote the baseline. (G) Migratory cells were counted in the HMGB1, supernatant and the control groups. Results were obtained from three independent experiments and are expressed as the mean ± standard deviation (n=3 in each experiment). *P<0.05. MSC, mesenchymal stem cell; HMGB1, high mobility group box 1 protein.

Isolation and culture expansion of human bone marrow MSCs. MSCs (Cyagen Biosciences Inc., Guangzhou, China) were commercially purchased. Adherent cells were trypsinized using 0.25% trypsin (Cyagen Biosciences Inc.) for ~30 sec and passaged after the cell confluence had reached ~80%, and the cells at passages 3 to 5 were used in the experiments detailed below. Typically, these cells exhibited the capacity of differentiation into osteoblasts, adipoblasts and chondrocytes under specific inductive conditions.

Transwell migration assay. Cell migration was performed with Transwell chambers (pore size, 8-μm diameter; Corning Costar, Inc., Corning, NY, USA). Complete™ medium (Cyagen Biosciences Inc.) containing 0.1% fetal calf serum (FCS; Cyagen Biosciences Inc.) was added into the wells of a 24-well plate, and subsequently, serum-starved MSCs ($1x10^5$) suspended in a volume of 100 μl Complete™ medium containing 0.1% FCS were added into the upper chamber. Prior to the addition of HMGB1, the transwell plate (with MSCs in the upper chamber and medium containing 0.1% FCS only in the lower chamber), was first incubated at 37˚C for 1 h. Following the addition of HMGB1, the plate was subsequently incubated at 37˚C for 3 h, followed by membrane fixation with 4% paraformaldehyde (Beyotime Institute of Biotechnology, Haimen, China) and staining with 0.1% crystal violet (Beyotime Institute of Biotechnology). The membrane was subsequently washed, and the cells on the underside of the membrane were observed under a light microscope (Leica DMI/LM; Leica Microsystems GmbH, Wetzlar, Germany). Numbers of cells were counted in five to ten random fields for each membrane.

Cell scratch assay. Cell migration was determined using a scratch assay. The cells were cultivated to 90% confluence on 12-well plates. The groups were as follows: Control, without HMGB1; treatment, MSCs cultured with 25 ng/ml HMGB1; supernatant, MSCs pretreated with 25 ng/ml HMGB1 and following 48 h, the supernatant of the MSCs was extracted to culture a fresh batch of MSCs. Subsequently, cell scrapers (Corning Costar Inc.) were used to scratch the confluent cells. The extent of cellular growth was observed at 0 and 48 h. All the experiments were repeated in triplicate.

Western blot analysis. Cells were harvested and lysed in radioimmunoprecipitation assay buffer containing proteinase inhibitors (Cyagen Biosciences Inc.). Following measurement of the protein concentration using a BCA kit (Beyotime Institute of Biotechnology), protein samples were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane by western blotting. The membranes were blocked in 5% skimmed milk for 1 h, and incubated with the following antibodies: Rabbit anti-human GTP-Rap1 (Active Rap1 Detection kit, cat. no. CST8818; dilution, 1:500) from Cell Signaling Technology, Inc., rabbit anti-human Rap1 (cat. no. ab47234; Abcam, Cambridge, MA, USA; dilution, 1:500) and rabbit anti-human β-actin (cat. no. sc-130301; Santa Cruz Biotechnology, Inc., Dallas,



A	B	C	D	E	F	G	H	I	J	K	L	M	N
POS1	POS2	POS3	NEG	NEG	ANG	BDNF	BLC	BMP-4	BMP-6	CKB 8-1	CNTF	EGT	CCL-11
POS1	POS2	POS3	NEG	NEG	ANG	BDNF	BLC	BMP-4	BMP-6	CKB 8-1	CNTF	EGT	CCL-11
CCL-24	CCL-26	FGF6	FGF7	FLT-3L	CX3CL1	GCP-2	GDNF	CSP2	I-309	IFN	IGBP-1	IGBP-2	IGBP-4
CCL-24	CCL-26	FGF6	FGF7	FLT-3L	CX3CL1	GCP-2	GDNF	CSP2	I-309	IFN	IGBP-1	IGBP-2	IGBP-4
IGF-1	IL-10	IL-13	IL-15	IL-16	IL-1a	IL-1b	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
IGF-1	IL-10	IL-13	IL-15	IL-16	IL-1a	IL-1b	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
Leptin	LIGHT	MCP1	MCP2	MCP3	MCP4	M-CSF	MDC	MG	MIP-1	MIP-3	NAP-2	NT-3	PARC
Leptin	LIGHT	MCP1	MCP2	MCP3	MCP4	M-CSF	MDC	MG	MIP-1	MIP-3	NAP-2	NT-3	PARC
PDGF-BB	RANTES	SCF	SDF-1	TARC	TGF-b1	TGF-b3	TNF-a	TNF-b	NEG	NEG	NEG	NEG	NEG
PDGF-BB	RANTES	SCF	SDF-1	TARC	TGF-b1	TGF-b3	TNF-a	TNF-b	NEG	NEG	NEG	NEG	NEG
POS1	POS2	POS3	NEG	NEG	Acrp30	AgRP	Angio-2	Amphire	Axi	bFGF	b-NGF	BTC	CCL-28
POS1	POS2	POS3	NEG	NEG	Acrp30	AgRP	Angio-2	Amphire	Axi	bFGF	b-NGF	BTC	CCL-28
CTACK	Dtk	EGF-R	ENA-78	Fas	FGF-4	FGF-9	GCSF	Ligand	GITR	GRO	GRO-a	HCC-4	HGF
CTACK	Dtk	EGF-R	ENA-78	Fas	FGF-4	FGF-9	GCSF	Ligand	GITR	GRO	GRO-a	HCC-4	HGF
ICAM-1	ICAM-3	IGFBP-3	IGFBP-6	IGF-ISR	IL-1 R4	IL-1 R	IL-11	IL-12 p40	IL-12 p70	IL-17	IL-2 R	IL-6 R	IL-8
ICAM-1	ICAM-3	IGFBP-3	IGFBP-6	IGF-ISR	IL-1 R4	IL-1 R	IL-11	IL-12 p40	IL-12 p70	IL-17	IL-2 R	IL-6 R	IL-8
I-TAC	Lymph	MIF	MIP-1a	MIP-1b	MIP-3b	MSP-a	NT-4	Osteo	Oncostain	PIGF	sgp 130	sTNF R2	sTNF R1
I-TAC	Lymph	MIF	MIP-1a	MIP-1b	MIP-3b	MSP-a	NT-4	Osteo	Oncostain	PIGF	sgp 130	sTNF R2	sTNF R1
TECK	TIMP-1	TIMP-2	Thrombo	TRAILR3	TRAILR4	uPAR	VEGF	VEGF-D	NEG	NEG	NEG	NEG	NEG
TECK	TIMP-1	TIMP-2	Thrombo	TRAILR3	TRAILR4	uPAR	VEGF	VEGF-D	NEG	NEG	NEG	NEG	NEG

Figure 2. Cytokine synthesis induced by HMGB1. The synthesis of cytokines in cell was measured using a Quantibody® array. The red square indicates the negative controls, whereas the green squares mark the positive controls. The spots were measured densitometrically. After subtracting the negative control and normalization to the positive control, the spots of stimulated MSCs were compared with the corresponding spots of the unstimulated MSCs. MSC, mesenchymal stem cell; HMGB1, high mobility group box 1 protein.

TX, USA), separately at 4˚C overnight. After an incubation with goat anti-rabbit peroxidase-linked secondary antibody (cat. no. 31210; Thermo Fisher Scientific, Inc., dilution, 1:5,000) at 25˚C for 3 h, immunoreactive proteins were visualized using an enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific, Inc.). Relative quantification of bands in the western blot was performed using ImageJ software [National Institutes of Health (NIH), Bethesda, MA, USA).

Antibody arrays. Soluble proteins in the medium of the stromal cell lines were measured using the Human Cytokine Array G1000 (AAH-CYT-G1000; RayBiotech, Inc., Norcross, GA, USA), according to the manufacturer's protocol. These arrays are able to detect up to 120 proteins. Stromal cells were plated 3 days prior to the experiment in Dulbecco's modified Eagle's medium (DMEM; Cyagen Biosciences Inc.) containing 10% FBS, and were 75-90% confluent when the cell lysis solution was collected and filtered. Medium containing 10% FBS was also hybridized to the arrays, and used subsequently for normalization. Ten technical and biological replicates were performed, and these demonstrated a very high correlation (correlation coefficient >0.9; data not shown). Hybridization was performed overnight at 4˚C. All slides were scanned using a GenePix® 4000B Microarray Scanner (Axon Instruments, Inc., Union City, CA, USA) and analysed using the software GenePix® Pro 6.0. The F532 median - 2B532 score was used, and averaged across triplicates on each array. The results were subsequently normalized using internal controls, and the values for cytokines in clear medium containing 10% FBS were subtracted.

Statistical analysis. Statistical significance was performed using the two-tailed Student's t-test, assuming equal variances. The chi-squared test was used to compare rates. $P<0.05$, $P<0.01$ and $P<0.001$ were taken to indicate statistically significant values.

Results

Effects of HMGB1 on the increasing migrational ability of MSCs. To investigate the effect of HMGB1 on the migration of MSCs, a scratch assay was performed (Fig. 1). The MSCs of all groups moved towards the blank area to a certain extent after 48 h, indicating a certain level of migrational ability. Compared with the control group, MSCs cultured with 25 ng/ml HMGB1 significantly surpassed the baseline ($P<0.05$), indicating that the migrational ability of MSCs was increased on stimulation with HMGB1 (Fig. 1B and C). In another experiment, MSCs were pretreated with 25 ng/ml HMGB1. After 48 h, the supernatant of the MSCs was extracted to culture a new batch of MSCs (Fig. 1D), and a scratch assay was performed. A greater number of the MSCs from the second treatment crossed the baseline compared with that observed in the group of MSCs directly cultured with HMGB1 ($P<0.05$). This confirmed that HMGB1 could significantly increase the migrational ability of MSCs (Fig. 1E).

Effects of HMGB1 on cellular chemokine synthesis by MSCs. To determine the influence of HMGB1 stimulation on human MSCs at the protein level, a Quantibody® array was performed. The treatment group was stimulated with 25 ng/ml HMGB1 and compared with non-stimulated cultures (Fig. 2).

Underlying our search criteria of a differential synthesis (>2.0-fold change or <0.5-fold change), the bioinformatics data analysis identified that seven cytokines were differentially synthesized between the two groups. Increased cytokine synthesis (relative level, >1.5-fold change) of bone morphogenetic protein-4 (BMP-4), neurotrophin-3 (NT-3), LIGHT [or tumor necrosis factor superfamily member 14 (TNFSF14)], monocyte chemoattractant protein 4 (MCP-4), Dtk and macrophage inflammatory protein-1β (MIP-1β) following induction with HMGB1 was measured (Fig. 3). A reduced level of cytokine synthesis (relative level, <0.5-fold change) was observed for β-nerve growth factor (β-NGF; Fig. 3A and B).

Hierarchical clustering of those seven cytokines with normalized cytokine synthesis values disclosed two distinct groups: The HMGB1-treated group and the non-stimulated control group. Six differentially secreted cytokines were visualized as being induced, and one cytokine as being repressed. Notably, among the seven cytokines, three of them were identified as being involved in the nuclear factor-κB (NF-κB) signaling pathway, namely, MCP-4, MIP-1β and LIGHT. Subsequently, two cytokines were identified as being involved in the neurotrophic factor-mediated Trk receptor signaling pathway: NT-3 and β-NGF. Seven differentially secreted cytokines were also revealed to be associated with the response to an external stimulus (7), cell migration (6), localization of the cell (6), regulation of programmed cell death (5) and the immune system process (5), according to Gene Ontology using the Database for Annotation, Visualization and Integrated Discovery (DAVID). As expected, numerous differentially synthesized cytokines were associated with more than one biological process.

Underlying our search criteria of a differential secretion, bioinformatics data analysis resulted in the selection of three cytokines (relative level, >2.5-fold control). These were visualized as being induced between the two groups. Increased levels of cytokine secretion were measured for chemokine ligand 4 (CCL4), CCL13 and TNFSF14 following induction with HMGB1. The detected differentially secreted cytokines serve roles in other molecular and cellular functions, including cellular growth and proliferation, cell death, cell morphology and cellular development. On the basis of the three differentially secreted cytokines, the cytokine-cytokine receptor

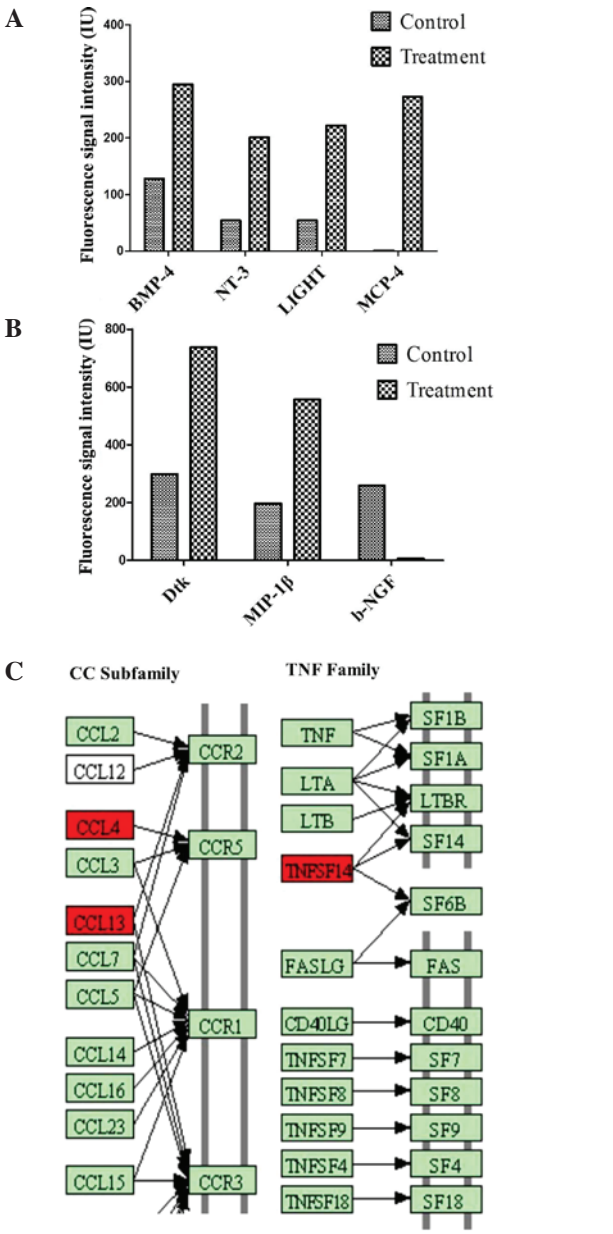


Figure 3. (A and B) Seven cytokines were differentially synthesized (>2.0-fold change or <0.5-fold change), based on the results of Quantibody® array. (C) Cytokine-cytokine receptor interaction between the synthesized cytokines. CCL4, CCL13 and TNFSF14 of the differentially secreted cytokines (>2.5-fold induction), involved in cellular movement, cellular development and cell death, are presented in their cytokine-cytokine receptor interaction pathway (CCR2, CCR5, CCR3, LTB R, SF14 and SF6B) according to the cytokine-cytokine receptor interaction pathway (0460hsa) in the Kyoto Encyclopedia of Genes and Genomes database (red indicates 'increased'). BMP-4, bone morphogenetic protein-4; NT-3, neurotrophin-3; MCP-4, monocyte chemoattractant protein 4; MIP-1β, macrophage inflammatory protein-1β; CCLx, chemokine ligand x; CCRx, chemokine receptor x; TNFSF14, tumor necrosis factor superfamily member 14; LTA/B(R), lymphotoxin-α/β (receptor).

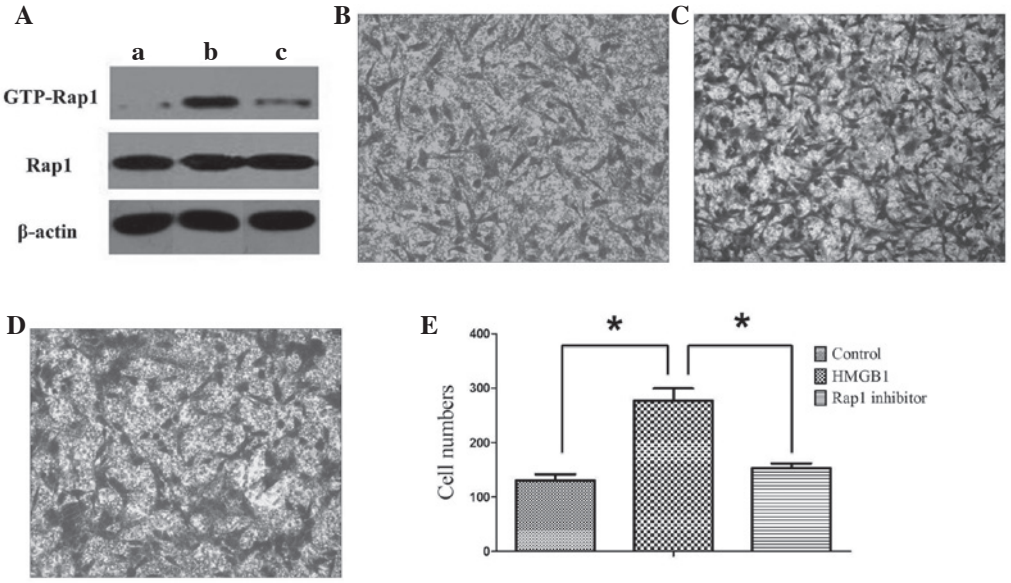


Figure 4. Rap1 activation is required for MSC migration. (A) Western blot analysis of the Rap1 signaling activity upon treatment with HMGB1 (25 ng/ml) and/or Rap1 inhibitor (note, β-actin was run during the same experiment, but on different parts of the gel). MSCs were maintained (A,a) in the basal medium alone, (A,b) with 25 ng/ml HMGB1 or (A,c) with 25 ng/ml HMGB1 and Rap1 inhibitor. (B-E) A transwell migration assay was performed. MSCs were maintained (B) in the basal medium alone, (C) with 25 ng/ml HMGB1 or (D) with 25 ng/ml HMGB1 and Rap1 inhibitor. (E) The migratory cells were counted in the HMGB1, inhibitor or control groups, respectively. Results were obtained from three independent experiments and the data are expressed as the mean ± standard deviation (n=3 in each experiment). *P<0.05. HMGB1, high mobility group box 1 protein.

interaction pathway (0460hsa in the Kyoto Encyclopedia of Genes and Genomes database) was the dominant pathway that was influenced by HMGB1. Thus, the secretion of CCL5 and CCL26 was induced (Fig. 3C).

HMGB1 induces MSCs to activate the Rap1 signaling pathway and enhance MSC migration. The present study identified that six cytokines, of the differentially synthesized cytokines, are involved in cell migration based on the results of the Quantibody® array. Subsequently, among the six cytokines, β-NGF was identified as being involved in the Rap1 signaling pathway. Therefore, the Rap1 signaling pathway was proposed to be significant to the current study. However, a limitation of the study is that the array was not repeated three times. Thus, to verify the hypothesis that the Rap1 signaling pathway of MSCs is also activated by HMGB1 stimulation, western blot analysis was performed. Compared with the expression of GTP-Rap1 in the control group, that in MSCs increased significantly following treatment with 25 ng/ml HMGB1. By contrast, the expression of GTP-Rap1 declined significantly following treatment with the Rap1 inhibitor. These results indicated that HMGB1 was able to activate the Rap1 signaling pathway in MSCs. However, the Rap1 inhibitor demonstrated marked inhibition of the Rap1 signaling pathway activated by HMGB1 (Fig. 4A).

To investigate whether activation of the Rap1 signaling pathway could increase the mobility of MSCs, a transwell migration assay was performed. The number of MSCs migrating towards the other side of the membrane increased markedly following treatment with HMGB1. However, following treatment with the Rap1 inhibitor, the number of migrating cells was markedly reduced compared with that in the group that was not treated with the inhibitor. This result suggested that activation and inhibition of the Rap1 signaling pathway significantly enhanced and suppressed the migration of MSCs, respectively (Fig. 4B-E).

Discussion

In the first scratch assay, it was observed that the mobility of MSCs treated with an appropriate concentration of HMGB1 was greater compared with that of the control group, suggesting that HMGB1 enhanced the migration of MSCs. In another experiment, the supernatant of MSCs induced with HMGB1 was used to culture a separate batch of MSCs. The migration of MSCs treated with the supernatant was enhanced compared with that of the MSCs of the control group and the group directly treated with an identical concentration of HMGB1. Despite the crude experimental design, these results suggested that certain cytokines in the supernatant were also able to enhance the migration of MSCs. Therefore, it was hypothesized that, following HMGB1 stimulation, MSCs synthesize certain cytokines to enhance their mobility further. However, this hypothesis requires further verification, since the present experiment did not control for all the potentially confounding variables.

To confirm whether the synthesis of specific cytokines by MSCs was enhanced following HMGB1 stimulation, and to identify the cytokines responsible for enhancing the mobility of MSCs, a Quantibody® array was performed, which revealed that the synthesis of CCL4, CCL13 and TNFSF14 increased markedly. TNFSF belongs to the family of type II transmembrane proteins, which contains 19 members, with approximately 150 homologous amino acids in the extracellular C-terminal domain. TNFSF is formed by ten β-strands, folded into a helical conformation, which forms a binding site for its corresponding receptors. The majority of the members of this family form trimers with their corresponding

receptors, and manifest their biological activities in the trimeric form (20). Following the binding of TNFSFs with their ligands, certain members activate the MAPK signaling pathway to stimulate cellular activities (21,22), whereas others recruit the death-induced signaling complex. These molecules subsequently activate caspases, and thereby induce cell apoptosis (23,24). The CCL subfamily members contains over 20 members, with two neighboring cysteine residues in the N-terminal domain. These predominantly activate monocytes and certain of the T cell subfamilies (25). CCL4, also termed MIP-1β, activates natural killer cells and multiple immune cells (26,27). CCL13, also termed MCP-4, is encoded by a gene located in human chromosome 17 within a large cluster of other CC chemokines. After binding with its receptors, it is involved in the body's allergic reaction (28-31). Most importantly, CCL4 and CCL13 have been revealed to enhance the migration of MSCs (32,33). Therefore, it was hypothesized that HMGB1 may enhance MSC migration by promoting the synthesis of CCL4 and CCL13, which would increase the mobility of MSCs.

To confirm our hypothesis about the Rap1 signaling pathway, the expression of relevant proteins of MSCs that are induced by HMGB1 was investigated using western blot analysis. The results indicated that the expression of GTP-Rap1 in MSCs increased markedly following HMGB1 stimulation. Since the level of GTP-bound Rap1 represents the activation of Rap1, it was deduced that HMGB1 activated the Rap1 signaling pathway of MSCs. Furthermore, the Rap1 inhibitor was used to block the Rap1 signaling pathway in MSCs. By means of the transwell migration assay, the mobility of MSCs was found to decrease substantially following treatment with the Rap1 inhibitor. This result suggested that HMGB1 activates the Rap1 signaling pathway, and enhances the migration of MSCs.

In the present study, a preliminary analysis of the Quantibody® array results was performed, and these results require further verification. Nevertheless, several of the results are intriguing, and merit further study. For example, several differentially synthesized cytokines were identified that are involved in two signaling pathways: The NF-κB signaling pathway and the neurotrophin signaling pathway. However, whether these two signaling pathways are activated by HMGB1 stimulation requires verification. Such a confirmation would help to elucidate the mechanism underlying the effect of HMGB1 on MSCs.

In conclusion, activation of the Rap1 signaling pathway has been shown to enhance the migration of MSCs. However, downstream signaling following activation of the Rap1 signaling pathway requires further investigation. It has been reported that Rap1 can activate β1 and β2 integrins in T cells, which could improve cell mobility (18,34). However, whether Rap1 in MSCs may also improve the synthesis of integrins, and whether the integrins have a direct effect on the migration of MSCs, remains to be elucidated. These questions will form the basis of our next research endeavours.

Acknowledgements

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References

1. Naglova H and Bucova M: HMGB1 and its physiological and pathological roles. Bratisl Lek Listy 113: 163-171, 2012.
2. Meng E, Guo Z, Wang H, Jin J, Wang J, Wang H, Wu C and Wang L: High mobility group box 1 protein inhibits the proliferation of human mesenchymal stem cells and promotes their migration and differentiation along osteoblastic pathway. Stem Cells Dev 17: 805-813, 2008.
3. Charoonpatrapong K, Shah R, Robling AG, Alvarez M, Clapp DW, Chen S, Kopp RP, Pavalko FM, Yu J and Bidwell JP: HMGB1 expression and release by bone cells. J Cell Physiol 207: 480-490, 2006.
4. Granero-Moltó F, Weis JA, Miga MI, Landis B, Myers TJ, O'Rear L, Longobardi L, Jansen ED, Mortlock DP and Spagnoli A: Regenerative effects of transplanted mesenchymal stem cells in fracture healing. Stem Cells 27: 1887-1898, 2009.
5. Glass GE, Chan JK, Freidin A, Feldmann M, Horwood NJ and Nanchahal J: TNF-alpha promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells. Proc Natl Acad Sci USA 108: 1585-1590, 2011.
6. Marsell R and Einhorn TA: The biology of fracture healing. Injury 42: 551-555, 2011.
7. Sidney LE, Kirkham GR and Buttery LD: Comparison of osteogenic differentiation of embryonic stem cells and primary osteoblasts revealed by responses to IL-1β, TNF-α and IFN-γ. Stem Cells Dev 23: 605-617, 2014.
8. Tso GH, Law HK, Tu W, Chan GC and Lau YL: Phagocytosis of apoptotic cells modulates mesenchymal stem cells osteogenic differentiation to enhance IL-17 and RANKL expression on CD4+ T cells. Stem Cells 28: 939-954, 2010.
9. Guo J, Jie W, Shen Z, Li M, Lan Y, Kong Y, Guo S, Li T and Zheng S: SCF increases cardiac stem cell migration through PI3K/AKT and MMP-2/-9 signaling. Int J Mol Med 34: 112-118, 2014.
10. Mingari MC, Moretta A and Moretta L: Regulation of KIR expression in human T cells: A safety mechanism that may impair protective T-cell responses. Immunol Today 19: 153-157, 1998.
11. Raaijmakers JH and Bos JL: Specificity in Ras and Rap signaling. J Biol Chem 284: 10995-10999, 2009.
12. Katagiri K, Maeda A, Shimonaka M and Kinashi T: RAPL, a Rap1-binding molecule that mediates Rap1-induced adhesion through spatial regulation of LFA-1. Nat Immunol 4: 741-748, 2003.
13. Rebstein PJ, Cardelli J, Weeks G and Spiegelman GB: Mutational analysis of the role of Rap1 in regulating cytoskeletal function in *Dictyostelium*. Exp Cell Res 231: 276-283, 1997.
14. Bos JL, Franke B, M'Rabet L, Reedquist K and Zwartkruis F: In search of a function for the Ras-like GTPase Rap1. FEBS Lett 410: 59-62, 1997.
15. McLeod SJ and Gold MR: Activation and function of the Rap1 GTPase in B lymphocytes. Int Rev Immunol 20: 763-789, 2001.
16. Katagiri K, Ohnishi N, Kabashima K, Iyoda T, Takeda N, Shinkai Y, Inaba K and Kinashi T: Crucial functions of the Rap1 effector molecule RAPL in lymphocyte and dendritic cell trafficking. Nat Immunol 5: 1045-1051, 2004.
17. Creasy CL and Chernoff J: Cloning and characterization of a member of the MST subfamily of Ste20-like kinases. Gene 167: 303-306, 1995.
18. Chen CP, Huang JP, Chu TY, Aplin JD, Chen CY and Wu YH: Human placental multipotent mesenchymal stromal cells modulate trophoblast migration via Rap1 activation. Placenta 34: 913-923, 2013.
19. Peng S, Zhou G, Luk KD, Cheung KM, Li Z, Lam WM, Zhou Z and Lu WW: Strontium promotes osteogenic differentiation of mesenchymal stem cells through the Ras/MAPK signaling pathway. Cell Physiol Biochem 23: 165-174, 2009.
20. Mauri DN, Ebner R, Montgomery RI, Kochel KD, Cheung TC, Yu GL, Ruben S, Murphy M, Eisenberg RJ, Cohen GH, *et al*: LIGHT, a new member of the TNF superfamily and lymphotoxin alpha are ligands for herpesvirus entry mediator. Immunity 8: 21-30, 1998.
21. Hsu TL, Chang YC, Chen SJ, Liu YJ, Chiu AW, Chio CC, Chen L and Hsieh SL: Modulation of dendritic cell differentiation and maturation by decoy receptor 3. J Immunol 168: 4846-4853, 2002.
22. Sugiyama T, Suzuki H and Takahashi T: Light-induced rapid Ca^{2+} response and MAPK phosphorylation in the cells heterologously expressing human OPN5. Sci Rep 4: 5352, 2014.

23. Yu KY, Kwon B, Ni J, Zhai Y, Ebner R and Kwon BS: A newly identified member of tumor necrosis factor receptor superfamily (TR6) suppresses LIGHT-mediated apoptosis. J Biol Chem 274: 13733-13736, 1999.
24. Kuai J, Nickbarg E, Wooters J, Qiu Y, Wang J and Lin LL: Endogenous association of TRAF2, TRAF3, cIAP1 and Smac with lymphotoxin beta receptor reveals a novel mechanism of apoptosis. J Biol Chem 278: 14363-14369, 2003.
25. Irving SG, Zipfel PF, Balke J, McBride OW, Morton CC, Burd PR, Siebenlist U and Kelly K: Two inflammatory mediator cytokine genes are closely linked and variably amplified on chromosome 17q. Nucleic Acids Res 18: 3261-3270, 1990.
26. Bystry RS, Aluvihare V, Welch KA, Kallikourdis M and Betz AG: B cells and professional APCs recruit regulatory T cells via CCL4. Nat Immunol 2: 1126-1132, 2001.
27. Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC and Lusso P: Identification of RANTES, MIP-1 alpha and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. Science 270: 1811-1815, 1995.
28. Garcia-Zepeda EA, Combadiere C, Rothenberg ME, Sarafi MN, Lavigne F, Hamid Q, Murphy PM and Luster AD: Human monocyte chemoattractant protein (MCP)-4 is a novel CC chemokine with activities on monocytes, eosinophils and basophils induced in allergic and nonallergic inflammation that signals through the CC chemokine receptors (CCR)-2 and -3. J Immunol 157: 5613-5626, 1996.
29. Naruse K, Ueno M, Satoh T, Nomiyama H, Tei H, Takeda M, Ledbetter DH, Coillie EV, Opdenakker G, Gunge N, *et al*: A YAC contig of the human CC chemokine genes clustered on chromosome 17q11.2. Genomics 34: 236-240, 1996.
30. Blanpain C, Migeotte I, Lee B, Vakili J, Doranz BJ, Govaerts C, Vassart G, Doms RW and Parmentier M: CCR5 binds multiple CC-chemokines: MCP-3 acts as a natural antagonist. Blood 94: 1899-1905, 1999.
31. Lamkhioued B, Garcia-Zepeda EA, Abi-Younes S, Nakamura H, Jedrzkiewicz S, Wagner L, Renzi PM, Allakhverdi Z, Lilly C, Hamid Q and Luster AD: Monocyte chemoattractant protein (MCP)-4 expression in the airways of patients with asthma. Induction in epithelial cells and mononuclear cells by proinflammatory cytokines. Am J Respir Crit Care Med 162: 723-732, 2000.
32. Lejmi E, Perriraz N, Clément S, Morel P, Baertschiger R, Christofilopoulos P, Meier R, Bosco D, Bühler LH and Gonelle-Gispert C: Inflammatory chemokines MIP-1δ and MIP-3α are involved in the migration of multipotent mesenchymal stromal cells induced by hepatoma cells. Stem Cells Dev 24: 1223-1235, 2015.
33. Zhang F, Wang C, Wang H, Lu M, Li Y, Feng H, Lin J, Yuan Z and Wang X: Ox-LDL promotes migration and adhesion of bone marrow-derived mesenchymal stem cells via regulation of MCP-1 expression. Mediators Inflamm 2013: 691023, 2013.
34. Burbach BJ, Medeiros RB, Mueller KL and Shimizu Y: T-cell receptor signaling to integrins. Immunol Rev 218: 65-81, 2007.