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Treatment Effects of rhBMP-2 on Invasiveness of Oral Carcinoma Cell Lines

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Objective: To determine if recombinant human bone morphogenetic protein-2 (rhBMP-2) has biological effects on the invasiveness of human oral squamous cell carcinoma (OSCCA) cell lines.

Study Design: Laboratory investigation using six human OSCCA cell lines, with three cell lines having baseline gene expression of BMP-2 and three cell lines without baseline gene expression of BMP-2.

Methods: The invasiveness of each cell line was measured using a matrigel invasion assay with or without stimulation by rhBMP-2. A tumor metastasis quantitative PCR array was used to establish whether observed findings from the invasion assay correlated to changes in gene expression.

Results: There was a significant increase in tumor cell invasion in response to rhBMP-2 in all BMP-2 positive cell lines but no change in the cell lines that did not express the BMP-2 gene. Quantitative PCR revealed that changes in gene expression were distinctly different based on the baseline gene expression of BMP-2 and favored a more metastatic genotype in the BMP-2-positive cells.

Conclusions: Recombinant human BMP-2 has an adverse biological effect on invasiveness of human OSCCA cell lines in vitro. This adverse effect is dependent on the baseline gene expression of BMP-2. Changes in expression of genes involved with tumor metastasis correlated to the invasion assay findings. These data raise concern for the safe application of rhBMP-2 for reconstruction of bone defects in oral cancer patients.

Key Words: Bone morphogenetic protein, oral cancer, squamous cell carcinoma.

Level of Evidence: 5 (bench research).

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INTRODUCTION

Human recombinant bone morphogenetic protein-2 (rhBMP-2) is a FDA-approved device for maxillary sinus floor and alveolar ridge augmentation. Based on this approval for use in orodental defects in addition to abundant preclinical data, rhBMP-2 has now been reported for use in segmental bone defects throughout the head and neck region.[1,2] As knowledge, technology, and experience advance in this area, there will be an inevitable paradigm shift in reconstructive techniques from current therapies using autologous bone grafts toward tissue engineering using BMPs.[3]

There are several limitations with the use of rhBMP-2 in craniofacial defects. One of the contraindications includes use in the vicinity of a resected tumor or in patients undergoing treatment for malignancy. Such use is currently contraindicated due to the unknown effects of this growth factor on malignancies. This is a significant consideration because the majority of segmental bone defects in the head and neck region are caused by resection of oral squamous cell carcinomas (OSCCA). Our previous data showed that rhBMP-2 did not have any adverse effects on proliferation or angiogenesis in OSCCA in vitro.[4] However, it has been reported that increased BMP-2 gene expression is associated with regional metastasis from OSCCA.[5] This could be related to BMP effects on invasiveness or migration ability of OSCCA cells, as has been shown for prostate, colon, and lung carcinomas.[6–8]

The primary objective of this study was to determine if rhBMP-2 has biological effects on the invasiveness of OSCCA. Secondary objectives were to determine if any observed effects correlate with baseline gene expression of BMP-2, and to establish if rhBMP-2 treatment changes expression profiles for genes involved with tumor metastasis.

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MATERIALS AND METHODS

Cell Culture and Media

Human oral squamous carcinoma cell lines UPCI:SCC032 (retromolar trigone), UPCI:SCC056 (oral tongue), and UPCI: SCC182 (retromolar trigone) were provided by Dr. Susanne M. Gollin (University of Pittsburgh, PA). Cells were maintained in MEM medium (GIBCO-Invitrogene, Carlsbad, CA) supplemented with Earle's salt, L-glutamine, 1× NEAA, 10% fetal bovine serum (FBS) and gentamicin. Human oral squamous carcinoma cell lines UMSCC-1 (floor of mouth), UMSCC-14A (floor of mouth), and UMSCC-74A (oral tongue) were provided by from Dr. Thomas E. Carey (University of Michigan at Ann Arbor, MI). The cells were grown in DMEM (Mediatech Inc., Manasses, VA) supplemented with 4.5 g/L glucose, L-glutamine, 10% FBS, 1× NEAA, and penicillin–streptomycin. Cultures were maintained at 37°C in an atmosphere of 5% CO_2.

BMP-2 Gene Expression Analysis

Total RNA was isolated by TRIzol Reagent (Invitrogene, Carlsbad, CA) according to manufacture's protocol. Reverse transcription to produce first-strand cDNA was performed with 2 μg of total RNA by High-Capacity cDNA Reverse Transcription kit with RNase inhibitor (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's protocols. The BMP-2 transcript was amplified from the cDNA by conventional PCR using gene-specific primers: forward primer (5′-CGAGGTCCTGAGCGAGTT CGAG-3′) and the reverse primer (5′-TGGCAGTAAAAGGCGTGA TACC-3′); amplicon = 837 bp; 35 cycles; cycle profile: 1 minute at 94°C, 1 minute at 60°C, 1 minute at 72°C.[9] The following GAPDH gene specific primers were used: forward primer (5′-ACCACAG TCCATGCCATCAC-3′) and the reverse primer (5′-TCCACCAC CCTGTTGCTGTA-3′); amplicon = 451 bp; 24 cycles; cycle profile: 30 seconds at 94°C, 30 seconds at 55°C, 30 seconds at 72°C.[10] Human normal oral mucosa was used as negative control, and MG-63, an osteosarcoma-derived osteoblastic cell line was used as positive control.[11,12] Each sample was tested in duplicate in three independent experimental runs.

Invasion Assay

Invasion assays were performed in BD BioCoat FluoroBlok Tumor Invasion System (BD Biosciences, Bedford, MA) according to the manufacturer's manual. This system consists of the 24-multiwell insert plate with an 8 micron pore size PET membrane that has been uniformly coated with BD Matrigel Matrix. Tumor cells were grown on 10-cm plates at the confluency of 70–90% and stained overnight with 7.5 μg/mL of BD $DiIC_{12}$ Fluorescent Dye in OPTIMEM medium (GIBCO-Invitrogene). On the following day, prelabeled cells were seeded to the top chamber at a density of 1.65–5.0 × 10^5 cells per well. OPTIMEM was used as a conditional medium. In the experimental wells rhBMP-2 (Cat: B3555. Sigma-Aldrich, St. Louis, MO) was added to the top and bottom chamber to the final concentration of 100 ng/mL. The 5% FBS in basal medium was used as a chemoattractant. The real-time fluorescence signals were detected by Synergy HT Multi-Detection Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT). Plate Reader was custom set up according to BD Biosciences technical bulletin #436 for a nonstandard 24-well plate dimension. Optic were set at excitation: 530/25, emission: 590/30 at the bottom reading position. Quantitative readings were done at 12 hours, 15 hours, 17 hours, and 19 hours after stimulation. Data were normalized on Blank-a chamber without cells, and Background- chambers with a cells and medium only. All experiments were done in triplicate.

An inverted fluorescent microscope/camera Nikon Eclipse TE2000-U was used to observe the cells on the membrane, and capture fluorescent images. AnalySIS software was utilized for handling and analysis of digital-microscopy images.

Quantitative PCR Array

Tumor cell lines were treated with 100 ng/mL of rhBMP-2 for 48 hours in OPTIMEM medium, control cells without rhBMP-2 were incubated for the same period of time. Total RNA was isolated by RNeasy Mini Kit (Qiagen, Valencia, CA) with addition of DNase I treatment step. Reverse transcription of 2.5 μg of total RNA was done by High-Capacity cDNA Reverse Transcription kit with RNase inhibitor (Applied Biosystems). All real-time PCR reactions were performed using an ABI Gene Amp 5700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA). Instrument-specific RT^2 Real-Time SYBR Green/Rox PCR master mix and custom 96-well Human RT^2 Profiler PCR Array Human Tumor Metastasis plates containing predispensed gene-specific primer sets were designed by SABiosciences (Frederick, MD). This PCR Array is specifically designed with 84 genes known to be involved in tumor metastasis. Two representative cell lines were tested; UMSCC-1 (BMP-2 negative gene expression), and UMSCC-74A (BMP-2 positive gene expression). A singleplex reaction mix was prepared according to the manufacturer's protocol. The thermal cycling conditions included an initial denaturation step at 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. To compare gene expressions between samples, the threshold cycle (C_T) was normalized using the mean C_T for reference genes. The RT^2 Profiler PCR Array Data Analysis software from SABiosciences (http://sabiosciences.com/pcr/arrayanalysis.php) based on the $2^{-\Delta\Delta CT}$ method was used to compare treatment and control and to determine fold changes in gene expression levels and statistical significance of these changes. Mean values from the three independent experiments were used in calculations. Each 96-well plate included five housekeeping gene panel (B2M, HPRT1, RPL13A, GAPDH, and β-Actin) to normalize PCR Array data and several controls: Genomic DNA Control (specifically detects nontranscribed genomic DNA contamination), Reverse Transcription Control (tests the efficiency of first strand kit), and Positive PCR Control (tests the efficiency of the polymerase chain reaction itself).

Statistical Analysis

Fluorescent readings were $\log_{10}$ transformed as typically done to minimize the scale effect and to improve fit of the models. Statistical analysis was performed using the PROC GLIMMIX procedure in SAS 9.1 (SAS Institute, Cary, NC). To evaluate differences in invasive activity, the following mixed models were used for each cell line: $Log_{10}(Fluorescence) = Treatment + Time + Treatment*Time + e$, where $Treatment \sim N(0, \sigma^2_{Treatment})$ is the effect of the BMP-2 treatment, $Time \sim N(0, \sigma^2_{Time})$ is the time effect, $Treatment*Time \sim N(0, \sigma^2_{T*T})$ is the effect of the treatment by time interaction, and $e \sim N(0, \sigma^2_R)$ is the *iid* random error. The covariance structure was modeled with the sp(pow) option that uses the anisotropic power law appropriate for correlations declining as a function of time and nonequal spacing between time points. The *P*-values at or below the .05 level were considered significant.

RESULTS

BMP-2 Gene Expression in Human OSCCA Cell Lines

RT-PCR was used to determine baseline BMP-2 gene expression in six oral carcinoma cell lines. Three cell lines were positive (UPCI:SCC 056, UMSCC-14A, UMSCC-74A) and the other three were negative (UMSCC-1, UPCI:SCC

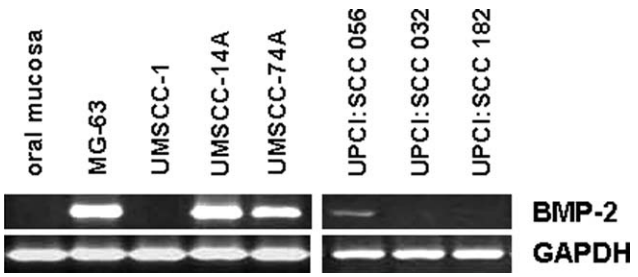

Fig. 1. BMP-2 gene expression in cell lines. RT-PCR experiments shown for normal human oral mucosa (negative control), MG-63 (positive control), and six oral squamous carcinoma cell lines.

032, UPCI:SCC 182) for BMP-2 expression (Fig. 1). All six cell lines expressed the genes for all BMP-receptors (data unpublished). These included BMPR-IA, BMPR-IB, BMPR-II, ActR-I, ActR-IIA, and ActR-IIB.

Effect of rhBMP-2 on Invasiveness of Human OSCCA Cell Lines

In response to treatment with rhBMP-2, there was a significant increase in tumor cell invasion through the Matrigel matrix in all BMP-2 positive cell lines. In contrast, no significant differences in invasiveness were observed in the cell lines that did not express BMP-2 (Table I). Comparisons between tumor cells with and without rhBMP-2 treatment were performed under stimulation with 5% FBS, which served as a chemoattractant. Dynamic changes of estimated means in treated and nontreated cells are presented in the $\log_{10}$-scale graphs for UMSCC-74A (Fig. 2B) and UPCI:SCC 182 (Fig. 2D) cell lines. To determine the extent to which rhBMP-2 alone could promote migration of tumor cells through a collagen matrix, rhBMP-2 was added to the bottom chamber without FBS. Only one cell line, UMSCC-74A, demonstrated increased invasive potential in the presence of BMP-2 and without chemoattractant (Fig. 2A).

Gene Expression Changes in Response to rhBMP-2

The Human Tumor Metastasis PCR Array was utilized for quantitative real-time PCR. Results for UMSCC-74A (positive BMP-2 gene expression) and UMSCC-1 (negative BMP-2 gene expression) are summarized in Table II. Changes in gene expression favored a more metastatic genotype in the BMP-2 positive cell line as opposed to the BMP-2 negative cell line. In particular, changes in IL1β, MTSS1, and TIMP-2 were positively correlated with the invasion assay findings of increased invasiveness for UMSCC-74A. MTSS1 and KISS1 are metastasis suppressor genes that were both upregulated in UMSCC-1.

DISCUSSION

Tissue invasion is one of the six acquired capabilities of cancer cells as described by Hanahan and Weinberg.[13] This in vitro study demonstrated that rhBMP-2 promotes the invasiveness of several human OSCCA cell lines, and that this biologic effect is dependent, in part, on the baseline gene expression of BMP-2. Specifically, this effect was only observed for cell lines that demonstrated gene expression of BMP-2. For the cell lines that did not express BMP-2, there did not appear to be any effect from the exogenous exposure to rhBMP-2. This cannot be explained by differences in expression for the BMP-receptors, because all these cell lines also express these genes. The finding that baseline BMP-2 expression level influences the biological behavior is not unique to this study.[14,15]

To determine if there were any molecular changes that correlated with the invasion assay findings, a real-time quantitative PCR array that included genes known to be involved with tumor metastasis was used on representative cell lines that expressed (UMCCC-74A) or did not express (UMSCC-1) BMP-2. In response to treatment with rhBMP-2, the Metastasis suppressor 1 (MTSS1) gene was significantly upregulated in UMSCC-1 and significantly downregulated in UMSCC-74A. These findings are consistent with the known function of this gene as a metastasis suppressor, and suggests that it might be a key modulator of invasive behavior of OSCCA.[16] Overexpression of MTSS1 has been shown to significantly suppress the invasive, migratory, growth, and adherence properties of human breast cancer cell lines.[16] Downregulation of MTSS1 is also associated with nodal metastasis and poor survival in patients with gastric cancer.[17]

Another metastasis suppressor, KISS1, was upregulated in UMSCC-1 in response to rhBMP-2. KISS1 encodes the protein metastin, which is a G-protein coupled receptor ligand for GPR54.[18] This gene product was initially described as a metastasis suppressor for melanoma, but more recently elevated expression has been shown to be a favorable prognostic factor for non-small cell lung cancer and pancreatic cancer.[19,20]

The IL-1β gene was upregulated in UMSCC-74A in response to rhBMP-2. This finding is consistent with other published data showing the involvement of this cytokine in tumor invasiveness and metastasis. Interleukin-1 expression at the site of tumor development enhances the expression of adhesion molecules on endothelial malignant cells, and facilitates the invasion of malignant cells into the circulation.[21] For IL-1β knockout mice, local tumors or lung metastasis of B16 melanoma cells were not observed compared with wild type.[22] These effects were not restricted to the

TABLE I.
rhBMP-2 Treatment Effect in Invasion Assay.

Cell Line	BMP-2 mRNA Level of Expression	Significance of the BMP-2 Treatment (*P*-Value)
UMSCC-1	–	.4978
UPCI:SCC 032	–	.0543
UPCI:SCC 182	–	.4959
UMSCC-14A	+	<.0001
UMSCC-74A	+	.0041
UPCI:SCC 056	+	.0024

The fluorescent readings between rhBMP-2 treated and nontreated cell lines were compared. *P*-values for the treatment effect are shown. *P*-values <.05 were considered significant.

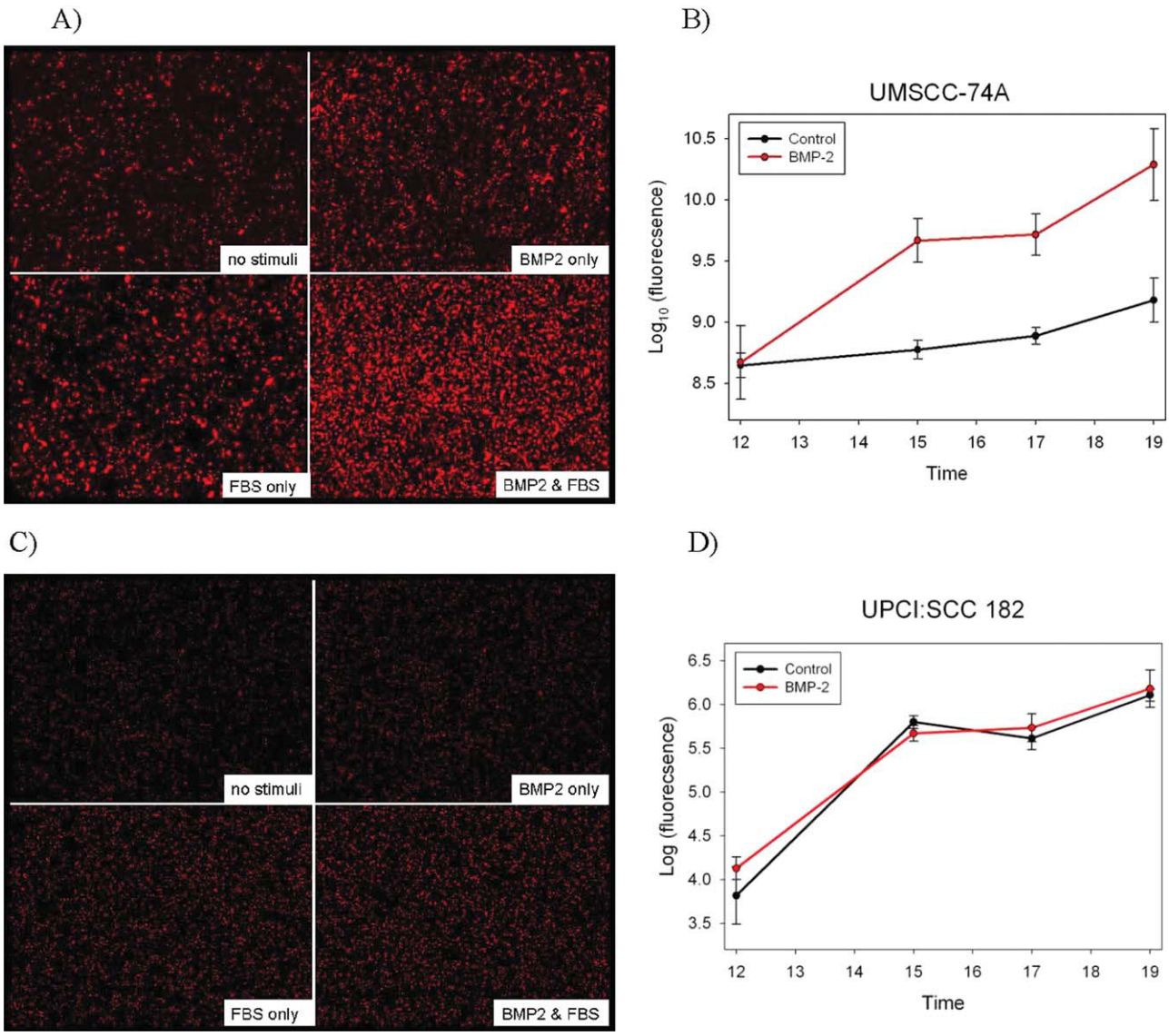

Fig. 2. Invasion assay. Images of oral carcinoma cell lines with positive (UMSCC-74A) (A) and negative (UPCI:SCC 182) (C) levels of BMP-2 mRNA expression were taken at 19 hours after stimulation (magnification 15×). The respective dynamic profiles of invasive activity represented by $\log_{10}$ of fluorescence levels (means of three independent measurements) are shown on B and D. Error bars correspond to standard deviations for each time point and treatment.

melanoma model, but were also observed in DA/3 mammary and prostate cancer cell models.

Interestingly, matrix metalloproteases (MMPs) were found to be upregulated in the cell line that did not exhibit increased invasiveness (MMP-3, MMP-10), whereas these genes were found to be downregulated in the cell line that demonstrated increased invasive behavior (MMP-9, MMP-11). These findings seem counterintuitive based on the general concept that MMPs mediate tumor progression and metastasis.[23] There is evidence in the literature, however, that MMPs also have a protective role in tumor progression.[24] The MMP functions in vivo are reliant on complex interactions between tumor cells and the stroma.[23] Our in vitro findings suggest that further mechanistic studies are needed to determine interactions between the BMP-2 signaling pathway and MMPs.

For the invasion assay, experiments were not performed to determine whether the findings were dependent on a dose response to rhBMP-2. The dose used for these experiments was a standard dose used in other published in vitro studies.[8,25] Another limitation is the absence of a treatment group with an antagonist of the BMP-2 pathway to see if the findings in the BMP-2-positive cells can be inhibited. Prior data has already shown that noggin or siRNA was able to inhibit the ability of rhBMP-2 to stimulate the migration of A549 lung carcinoma cells or liver cancer cells, respectively.[8,26] Last, only one cell line was used for each group (BMP-2-positive vs. BMP-2-negative gene expression) for the quantitative PCR array. We chose these representative cell lines to generate hypotheses for future studies.

Our in vitro quantitative PCR data and invasion assay analysis could not adequately answer all the

TABLE II.
Real-Time Quantitative PCR Data.

BMP-2 positive Expression (UMSCC-74A)			BMP-2 Netagive Expression (UMSCC-1)		
Gene	Fold Regulation	*P*-Value	Gene	Fold Regulation	P-Value
IL1B	1.89	.0142			
MMP-9	−4.19	.0378			
MTSS1	−3.57	.0022			
HGF	−3.18	.0117			
MMP-11	−1.79	.0161	MMP-10	2.87	.0087
MCAM	−1.76	.0043	MMP-3	2.35	.0029
PLAUR	−1.59	.0041	KISS1	1.87	.0043
TIMP-2	−1.55	.0095	MTSS1	1.52	.0023

Fold changes in expression levels of genes after rhBMP-2 treatment for two representative OSCCA cell lines. Only genes showing statistically significant changes (p-values $< .05$) are shown.

questions concerning invasive phenotype in OSCCA tumors. More detailed protein analysis and in vivo studies are required. However, the complexity and diversity of BMP receptors and their signal transduction mechanisms make it likely that BMP-2 will exert a variety of interdependent actions in tumor invasion and progression.

CONCLUSION

Recombinant human BMP-2 has an adverse biological effect on invasiveness of human OSCCA cell lines in vitro. This adverse effect appears to be dependent on the baseline gene expression of BMP-2. We also detected changes in expression of genes involved with tumor metastasis that correlated to the invasion assay findings.

Collectively, these data raise concern for the safe application of rhBMP-2 for reconstruction of bone defects in oral cancer patients and support the need for in depth in vivo analysis of these potential untoward effects.

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