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Expression of *TNF-α*, *VEGF*, and *MMP-3* mRNAs in synovial tissues and their roles in fibroblast-mediated osteogenesis in ankylosing spondylitis

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ABSTRACT. The aim of this study was to explore the mRNA levels of tumor necrosis factor-α (*TNF-α*), vessel endothelial growth factor (*VEGF*), and matrix metalloproteinase-3 (*MMP-3*) in synovial tissues in ankylosing spondylitis (AS), and to analyze the functions of these proteins in the differentiation of AS synovial tissue fibroblasts into osteoblasts (OB) and osteoclasts. Synovial tissue samples from 22 AS patients and 22 normal individuals were collected. *In situ* hybridization was utilized to detect *TNF-α*, *VEGF*, and *MMP-3* transcripts. After counting numbers of positive cells, Spearman analysis was used to determine the correlation between transcriptional levels of the three mRNAs and the AS disease activity index (BASDAI) and the C-response protein (CRP) levels. With the addition of TNF-α, VEGF, or both factors into cultured normal synovial fibroblasts, osteocalcin (bone gla protein, BGP) secretion levels were compared. We found that expression of *TNF-α*, *VEGF*, and *MMP-3* was identified exclusively in the disease group. mRNA levels were significantly positively

correlated with BASDAI (r = 0.42, 0.38, and 0.47, respectively; P < 0.05) and CRP (r = 0.44, 0.34, and 0.47 respectively; P < 0.05) scores. The secretion level of BGP in normal synovial fibroblasts increased progressively with increasing concentrations of VEGF or TNF-α (P < 0.01 compared to levels before treatment). Furthermore, co-incubation using both VEGF and TNF-α significantly elevated BGP levels compared to the single addition of VEGF or TNF-α (P < 0.01). These results suggest TNF-α, VEGF, and MMP-3 might directly participate in the differentiation of fibroblasts into OBs.

Key words: Ankylosing spondylitis; Synovial tissues; Osteoclast; Tumor necrosis factor-α; Vessel endothelial growth factor; Matrix metalloproteinase-3

INTRODUCTION

Ankylosing spondylitis (AS) has an insidious onset, involving the axial skeleton at an early stage, and affecting the activity of peripheral joints, and thus severely impairs the quality of patient life. Clinical studies have demonstrated that osteoclasts (OCs) are the primary effector of bone destruction, whereas the ossification and rigidity of normal ligament and synovial are indispensable for differentiation and maturation of OCs (Briolay et al., 2013). However, the conditions required for differentiation and related mechanisms of OC precursors are not yet understood, which impedes the prevention and treatment of this disease. As an important inflammatory initiating factor, tumor necrosis factor-α (TNF-α) plays a critical role in the differentiation of multiple inflammatory and tumor cells (Scudiero et al., 2012). Vessel endothelial growth factor (VEGF) works as an angiogenesis regulating factor and has shown a powerful accelerating function of fracture healing in some studies (Won et al., 2012). Matrix metalloproteinase-3 (MMP-3) can specifically degrade the extracellular matrix (Fujita et al., 2012). We hypothesized that these three factors might be related to the development of osteoblast (OB) and OC cells, and conducted this study to examine whether this relationship could be substantiated.

MATERIAL AND METHODS

Tissue samples

Synovial tissue samples were collected from 22 patients with AS admitted in Yantaishan Hospital (Shandong, China) between January 2010 and May 2014. The diagnostic criteria adapted the recommendations of the American College of Rheumatology (ACR) (van der Linden et al., 1984). All samples were collected from CT-guided puncture of sacroiliac joints and were included in the disease group. An additional 22 normal synovial tissue samples were collected from patients with traumatic femoral head fracture who had undergone open reduction and internal fixation treatment. Written consents were obtained from all patients. This study was pre-approved by the ethical committee of Yantaishan Hospital.

General research method

Patient medical records were collected and analyzed for parameters such as the Bath

ankylosing spondylitis disease activity index (BASDIA) and C-reactive protein (CRP) scores. Samples were frozen, sectioned, subjected to *in situ* hybridization (ISH) for detection of *TNF-α*, *VEGF*, and *MMP-3* mRNA transcripts. AS synovial fibroblasts were enzymatically digested and cultured to detect BGP production levels under different culture conditions using radioimmunoassay.

ISH staining

ISH staining utilized the following equipment and reagents: freezing microtome (Microm International, Walldorf, Germany), imaging analysis system (Leica, Wetzlar, Germany), and an ISH test kit (Boshide Corp., Wuhan, China). Other reagents such as hydrogen peroxide, pure methanol, and citric acid were prepared in-house. The test was performed according to the instruction manual of the assay kit. In brief, samples were sectioned and deactivated using H_2O_2 and methanol, followed by pepsin digestion and fixation in paraformaldehyde. After washing 3 times in distilled water, tissue samples were first incubated in pre-hybridization buffer at 40°C, and then hybridized with oligonucleotide probes in hybridization buffer at 40°C overnight. On the next day, the blocking buffer was applied, followed by incubation with mouse anti-DIG antibody conjugated with biotin, and washed with phosphate buffered saline (PBS). Samples were then processed in streptavidin-biotin-peroxidase complex (SABC), washed in PBS, developed using DAB substrate, washed in distilled water, and counter-stained using hematoxylin. Finally, samples were dehydrated, hyalinized, mounted, and observed under the microscope. The ImageJ software was used to quantify positive stained cell numbers and average gray values.

Primary culture of AS synovial fibroblasts

Major equipment and reagents included: osteocalcin assay kit (provided by the Chinese Nuclear Power Institute, Chengdu, Sichuan, China), and a VEGF and TNF-α assay kit (Jingmei Corp., Shenzhen, China). In brief, samples were cultured in 20% fetal bovine serum medium at 37°C with 5% CO_2 in a humidified chamber. The 3rd generation of cultured cells were counted and inoculated into 96-well plates (1 x 10^5/mL per well) with 6 replicates for each treatment group. A total of 16 treatment conditions were applied, including VEGF at 0, 5, 10, and 20 ng/mL, TNF at 0, 10, 100, and 500 U/mL, or co-treatment of both factors. After 5 days incubation, the content of BGP in the upper supernatant was quantified by a BGP radioimmunoassay kit (Yuanye Biotech, China).

Statistics

The SPSS 19.0 software package (IBM Corp., US) was utilized to process all collected data, which were presented as means ± standard error (SE), unless otherwise specified. Student *t*-test was applied for between-group-comparisons while the F-test was used in the case of multiple group comparisons. The Spearman correlation test was employed to analyze the correlation between mRNA transcripts of *TNF-α*, *VEGF*, and *MMP-3* and BASDAI and CRP indices. Statistical significance was defined as $P < 0.05$.

RESULTS

General information of patients and controls

No significant differences regarding sex ratio and average age existed between the disease and control groups (P > 0.05, Table 1). Therefore, the samples included in this study were comparable.

Table 1. General sample information.

Group	N	Male: Female	Average age (years)
Disease	22	15:7	26.2 ± 5.8
Control	22	16:6	26.8 ± 5.5
χ^2/t		0.10	-0.35
P value		>0.05	>0.05

mRNA transcripts of *TNF-α*, *VEGF*, and *MMP-3*

Numbers of cells positive for *TNF-α*, *VEGF,* and *MMP-3* mRNA in the disease group were all significantly higher than those in the control group, while their gray values were significantly lower than the control group. All these differences were of statistical significance (P < 0.05, Table 2).

Table 2. mRNA expression levels of *TNF-α*, *VEGF*, and *MMP-3*.

Group (N)	*TNF-α* mRNA		*VEGF* mRNA		*MMP-3* mRNA	
	Positive cell	Gray value	Positive cell	Gray value	Positive cell	Gray value
Disease (22)	72.2 ± 5.6	148.5 ± 7.1	51.8 ± 6.8	142.6 ± 6.7	88.7 ± 6.2	141.3 ± 5.8
Control (22)	6.4 ± 2.2	186.6 ± 5.8	7.4 ± 2.6	183.8 ± 7.2	3.1 ± 1.2	182.2 ± 6.7
t value	51.30	-19.49	28.61	-19.65	63.58	-21.65
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Correlative analysis between mRNA levels of *TNF-α*, *VEGF*, and *MMP-3* and clinical factors

The disease group had CRP levels of 44.3 ± 25.2 mg/L and BASDAI scores of 3.4 ± 2.0 while the control group had CRP concentration of 5.1 ± 0.6 mg/L and a BADSAI score of 0.6 ± 0.3. The Spearman analysis showed a significantly positive relationship between *TNF-α* mRNA and CRP levels (r = 0.47, P < 0.05) as well as BASDAI scores (r = 0.42, P < 0.01). It also showed that *VEGF* mRNA was significantly positively correlated with CRP (r = 0.34, P < 0.05) and BASDAI (r = 0.38, P < 0.05) scores. Furthermore, *MMP-3* mRNA displayed a significantly positive relationship with CRP (r = 0.44, P < 0.05) and BASDAI (r = 0.47, P < 0.01) scores.

Effect of TNF-α and VEGF on synovial fibroblast BGP levels

BGP levels were elevated upon addition of higher dosages of TNF-α or VEGF into the culture medium. A cross-interaction occurred with the co-incubation of both TNF-α and

VEGF, which significantly increased BGP concentration compared to those cells with addition of only TNF-α or VEGF. All differences were of statistical significance ($P < 0.05$, Table 3).

Table 3. Effects of TNF-α or VEGF on BGP concentration in synovial fibroblasts.

VEGF concentration (ng/mL)	TNF-α concentration (U/mL)					
	0	10	100	500	F value	P value
0	0.47 ± 0.22	1.08 ± 0.15[a]	1.28 ± 0.31[a]	1.53 ± 0.25[a]	48.17	<0.001
5	0.95 ± 0.20[b]	1.57 ± 0.35[c]	2.05 ± 0.33[c]	2.08 ± 0.22[c]	34.02	<0.001
10	1.28 ± 0.12[b]	1.68 ± 0.21[c]	2.28 ± 0.52[c]	2.48 ± 0.17[c]	25.32	<0.001
20	1.93 ± 0.55[b]	2.08 ± 0.25[c]	2.53 ± 0.31[c]	2.62 ± 0.45[c]	18.31	<0.001
F value	32.14	28.31	24.31	17.22		
P value	<0.001	<0.001	<0.001	<0.001		

[a]Compared to cells with 0 ng/mL VEGF, $P < 0.05$; [b]compared to cells with 0 U/mL TNF-α, $P < 0.05$; [c]compared to cells with only TNF-α or VEGF, $P < 0.05$.

DISCUSSION

Clinical studies have demonstrated a close relationship between OCs and progression of AS disease (Sieper et al., 2009). As a major cell type responsible for bone reabsorption, OCs are formed in the mononuclear macrophage system and directly involved in the bone reformation (Eeles et al., 2015). Cellular research suggested that tartrate resistant acid phosphatase (TRAP) staining showed a positive response only after the differentiation and maturation of OC (Grcevic et al., 2010; Won et al., 2012). Other studies in AS-related hyperplasia also demonstrated the existence of large amounts of TRAP-positive mono- or poly-nuclear cells adhesive in the synovial tissue near the disease site, whereas no TRAP signal could be detected in cells near normal synovial tissues. These results suggested that OC precursors might consist of macrophages near synovial tissues.

This study aimed to investigate the mechanism related trophic factors underlying the transformation of macrophages into OCs. Based on previous knowledge, we first deduced a potential role of MMP-3, VEGF, and TNF-α in OC formation. As a Zn^{2+}-dependent endopeptidase, MMP-3 has been reported to have elevated expression in the synovial tissues of patients with AS (Fujita et al., 2012; Wendling et al., 2012). Consistent with these studies, the current research also showed a higher number of MMP-3 positive cells in synovial tissues from the disease group compared to the control group, and identified a significantly positive relationship between *MMP-3* transcript levels and CRP or BASDAI indices. As a major body inflammatory factor, CRP can reflect the severity of AS while the BASDAI score has been widely used to directly evaluate disease progression (van der Linden et al., 1984; Wong et al., 2012). We suggest that this relationship is due to the fact that MMP-3 directly participates in the degradation of the extracellular matrix, and its elevation can activate multiple MMP factor precursors including MMP-1, -9, and -13 to further accelerate the degradation of joint cartilage matrix, destroy cartilage, and advance the progression of AS (Markel et al., 2007; Kawamura et al., 2008).

The differentiation and maturation of OCs can only occur when large numbers of OBs are present (Suzuki et al., 1998; Raidl et al., 2007). While no OBs have been found in normal synovial tissues, we hypothesized that some OBs might exist in AS patient synovial tissues in order to support the differentiation and maturation of OCs. We therefore performed further studies to quantify the levels of osteocalcin to substantiate the existence of OBs in AS synovial

tissues. Our study has also demonstrated the facilitation of osteocalcin secretion by TNF-α and VEGF, as higher concentrations of those two factors induced larger amounts of osteocalcin secretion in addition to demonstrating a synergetic effect between the two factors in facilitation of osteocalcin. In previous studies in AS patient synovial tissues, we confirmed higher expression levels of *TNF-α* and *VEGF* mRNA, which had a significantly positive relationship with the CPR and BASDAI indices. Therefore, we suggest that both TNF-α and VEGF might participate in the differentiation of fibroblasts into OBs, which provides additional basis for the formation of OCs (Pedersen et al., 2010). As an early-stage inflammatory trophic factor, TNF-α has multiple inflammatory responses and immune reactivities while VEGF can accelerate the recovery from bone fracture due to its ability as an angiogenesis regulation factor (Bottomley et al., 1999; Appel et al., 2010). The physiological function of VEGF underlies its potency in the progression of AS, as directly demonstrated by this study.

The current research, however, did not demonstrate the direct involvement of TNF-α and VEGF in OB formation. More illustrative evidence can only be obtained after the application of related antibodies to directly decrease BGP secretion (Genevay et al., 2009). Some limitations also exist in the current study, as only mRNA transcripts but not related protein expression levels have been quantified, thus impairing the overall representativeness. Furthermore, our research only focused on TNF-α, VEGF, and MMP-3, and thus cannot fully illustrate the differentiation of synovial fibroblasts (Hamed et al., 2004; Crisostomo et al., 2007). Other factors such as IL-12B might also be related to AS progression (Kolomecki et al., 2001; Bidad et al., 2012). These questions require further analysis.

In summary, our results suggest that TNF-α and VEGF might directly participate in the differentiation of fibroblasts into OBs, while mature OCs might express MMP-3 to a large degree to accelerate the degradation of cartilage.

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