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Abstract

Angiogenesis is an essential event in the development of synovial inflammation in rheumatoid arthritis (RA). The aim of the current study was to investigate the expression of vasohibin-1, a novel endothelium-derived vascular endothelial growth factor (VEGF)-inducible angiogenesis inhibitor, in the RA synovium, and to test the effect of inflammatory cytokines on the expression of vasohibin-1 by RA synovial fibroblasts (RASFs). Synovial tissue samples were obtained at surgery from patients with osteoarthritis (OA) and RA, and subjected to immunohistochemistry to investigate the expression and distribution of vasohibin-1 relevant to the degree of synovial inflammation. In an in vitro analysis, RASFs were used to examine the expression of vasohibin-1 and VEGF mRNA by real-time PCR after stimulation with VEGF or inflammatory cytokines under normoxic or hypoxic conditions. The immunohistochemical results showed that vasohibin-1 was expressed in synovial lining cells, endothelial cells, and synovial fibroblasts. In synovial tissue, there was a significant correlation between the expression of vasohibin-1 and histological inflammation score (p0.002, r0.842). In vitro, stimulation with VEGF induced the expression of vasohibin-1 mRNA in RASFs under normoxic conditions, and stimulation with cytokines induced vasohibin-1 mRNA expression under a hypoxic condition. These results suggest that vasohibin-1 was expressed in RA synovial tissue and might be regulated by inflammatory cytokines.

KEYWORDS: angiogenesis, vasohibin-1, rheumatoid arthritis, synovial membrane, VEGF

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Original Article

Inflammatory Cytokine-induced Expression of Vasohibin-1 by Rheumatoid Synovial Fibroblasts

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Angiogenesis is an essential event in the development of synovial inflammation in rheumatoid arthritis (RA). The aim of the current study was to investigate the expression of vasohibin-1, a novel endothelium-derived vascular endothelial growth factor (VEGF)-inducible angiogenesis inhibitor, in the RA synovium, and to test the effect of inflammatory cytokines on the expression of vasohibin-1 by RA synovial fibroblasts (RASFs). Synovial tissue samples were obtained at surgery from patients with osteoarthritis (OA) and RA, and subjected to immunohistochemistry to investigate the expression and distribution of vasohibin-1 relevant to the degree of synovial inflammation. In an *in vitro* analysis, RASFs were used to examine the expression of vasohibin-1 and VEGF mRNA by real-time PCR after stimulation with VEGF or inflammatory cytokines under normoxic or hypoxic conditions. The immunohistochemical results showed that vasohibin-1 was expressed in synovial lining cells, endothelial cells, and synovial fibroblasts. In synovial tissue, there was a significant correlation between the expression of vasohibin-1 and histological inflammation score ($p = 0.002$, $r = 0.842$). *In vitro*, stimulation with VEGF induced the expression of vasohibin-1 mRNA in RASFs under normoxic conditions, and stimulation with cytokines induced vasohibin-1 mRNA expression under a hypoxic condition. These results suggest that vasohibin-1 was expressed in RA synovial tissue and might be regulated by inflammatory cytokines.

Key words: angiogenesis, vasohibin-1, rheumatoid arthritis, synovial membrane, VEGF

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by progressive bone and joint destruction. In RA, angiogenesis plays a pivotal role in the pathogenesis of synovial inflammation, maintaining the inflammatory synovial tissues, and providing the condition for inflammatory cell infiltration from the early stage of the disease [1, 2]. Newly formed blood vessels allow the synovial tissue to cope with increased demand of oxygen and nutrients by infiltrating CD4+ T cells, B cells, macrophages, and the proliferated synovial lining cells. In fact, the hypoxic nature of the RA synovium has been confirmed by measuring oxygen tension in samples of synovial fluids obtained from patients with RA [3, 4]. This

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hypoxic condition stimulates the activation of transcriptional regulators, such as hypoxia inducible factor (HIF) [5]. HIF induces angiogenesis in arthritis [6] by directly inducing the expression of a broad range of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) and its receptors flk-1, flt-1, and angiopoietin, as well the angiopoietin receptor tie-2, all of which are upregulated in the RA synovium [6–8].

Among the various growth factors promoting angiogenesis, VEGF plays perhaps the important roles in the process of angiogenesis by promoting endothelial cell migration, proliferation, and vascular permeability in association with inflammation [9]. VEGF secretion by RA synovial fibroblasts (RASFs) and PBMCs from RA patients is up-regulated by inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) or interleukin-1-beta (IL-1β), both of which are considered to be key cytokines in the pathogenesis of RA [10, 11].

A number of pro-angiogenic and anti-angiogenic factors regulate VEGF expression [12]. Vasohibin-1 was originally identified from a microarray analysis designed to examine genes up-regulated by VEGF in endothelial cells [13], and was considered to act as an endogenous negative feedback regulator of angiogenesis. Vasohibin-1 is induced by VEGF-A and FGF-2, and inhibits angiogenesis in an autocrine manner [13, 14]. Vasohibin-1 is expressed in the tissues of patients with endometrial cancer [15] and breast cancer [16], and in the retina of patients with diabetic retinopathy [17]. These recent findings suggest that the levels of vasohibin-1 may be associated with the clinical activities and severity of various disorders. In addition, therapeutic effects of vasohibin-1 in tumor growth, atherosclerosis and proliferative retinopathy models have also been reported [13, 18, 19].

In the present study, we investigated the expression of vasohibin-1 in human synovial tissue for the first time. To examine the association between synovial inflammation and vasohibin-1 expression, we investigated samples from patients with RA and also patients with osteoarthritis (OA) as a low-grade inflammation control. Interestingly, the expression of vasohibin-1 was observed in both the OA and RA synovium, and the number of vasohibin-1-positive cells correlated with the degree of tissue inflammation. These findings further prompted us to investigate the mechanism of vasohibin-1 induction in synovial fibroblasts. The results suggested that vasohibin-1 expression might be up-regulated by VEGF under a normoxic condition, and by the inflammatory cytokines under a hypoxic condition.

Materials and Methods

Immunohistochemistry. For the immunohistochemical analysis, fresh synovial tissues were obtained from the knee, wrist or elbow joints of 12 patients with RA (11 women and 1 man; mean age, 63.9 ± 8.1 years old) and 9 patients with OA (5 women, 2 men, and data not available in 2 samples; mean age, 69.7 ± 8.7 years old) who were undergoing total knee, wrist or elbow arthroplasty. Informed written consent was obtained from all patients. The diagnosis of RA was determined according to the revised 1987 criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) [20]. Immunohistochemistry was performed as previously described [21] utilizing paraffin-embedded sections. Serial paraffin sections of the synovium (4μm) were deparaffinized, rehydrated, and incubated with 3% H_2O_2 for 30 min to block endogenous peroxidase activity. Sections were then incubated for 30 min at room temperature (RT) in a blocking solution of 10% rabbit serum (Nichirei Biosciences, Tokyo, Japan), and then incubated with mouse anti-human vasohibin-1 monoclonal antibody (4.0μg/ml; Tohoku University, Sendai, Japan) [13], anti-VEGF-A antibody (Millipore, Billerica, MA, USA; 1 : 800 dilution), and anti-CD34 antibody (Dako, Copenhagen, Denmark; 1 : 200 dilution) overnight at 4℃, followed by incubation with biotinylated secondary antibodies (Nichirei Biosciences) for 60 min at RT. Immunoperoxidase staining was conducted using a Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA, USA). Diaminobenzidine was used as a chromogen. All slides were counterstained with hematoxylin.

Histological examination. Following immunostaining, each section was evaluated under a light microscope in 5 randomly selected areas of the synovium at a magnification of $\times 100$ and the means of the inflammation scores and vasohibin-1 scores were calculated. The inflammation score was calculated as described previously [22] (Table 1). The vasohibin-1

Table 1 The grading system of the synovial membrane inflammation

Score	Synovial lining cell hyperplasia	Cellular infiltration	Fibrosis of the lining cell layer
0	1-2 layers of cells	Not present	Normal
1	3-4 layers	Mild	Mild
2	5-6 layers	Moderate and focal	Moderate
3	6-7 layers	Moderate and diffuse	Marked
4	Over 7 layers	Marked and diffuse	Marked

score in each area was determined according to the previously described method with some modifications [23]. In brief, the number of positively stained cells was counted and evaluated using a 4-point scale: 0 = no staining, 1 = localized staining, 2 = positively stained in more than 20% of the cells, 3 = positively stained in more than 50% of the cells, and 4 = widespread, total staining of the synovial tissue. The amount of VEGF was similarly determined in the same 5 areas on the serial sections used for vasohibin-1 staining. The number of microvessels positively stained with CD34 was also counted in the same 5 areas on the next serial section.

Isolation and culture of human RASFs. With the patients' written consent, fresh synovial tissues were obtained from the knee, elbow or wrist joints of 4 RA patients (4 women; mean age, 65.7 ± 6.0 years old) who underwent total knee, elbow or wrist arthroplasty. Tissues were minced and digested immediately with collagenase (Wako, Osaka, Japan) and DNase (Sigma-Aldrich, St. Louis, MO, USA) at 37℃, as previously described [24]. Tissue debris was removed with a cell strainer, and cells were washed twice with medium consisting of Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Rockville, MD, USA), 100 IU/ml of penicillin, and 100 mg/ml of streptomycin (Life Technologies). The resultant single cells were dispensed into the wells of a 24-well microtiter plate (Costar, Cambridge, MA, USA) at a density of 2×10^6 cells/ml in 2 ml of DMEM supplemented with 10% FCS, 100 IU/ml of penicillin, and 100 mg/ml of streptomycin. The plates were incubated at 37℃ in a humidified atmosphere containing 5% CO_2. Synovial tissue cell cultures were divided once weekly until the primary cultures had reached confluence. After the third passage, the cells appeared to be morphologically homogeneous fibroblast-like cells.

Real-time polymerase chain reaction (PCR) for the quantitative detection of vasohibin-1 and VEGF messenger RNA (mRNA). The cells were seeded at a density of 5×10^5/dish into a 6 cm-dish (Costar) containing 3 ml of DMEM and 10% FCS and were allowed to adhere at least 12 h. The cells were then incubated with FCS-free DMEM for 24 h. Next, the cells were stimulated with recombinant human VEGF (0.1 nM; R&D Systems, Minneapolis, MN, USA), or either or both recombinant human TNF-α (1 ng/ml; R&D Systems) or recombinant human IL-1β (10 ng/ml; R&D Systems) and incubated with or without hypoxia (1% O_2) under an atmosphere of 5% CO_2 for the time periods indicated below. Cell viability in the 6 cm-dish was evaluated at 12, 24, and 48 h after cytokine or VEGF treatment. Total RNA was isolated from cultured cells with an RNA isolation kit (RNeasy Mini kit; Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The RNA was reverse-transcribed using ReverTra Ace (Toyobo, Tokyo, Japan). The primer pair for vasohibin-1 was purchased from Nihon Idenshi Kenkyujo (Sendai, Japan) and was as follows: 5'-CCATACCGAGTGTGCCTAC-3' (forward) and 5'-AGAACTGTGTCCCTG-TGTGA-3' (reverse). Primers for beta-actin and VEGF were purchased from Roche Diagnostics. Real-time quantitative PCR reactions were performed on a LightCycler instrument (Roche Diagnostics) using a LightCycler Fast-Start DNA Master SYBR Green I kit (Roche Molecular Biochemicals, Mannheim, Germany) as recommended by the manufacturer. The final expression value was calculated by dividing the level of vasohibin-1 or VEGF mRNA expression by the level of beta-actin mRNA expression, and each value at the basal time point was set as 1. For all real-time PCR experiments, cDNA samples were obtained from 3 distinct lines of RASFs derived from three individual patients at passages 3–5, and real-time PCR reactions

were repeated three times for each cDNA sample obtained from RASF.

Statistical analysis. Data were expressed as the means ± SD. Statistical analysis was performed using a one-way analysis of variance followed by either Fisher's least significant difference test or the Mann-Whitney U test, using Statview-J 5.0 software (SAS Institute, Cary, NC, USA); values of $p < 0.05$ were considered statistically significant.

Results

Expression of vasohibin-1 in OA and RA synovial tissues. The inflammation scores of OA and RA synovial tissues were 2.60 ± 0.94 and 5.68 ± 1.96, respectively (Table 2). The inflammation score was significantly different between the two groups ($p = 0.003$). Immunoreactivity for vasohibin-1 was detected in the synovial lining cells, endothelial cells, and synovial fibroblasts (Fig. 1A). The vasohibin-1 scores of OA and RA synovial tissues were 1.31 ± 0.42 and 2.17 ± 0.7, respectively (Fig. 1B). The vasohibin-1 score was significantly different between the two groups ($p = 0.0048$). The inflammation score was positively and significantly correlated with the vasohibin-1 score in the RA synovium ($r = 0.842$, $p = 0.002$, n=12), but not in the OA synovium ($r = 0.842$, $p = 0.09$, $n = 9$; Fig. 1C). These results suggest that vasohibin-1 is preferentially expressed in the RA synovium, in association with the severity of inflammation.

Expression of VEGF in OA and RA synovial tissues. Marked immunoreactivity for VEGF was observed in infiltrating cells, and lesser VEGF immunoreactivity was seen in synovial lining cells, synovial fibroblasts, and endothelial cells in the RA synovium (Fig. 2A). The VEGF scores of OA and RA synovial tissues were 1.47 ± 0.53 and 2.30 ± 0.88, respectively, and these values were significantly different ($p = 0.02$, Fig. 2B). In the synovial samples from patients with RA, the VEGF score was positively and significantly correlated with the vasohibin-1 score ($r = 0.736$, $p = 0.005$; Fig. 2C). These results suggest that VEGF was up-regulated in the RA synovium, in association with the levels of vasohibin-1.

CD34-positive microvessel density in the OA and RA synovium. The immunohistochemical analysis revealed the presence of CD34-positive endothelial cells in blood vessels in the OA and RA synovium (Fig. 3A). The CD34-positive microvessel densities (vessel number per × 100 field) were not statistically different between OA and RA (19.2 ± 11.9 and 24.5 ± 11.0, respectively; $p = 0.30$; Fig. 3B). In the RA synovial samples, the microvessel density was not significantly correlated with the vasohibin-1 score ($r = 0.542$, $p = 0.069$; Fig. 3C). Thus the microvessel density was not associated with the expression of vasohibin-1in the RA synovium, in contrast to the levels of VEGF, which exhibited a positive correlation with vasohibin-1 expression.

VEGF-stimulated expression of vasohibin-1 in RASFs. We next examined the potential induction of vasohibin-1 by VEGF under normoxia or hypoxia in RASFs. RASFs were stimulated with VEGF (0.1 and 1.0 nM) for the indicated periods and real-time PCR was performed to investigate vasohibin-1 mRNA expression. The levels of vasohibin-1 mRNA were significantly increased by VEGF at 48 h under normoxia by 0.1 nM of VEGF, but not under a hypoxic condition (Fig. 4). Dose-dependent effects of VEGF on the levels of vasohibin-1 mRNA were not observed under either a normoxic or hypoxic condition (Fig. 4).

Cytokine-stimulated expression of vasohibin-1 in RASFs. We next examined the potential induction of vasohibin-1 by inflammatory cytokines in RASFs. Stimulation of RASFs with TNF-α (1 ng/ml) significantly down-regulated vasohibin-1 mRNA expression at 12–48 h under a normoxic condition ($p < 0.01$). Stimulation by IL-1β (10 ng/ml) under a nor-

Table 2 The synovial inflammation score of the OA and RA synovial membrane (mean ± SD). The inflammation score was significantly different between the two groups ($p < 0.003$). $^{*}p < 0.05$ vs. OA. $^{**}p < 0.01$ vs. OA.

	Synovial lining cell hyperplasia	Cellular infiltration	Fibrosis of the lining cell layer	Inflammation score (total)
OA	0.47±0.35	0.98±0.49	1.16±0.34	2.60±0.94
RA	1.22±0.83*	2.32±0.95**	2.15±0.62**	5.68±1.96**

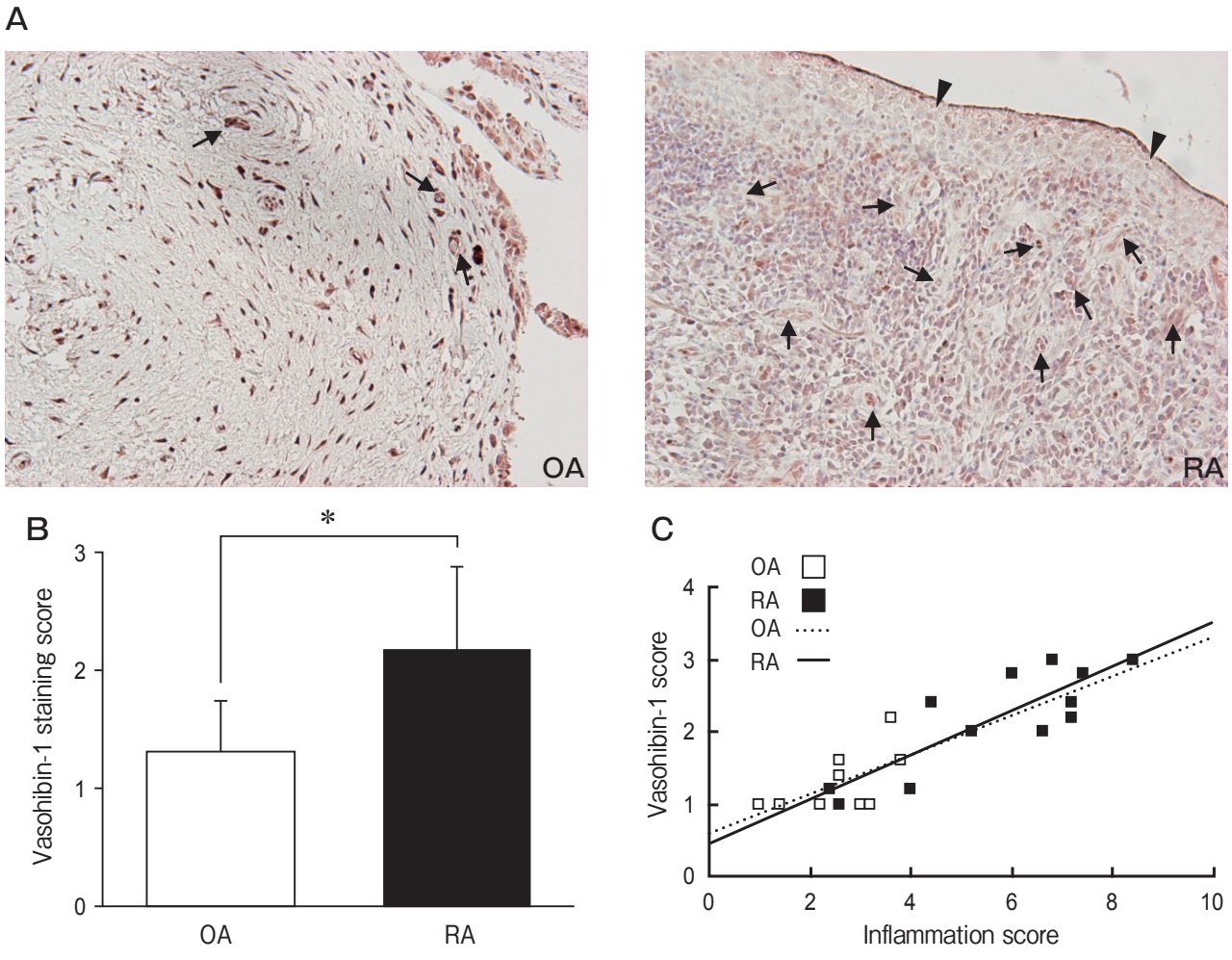

Fig. 1 **A**, Comparison of the histologic features of synovial samples derived from patients with rheumatoid arthritis (RA) and osteoarthritis (OA). Immunohistochemical analysis was performed to detect vasohibin-1 in OA and RA synovial samples. In OA samples, immunoreactivity for vasohibin-1 was observed in a few endothelial cells (arrows). In RA samples, immunoreactivity for vasohibin-1 was observed in synovial lining cells (arrowheads), endothelial cells (arrows), synovial fibroblasts, and infiltrating inflammatory cells. In OA samples, the inflammation score was 2 (synovial lining cell hyperplasia 0, cellular infiltration 0, fibrosis of the lining cell layer 2), the vasohibin-1 score 1, and the VEGF score 1. In the RA samples, the inflammation score was 10 (synovial lining cell hyperplasia 2, cellular infiltration 4, fibrosis of the lining cell layer 4), the vasohibin-1 score 3, and the VEGF score 4; **B**, The vasohibin-1 score of synovial tissues. The vasohibin-1 scores of OA and RA synovial tissues were 1.31 ± 0.42 and 2.17 ± 0.7, respectively ($^*p = 0.0048$), as determined by the method described in the Materials and Methods. The values in each column are the means $\pm$ SD; **C**, Correlation between inflammation scores and vasohibin-1 scores. The inflammation score was also positively correlated with the vasohibin-1 score in the RA synovium ($r = 0.842$, $p = 0.002$, $n = 12$), but not in the OA synovium ($r = 0.842$, $p = 0.09$, $n = 9$).

moxic condition down-regulated vasohibin-1 expression at 48 h ($p < 0.01$). Stimulation by the combination of IL-1β (10 ng/ml) and TNF-α (1 ng/ml) under a normoxic condition also down-regulated vasohibin-1 expression at 48 h ($p < 0.01$) (Fig. 5A). Under a hypoxic condition, TNF-α (1 ng/ml) stimulation did not up-regulate vasohibin-1 expression. However, IL-1β (10 ng/dl) stimulation under a hypoxic condition up-regulated vasohibin-1 expression at 12 h ($p < 0.05$), and significantly down-regulated vasohibin-1 expression at 48 h ($p < 0.01$). Stimulation by the combination of IL-1β (10 ng/ml) and TNF-α (1 ng/ml) under a hypoxic condition further enhanced the mRNA expression of vasohibin-1 (Fig. 5B) up to 24 h ($p < 0.05$; Fig. 5B).

Cytokine-stimulated expression of VEGF in

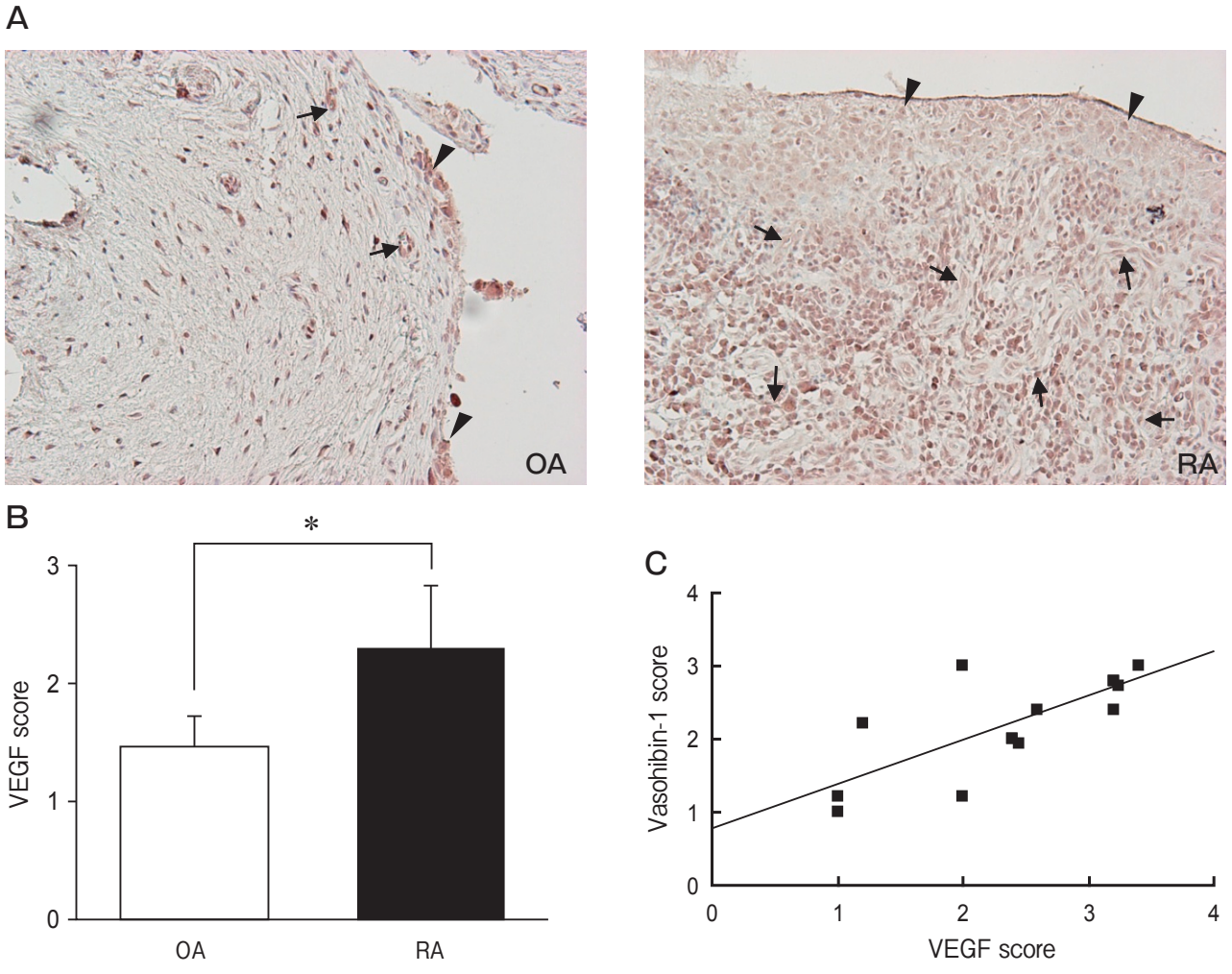

Fig. 2 **A**, Immunohistochemistry for VEGF in synovial samples from patients with OA and RA. In OA samples, immunoreactivity for vasohibin-1 was observed in a few synovial lining cells (arrowheads) or endothelial cells (arrows). In RA samples, immunoreactivity for VEGF was strongly observed in infiltrating cells, synovial lining cells (arrowheads), synovial fibroblasts, and endothelial cells (arrows); **B**, The VEGF score of synovial tissues. The VEGF scores of RA and OA synovial tissues were 1.47 ± 0.53 and 2.30 ± 0.88, respectively, and these values were significantly different ($^{*}p = 0.02$), as determined by the method described in the Materials and Methods. The values in each column are the means $\pm$ SD; **C**, Correlation between VEGF scores and vasohibin-1 scores in the RA synovium ($n = 12$). The VEGF score was positively correlated with the vasohibin-1 score ($r = 0.736$, $p = 0.005$).

RASFs. We next examined the levels of VEGF mRNA in RASFs by stimulation with cytokines under a normoxic or hypoxic condition. VEGF mRNA expression was not up-regulated by TNF-α (1 ng/ml), but was up-regulated by IL-1β (10 ng/ml) under a normoxic condition. Stimulation of the cells by the combination of IL-1β (10 ng/ml) and TNF-α (1 ng/ml) under a normoxic condition also enhanced mRNA expression of VEGF (Fig. 6A). VEGF expression was markedly up-regulated by TNF-α (1 ng/ml) or IL-1β (10 ng/ml) under a hypoxic condition. Stimulation of the cells by the combination of IL-1β (10 ng/ml) and TNF-α (1 ng/ml) under a hypoxic condition also markedly enhanced the mRNA expression of VEGF (Fig. 6B).

Discussion

Angiogenesis plays a key role in normal vascular development, is a decisive factor in cancer, wound

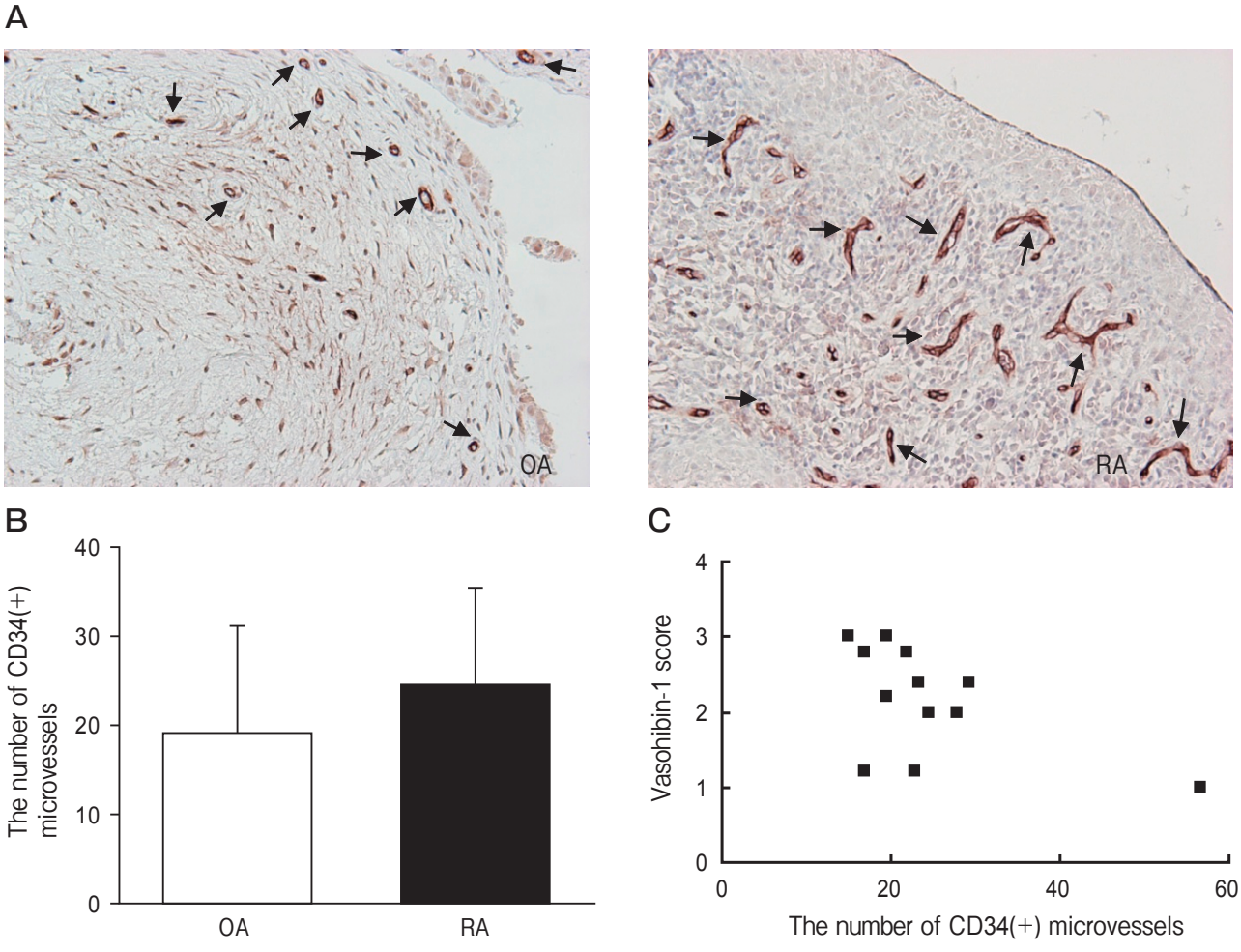

Fig. 3 **A**, Immunohistochemistry for CD34. CD34-positive endothelial cells were observed in blood vessels (arrows); **B**, CD34(+) microvessel densities (vessel number per × 100 field). Microvessel densities were not statistically different between the OA and RA synovium (19.2 ± 11.9 and 24.5 ± 11.0, respectively; $p = 0.30$); **C**, Correlation between the CD34(+) microvessel density (counts per × 100 field) and vasohibin-1 score. The microvessel density was not statistically correlated with the inflammation score in RA ($r = -0.542$, $p = 0.069$).

healing, and inflammation [25], and is regulated by the local balance between angiogenic stimulators and inhibitors. As the pathogenesis of RA is highly influenced by angiogenesis in the process of forming and maintaining inflammatory synovial tissues, the application of angiogenesis inhibitors for the treatment of RA has been expected. A number of angiogenesis inhibitors have been investigated and identified, including angiostatin, endostatin, platelet factor-4 (PF4), thrombospondin-1 (TSP-1) [26] and tumstatin [27]. Vasohibin-1 is a newly identified negative feedback regulator of angiogenesis. In humans, the levels of vasohibin-1 have been investigated in angiogenesis-associated disorders such as endometrial cancer, or in choroidal neovascular membranes [15, 28], and a potential association with disease activity was demonstrated. The results of the immunohistochemistry in the present study suggested that vasohibin-1 is expressed in synovial lining cells, endothelial cells, and synovial fibroblasts in RA synovial tissue. The intensity of the immunoreactivity for vasohibin-1 was correlated with the inflammation score and VEGF score, and was significantly higher in the RA synovium than the OA synovium. In fact, Tamaki *et al.* demonstrated an association between vasohibin-1 expression and inflammation in human breast lesions

[16], consistent with the present results in patients with RA. On the other hand, the vessel densities in synovial tissue in patients with RA were not significantly different from those in patients with OA as detected by immunohistochemistry for CD34, probably because blood vessels may not be easily eliminated even after the suspension of inflammatory stimuli.

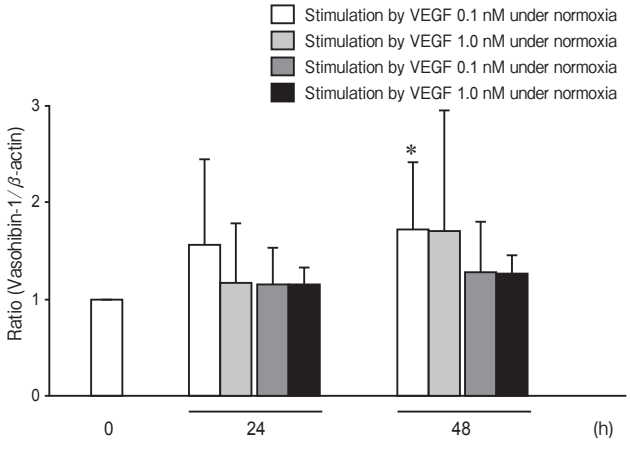

Fig. 4 Time course of the effects of VEGF (0.1 and 1.0 nM) on the expression of vasohibin-1 mRNA (normoxia). Cells were stimulated with VEGF (0.1 and 1.0 nM) for 48 h under a normoxic or hypoxic (O_2 1%) condition as described in the *Materials and Methods*. VEGF (0.1 nM) up-regulated the expression of vasohibin-1 at 48 h under normoxia, but VEGF-stimulated induction of vasohibin-1 mRNA expression was not observed under hypoxia. $^*p <$ 0.01 vs 0 h. The values in each column are the means ± SD.

Hypoxia caused by synovial inflammation in RA stimulates HIF. In their immunohistochemical analysis, Hollander *et al.* observed that HIF-1α was up-regulated in synovial CD68+ macrophages prepared from biopsy samples of patients with RA and OA in comparison with healthy controls [29]. In addition, Manabe *et al.* demonstrated that inflammatory cytokines further induced HIF-1 expression in RASFs *in vitro* [23]. Since HIF induces the expression of a broad range of pro-angiogenic factors, hypoxia and inflammatory cytokines are closely associated with angiogenesis in RA. Previous reports utilizing HUVEC demonstrated that vasohibin-1 expression was up-regulated by exogenous VEGF in a time and concentration-dependent manner [13]. Interestingly, hypoxia is known to act as a trigger of both physiological and pathological angiogenesis by inducing VEGF, mediated via HIF [30, 31]. However, a previous study reported that hypoxia did not affect the expression of vasohibin-1, and rather inhibited up-regulation of vasohibin-1 mRNA as well as protein levels upon stimulation by VEGF in ECs [13]. Our results demonstrated that up-regulation of vasohibin-1 was abolished under a hypoxic condition in RASFs, consistent with previous findings in ECs. On the other hand, the expression of vasohibin-1 was down-regulated by inflammatory cytokines (*e.g.*, TNF-α and IL-1)

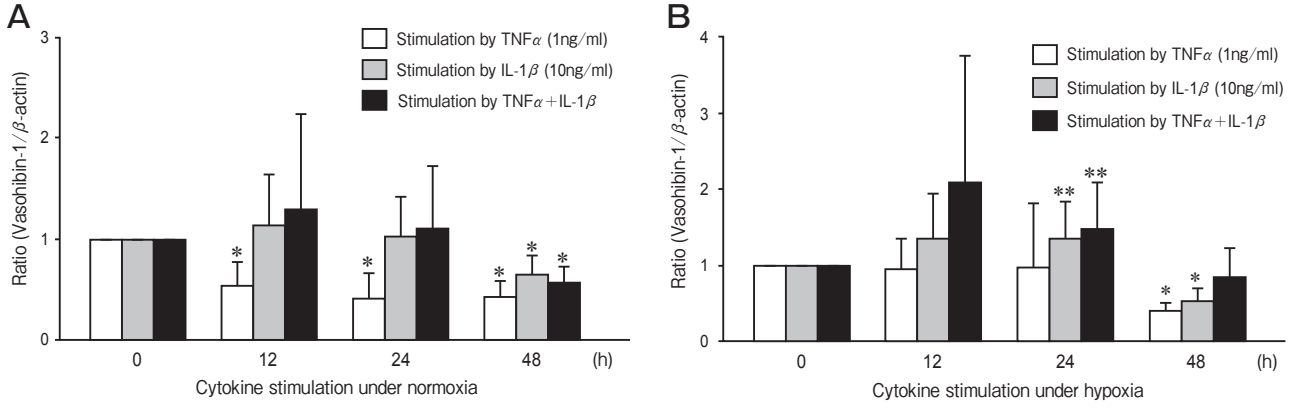

Fig. 5 **A**, Time course of the effects of cytokines on the expression of vasohibin-1 mRNA (normoxia). Cells were stimulated with TNF-α (1 ng/ml) and/or IL-1β (10 ng/ml) for 48 h under a normoxic condition as described in the Materials and Methods. Stimulation by cytokines down-regulated the expression of vasohibin-1; **B**, Time course of the effects of cytokines on the expression of vasohibin-1 mRNA (hypoxia). Cells were stimulated with TNF-α (1 ng/ml) and/or IL-1β (10 ng/ml) under a hypoxic condition (1% O_2) as described in the Materials and Methods. IL-1β with and without TNF-α significantly up-regulated vasohibin-1 expression at 24 h, and TNF-α or IL-1β down-regulated vasohibin-1 expression at 48 h under a hypoxic condition. $^*p < 0.01$ vs 0 h. $^{**}p < 0.05$ vs 0 h. The values in each column are the means ± SD.

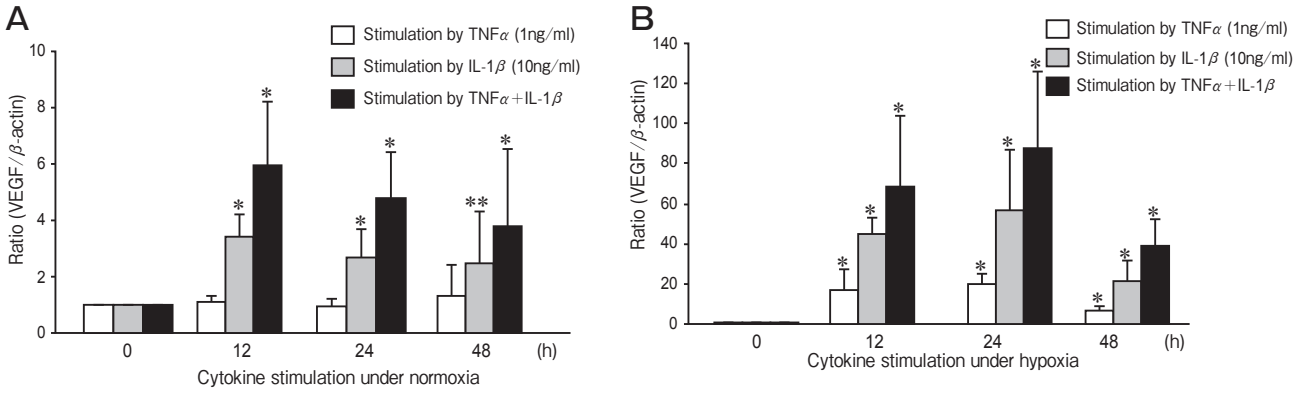

Fig. 6 **A**, Time course of the effects of cytokines on the expression of VEGF mRNA (normoxia). Cells were stimulated with TNF-α (1ng/ml) and/or IL-1β (10ng/ml) for 12–48h. IL-1β with or without TNF-α significantly up-regulated the expression of VEGF mRNA; **B**, Time course of the effects of cytokines on the expression of VEGF mRNA (hypoxia). Cells were stimulated with TNF-α and/or IL-1β for 48h under a hypoxic condition (1% O_2). TNF-α and/or IL-1β markedly up-regulated the expression of VEGF for 12–48h under a hypoxic condition. $^{*}p < 0.01$ vs 0h. $^{**}p < 0.05$ vs 0h. The values in each column are the means ± SD.

alone and was further down-regulated in the presence of VEGF in ECs [13, 32]. In the present study, the expression of vasohibin-1 in RASFs was down-regulated by stimulation with TNF-α or IL-1β under normoxia, consistent with the results observed in ECs [13]. However, the levels of VEGF mRNA were markedly increased by TNF-α and IL-1β under a hypoxic condition, accompanied by an increase in vasohibin-1 mRNA. The discrepancy in regard to the response to cytokines under hypoxia between endothelial cells and RASFs may be, at least in part, attributed to the distinct cell-types. On the other hand, Shen *et al.* reported that increased expression of VEGF in the ischemic retina was accompanied by increased levels of vasohibin-1 mRNA in a murine retinal neovascular model [19]. Sato *et al.* observed a statistically significant correlation between the vitreous concentration of vasohibin-1 and VEGF in patients with proliferative diabetic retinopathy [17]. These results suggest that increased "endogenous" VEGF induced by hypoxia in combination with inflammatory cytokines may up-regulate vasohibin-1 expression in spite of the hypoxic condition, as observed in the RA synovium and in proliferative retinopathy.

Since the expression of vasohibin-1 was correlated with inflammation, the biological roles of vasohibin-1 in regulating anti-angiogenic activity in inflammation of the synovium and joint destruction would seem to be an attractive research topic. Shen *et al.* reported that intraocular injection of recombinant vasohibin-1 or an adenoviral vector containing a vasohibin-1 expression cassette strongly suppressed retinal neovascularization in mice with ischemic retinopathy [19]. Sato *et al.* reported that transfection of Lewis lung carcinoma (LCC) cells with the vasohibin-1 gene did not affect the proliferation of cancer cells *in vitro*, but did inhibit tumor growth and tumor angiogenesis *in vivo* [13]. Thus, the therapeutic application of vasohibin-1 targeting VEGF and angiogenesis in animal models of RA may clarify the biological role of this factor in the progression of rheumatoid arthritis.

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