Hypoxia-inducible Factor Mediates Hypoxic and Tumor Necrosis Factor-α-induced Increases in Tumor Necrosis Factor-α Converting Enzyme/ADAM17 Expression by Synovial Cells*

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Chronic hypoxia and inflammatory cytokines are hallmarks of inflammatory joint diseases like rheumatoid arthritis (RA), suggesting a link between this microenvironment and central pathological events. Because TACE/ADAM17 is the predominant protease catalyzing the release of tumor necrosis factor α (TNFα), a cytokine that triggers a cascade of events leading to RA, we examined the regulation of this metalloprotease in response to hypoxia and TNFα itself. We report that low oxygen concentrations and TNFα enhance TACE mRNA levels in synovial cells through direct binding of hypoxia-inducible factor-1 (HIF-1) to the 5′ promoter region. This is associated with elevated TACE activity as shown by the increase in TNFα shedding rate. By the use of HIF-1-deficient cells and by obliterating NF-κB activation, it was determined that the hypoxic TACE response is mediated by HIF-1 signaling, whereas the regulation by TNFα also requires NF-κB activation. As a support for the in vivo relevance of the HIF-1 axis for TACE regulation, immunohistological analysis of TACE and HIF-1 expression in RA synovium indicates that TACE is up-regulated in both fibroblast- and macrophage-like synovial cells where it localizes with elevated expression of both HIF-1 and TNFα. These findings suggest a mechanism by which TACE is increased in RA-affected joints. They also provide novel mechanistic clues on the influence of the hypoxic and inflammatory microenvironment on joint diseases.

Tumor necrosis factor-α converting enzyme (TACE)2 or ADAM17 was initially described as the predominant enzyme responsible for the physiological cleavage of membrane-anchored tumor necrosis factor-α (TNFα), releasing it in soluble form (1, 2). This enzyme belongs to the ADAM (a disintegrin and metalloprotease domain) family of transmembrane, multidomain zinc metalloproteinas (3) and is expressed in a wide variety of cell types, including TNFα non-producing cells (1). Beside TNFα, TACE was also shown to solubilize a wide variety of proteins including the receptors TNFR-I and TNFR-II (4), interleukin-1RII (5), interleukin-6R (6), and macrophage/colony-stimulating factor-R (7), the cytokine transforming growth factor-α (4), members of the membrane-bound epidermal growth factor family (4), the Notch receptor (8), the chemokine fractalkine (9), L-selectin (4), and the β-amloid precursor protein (10). The importance of TACE substrates in a variety of physiological functions, including development, is underscored by the fact that in vivo inhibition of TACE or disruption of the TACE gene results in the death of mice between embryonic day 17.5 and the first day after birth, due to a number of developmental defects. In addition, the implication of TACE substrates in immunoregulation has made this enzyme an efficient therapeutic target in the treatment of a number of pathological conditions including airway inflammation, cancer, and arthritis.

Because of the pathophysiological importance of TACE-mediated shedding, several studies have addressed the mechanism of TACE regulation. Surprisingly, few agents, which are known to enhance ectodomain shedding of proteins have been documented for their role in the regulation of TACE expression. Increased levels of TACE mRNA were observed in murine retinal endothelial cells or human endothelial cells exposed to vascular endothelial growth factor (11) or TNFα (12), respectively. In addition, several studies have shown that TACE expression is elevated under pathological conditions. In mammary tumor tissues, TACE protein levels are higher than in normal tissues (13, 14). Also, elevated levels of TACE mRNA expression were found in osteoarthritis (15) and rheumatoid arthritis (RA)-affected cartilage as compared with normal cartilage (16), suggesting that abnormal TACE activity may contribute to the development of several pathological conditions, including RA. Despite this, the mechanisms involved in TACE regulation under pathological conditions remain unknown.

It has been known for many decades that hypoxic conditions prevail in RA-affected joints. In fact, even in normal joints, the
high cavity pressure upon movement and the scarcity of blood vessels make the oxygen tension lower than in other tissues (17, 18). Also, due to inflammation in the growing synovial membrane during RA development, there is increasing metabolic needs in the arthritic joints, resulting in insufficient oxygen uptake (17, 19). It is well known that hypoxia induces the expression of a number of transcription factors involved in arthritis, such as ETS-1 (20), nuclear factor-κB (NF-κB) (21), and hypoxia-inducible factor-1 (HIF-1) (22). Among these, HIF-1 was shown to regulate the expression of a variety of genes implicated in arthritis, an important one being vascular endothelial growth factor, a growth factor involved in the perpetuation of the abundant vasculature found in the synovia (23). HIF-1 is composed of two subunits, HIF-1α and HIF-1β/ARNT. Both subunits are members of the basic-loop-helix-PAS protein family (24). Whereas HIF-1β is constitutively expressed, HIF-1α undergoes proteasomal degradation under normoxic conditions. On the other hand, it is stabilized under oxygen deprivation to eventually translocate to the nucleus where it heterodimerizes with HIF-1β. The resulting HIF-1 complex binds to hypoxia-responsive elements (HRE), composed of the HIF-1 binding sequence and HIF-1 ancillary sequence, to regulate gene expression (25, 26).

Beside hypoxia, several lines of evidence indicate that HIF-1α can accumulate and be transcriptionally activated during normoxia by cytokines, growth factors, hormones, nitric oxide, and loss of the tumor suppressor protein pVHL or PTEN (27). Whereas HIF-1 is composed of two subunits, HIF-1α and HIF-1β/ARNT. Both subunits are members of the basic-loop-helix-PAS protein family (24). Whereas HIF-1β is constitutively expressed, HIF-1α undergoes proteasomal degradation under normoxic conditions. On the other hand, it is stabilized under oxygen deprivation to eventually translocate to the nucleus where it heterodimerizes with HIF-1β. The resulting HIF-1 complex binds to hypoxia-responsive elements (HRE), composed of the HIF-1 binding sequence and HIF-1 ancillary sequence, to regulate gene expression (25, 26).

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EXPERIMENTAL PROCEDURES

Cell Culture—The murine macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (FBS) (Sigma), 1 mm sodium pyruvate (HyClone, Logan, UT), and 40 μg/ml garamycin (Schering Canada). During following passages, FBS concentrations were gradually decreased to 10%.

The Hepa-1 c1c7, derived from Hepa-1 c1c7, was a generous gift from Dr. Oliver Hankinson (University of California, Los Angeles, CA). All these cell lines were cultured in minimal essential medium (Invitrogen) supplemented with 10% FBS (Sigma) and 40 μg/ml garamycin (Schering Canada).

The protocol used for the isolation of rat synovial cells is a modified version of previously described methods (30). Briefly, the synovial membranes were isolated from the knees of healthy specific pathogen-free inbred Lewis female rats (Harlan Sprague-Dawley) under sterile conditions, and were digested in a phosphate-buffered saline (PBS) collagenase type IV (0.2 mg/ml) solution (Sigma) for 2 h in a humidified incubator containing 5% CO2 at 37 °C. The cells were then washed by centrifugation in sterile PBS. The synovial cells were allowed to adhere to 100-mm Petri dishes containing Dulbecco’s modified Eagle’s medium/F-12 (Invitrogen), 20% FBS (Sigma), and 40 μg/ml garamycin (Schering Canada). During following passages, FBS concentrations were gradually decreased to 10%.

All cell cultures were kept in a humidified 95% O2, 5% CO2 incubator at 37 °C. For hypoxic experiments, cells were serum starved and placed in a sealed humidified chamber maintained at 1% O2, 5% CO2 and balanced in N2 for different time periods as indicated in the figure legends.

Plasmids for Transient Transfections—The vectors pcDNA3-HIF-1α, pcDNA3-HIF-1β, and (HRE)3-Luc were generously provided by Dr. Darren Richard (Centre de Recherche de l’Hôpital-Dieu). The IκBSR was kindly provided by Dr. C. Jobin (University of North Carolina, Chapel Hill, NC); pNFκB-Luc was from Stratagene. The pcDNA3-hTNFα was a generous gift from Dr. Franck Perietti (Laboratoire d’hématologie, Marseille, France).

The TACE promoter sequence (−2304 to −1 bp) was amplified by PCR using the following oligonucleotide pair: (forward) 5′-GGCCGAGCTCGACTAAGATAAAGGCTTGTAGCC-3′ (the underline indicates SacI cleavage site) and (reverse) 5′-GGCCCTCGAGGTTCCCGTCCACCACCTCTTCTTG-3′ (the underline indicates XhoI cleavage site). The DNA extracted from C57BL/6J mouse cells was used as template. PCR conditions were: initial denaturation at 95 °C for 5 min, 35 cycles of amplification with a denaturation step at 95 °C for 15 s, an annealing temperature of 59 °C for 60 s, and an extension step at 72 °C for 2 min, followed by a final extension at 72 °C for 8 min. The resulting fragment was inserted into the pGKL basic vector (Promega) and sequenced. TACE promoter 5′ deletants, pTACE-1567, -991, -903, and -410 were generated by digestion of the pTACE-Luc with the appropriate restriction enzymes. The shorter TACE promoter, pTACE-H6, was generated by PCR amplification using the same technique as described above for the pTACE-Luc vector. The primer pair used was: (forward) 5′-GGCCGAGCTCGGCGGCGGCGGAGGGAAGGT-3′ (the underline indicates SacI cleavage site) and (reverse) 5′-GGCCCTCGAGGTTCCCGTCCACCACCTCTTCTTG-3′ (the underline indicates XhoI cleavage site) and sequenced.

The H3-HIF-1 binding and H4-HRE sequences located at positions −960 and −607 bp, respectively, within the TACE promoter, were mutated by PCR. Distinct mutations were generated by replacing the H3 motif 5′-AATGTCG-3′ by 5′-AATGGCC-3′ and/or the H4 motif 5′-GCACACCCGATCCG-
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CGTGG-3’ by 5’-GGCCACCCGATCCCCGGTGG-3’ (the mutated base pairs are underlined). The integrity of these mutants was verified by sequencing.

**Luciferase Assays**—Cells were transiently transfected by the CaPO₄ precipitation technique using a Mammalian Cell Transfection Kit (Specialty Media, Lavallette, NJ) as previously described (31). Briefly, cells were transfected with 2 μg/well of vectors as indicated in the figure legends. Twenty-four hours following transfection, cells were serum starved 3 h prior to overnight stimulation in hypoxic conditions (1% O₂) with or without murine TNFα (Peprotech Canada Inc., Ottawa, ON, Canada) as indicated in the figure legends. Cell lysates were assayed for luciferase activity as previously described (31). The control pGL2-Basic vector was routinely used as an internal control of transfection.

**Western Blot Analysis**—Cells were seeded in 100-mm Petri dishes at a density of 1 x 10⁶. The following day, they were serum starved prior to stimulation with 20 ng/ml of murine TNFα (Peprotech Canada Inc.) under normoxic (21% O₂) or hypoxic (1% O₂) conditions for 16 h. For nuclear extract preparation, we used a modification of the protocol described by Dignam et al. (32, 33). Total cell lysates and immunoblotting were performed as previously described (34). The membranes were probed overnight with the following primary antibodies: anti-TACE at 1:1000 (Chemicon International, Temecula, CA), anti-HIF-1α at 1:250 (Novus Biologicals, Littleton, CO), anti-actin at 1:5000 (Sigma), or anti-nucleoporin p62 (Nup62) at 1:1000 (BD Transduction Laboratories). The secondary antibodies were peroxidase-conjugated anti-rabbit for TACE and actin or peroxidase-conjugated anti-mouse for HIF-1α and Nup62 (Amersham Biosciences, Baie d’Urfe, QC, Canada). Blots were developed using ECL Western blotting detection reagents (Amersham Biosciences).

**RT-PCR and Quantitative Real-time PCR**—One million cells were seeded in 100-mm Petri dishes. The following day, they were serum starved prior to stimulation with 20 ng/ml of murine TNFα or incubation in normoxia (21% O₂) or hypoxia (1% O₂) for the indicated time periods. In selected experiments, cells were pretreated with 20 μg of actinomycin D (Merck, Freiburg, Germany) 30 min before stimulation. Total RNA was isolated using the TRI-Reagent protocol as previously described (35). Reverse transcription was achieved using random decamer primers (Ambion Inc., Austin, TX). The PCR were performed using the following primer pairs: TACE (forward) 5’-ATCCATGAGCTCTTGGTGAAA-3’; (reverse) 5’-ATCCATGAGCTCTTGGTGAAA-3’; ADAM10 (forward) 5’-GTGCCAGTACAGGCTCTTGC-3’; (reverse) 5’-CACAGTACCTCCTGGAATGTC-3’; MMP-1 (forward) 5’-TCTGGGAATGTGACACCCGC-3’; (reverse) 5’-GGGCCTGTGGGAGATGTGTA-3’; MMP-13 (forward) 5’-GATGATCCCACCTTAGG-3’; (reverse) 5’-GATGATCCCACCTTAGG-3’; ADAM10 (forward) 5’-GATGATCCCACCTTAGG-3’; (reverse) 5’-GATGATCCCACCTTAGG-3’. 40 cycles of amplification with a denaturation step at 95 °C for 15 s, an annealing temperature of 60 °C (MMP-1 and MMP-13) or 68 °C (TACE and ADAM10) for 45 s, and an extension step at 72 °C for 30 s.

**Confocal Microscopy**—Synoviocytes were cultured on coverslips in complete medium. Cells were serum starved for 3 h before overnight stimulation in normoxia or hypoxia (1% O₂) in the presence or absence of 20 ng/ml of murine TNFα. Cells were then prepared for immunofluorescence staining as previously described (34). Briefly, cells were washed with cooled PBS and then fixed for 15 min in precooled (−20 °C) methanol/acetone (30/70). After a 15-min rehydration in PBS, cells were permeabilized in PBS with 0.1% Triton for 5 min and nonspecific binding was blocked by a 20-min incubation step in PBS containing 2% bovine serum albumin. Fixed cells were then incubated overnight with anti-TACE primary antibody (1:100), washed in PBS, and incubated for 1 h with anti-Rb-Cy2 secondary antibody (1:200). Cellular localization of TACE was examined with a scanning confocal microscope (NORAN Instruments Inc., Middleton, WI) equipped with a krypton/argon laser. Images were obtained and treated with INTERVISION software (NORAN Instruments Inc.) on a Silicon Graphics O2-work station. Image processing and surface quantification of pixel intensities were done using the ImageJ public domain software (Wayne Rasband, National Institutes of Health, Bethesda, MD). For fluorescence quantification, images were processed with ImageJ software for a total of 35 slices per cell. Data were expressed as the sum of pixel intensities (SPI) for 5 cells/experimental condition.

**TNFα Measurement**—Synoviocytes were transiently transfected by electroporation using the NucleoFector System from Amasa Biosystems. Five μg of human pcDNA3-TNF were transfected in 2 x 10⁶ cells using the U23 nucleofection program. Twenty-four hours after transfection, cells were serum starved and incubated overnight under normoxic or hypoxic conditions (1% O₂) with or without 20 ng/ml of murine TNFα. Supernatants were harvested and cell lysates were prepared as described above. Amounts of human TNFα were measured in cell lysates and in culture media using an enzyme-linked immunosorbent assay kit, according to the manufacturer’s protocol (R&D Systems, Minneapolis, MN).

**Chromatin Immunoprecipitation**—Synoviocytes were seeded at a density of 2 x 10⁶ cells in Petri dishes, serum starved, and exposed or not to either TNFα or hypoxia for 16 h. The chromatin immunoprecipitation assays were performed using the EZ ChIP assay kit (Upstate, Lake Placid, NY) according to the manufacturer’s protocol. Briefly, cross-linking was achieved by adding formaldehyde to a final concentration of 1% and incubating at room temperature for 10 min. Cells were washed with cold PBS, collected, pelleted, and resuspended in lysis buffer. Cell lysates were sonicated to achieve DNA sizes ranging from 200 to 1000 bp. Diluted supernatants were then pre-cleared by incubating with salmon sperm DNA/protein A-agarose, 50% slurry for 60 min at 4 °C and treated with anti-HIF-1α antibodies (Novus Biologicals number 100–123) or isotype-matched antibodies (Sigma) overnight at 4 °C. Immunocomplexes were collected with salmon sperm DNA/protein A-agarose, 50% slurry, washed, eluted, and cross-linkage was
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FIGURE 1. Regulation of the TACE gene by TNFα and hypoxia. Synoviocytes were serum starved and exposed to normoxia (Norm) (21% O2) or hypoxia (Hyp) (1% O2) in the presence or absence of TNFα (20 ng/ml). mRNA was extracted, reverse transcribed, and real-time PCR was performed. A, cells were stimulated for 4, 8, and 16 h and TACE mRNA levels determined by real-time RT-PCR. B, cells were pretreated with 20 μg/ml actinomycin D, 30 min before exposition to the indicated stimuli for 16 h and TACE mRNA levels were determined by real-time RT-PCR. C, luciferase activity of synovial cells transfected with a full-length TACE-luciferase reporter and exposed to normoxic or hypoxic environments with 0, 2, or 20 ng/ml of TNFα for 16 h. A and B, data are expressed as -fold increase relative to normoxic control cells. Error bars represent the mean ± S.E., n = 3, * p < 0.05; **, p < 0.005, compared with normoxic cells.

RESULTS

Hypoxia and TNFα Increase TACE Protein Levels and Activity by Up-regulating Its Transcription—Few stimuli are known to modulate TACE mRNA expression. Among these, TNFα was shown to increase TACE mRNA levels in endothelial cells, suggesting a role for this cytokine in the regulation of TACE in other cell types (12). It is well known that large amounts of TNFα are present in the synovial membrane of arthritic joints, a diseased tissue also characterized by the presence of numerous hypoxic areas (37, 38). To define the impact of such an inflammatory/hypoxic milieu on TACE expression, cultured synovial cells were exposed to low oxygen concentrations in the presence or absence of TNFα, and TACE mRNA expression was assessed by real-time PCR. Results indicated that although reversed by heating at 65°C. The eluates were then digested with proteinase K-treated DNA as a template for PCR amplification of the target sites in TACE promoter. Primer sequences used for amplification of relevant HRE sites-containing regions were: (forward) 5’-AGTGAACATTATACCTCGG-3’ and (reverse) 5’-ACTGACATTATAGCCCTGGT-3’ for the H3 region and (forward) 5’-GTGACATTATATGCTTG-3’ and (reverse) 5’-CGTATTAGAGTCCCCCCTTGC-3’ for the H4 site.

Immunohistochemistry—Human synovial tissue samples were obtained from three patients who fulfilled the American College of Rheumatology revised criteria for RA (36) and one osteoarthritis patient undergoing total joint replacement surgery, with the collaboration of Dr. Manuela Pelmus from the Department of Pathology (Faculty of Medicine, Université de Sherbrooke). The rheumatoid arthritis patients, one male and two females, were positive for the rheumatoid factor. Paraffin-embedded synovial tissue sections were deparaffinized and endogenous peroxidase was quenched with 3% H2O2 in methanol for 30 min. Sections were permeabilized in 2% saponin for 30 min at 4°C, boiled for 10 min for antigen retrieval, and allowed to cool for another 10 min. Bovine serum albumin 2% blocking solution (supplemented with 5% milk proteins and 10% goat anti-serum for TACE) was added for 30 min before incubation with primary antibodies for 90 min: anti-TACE 1:300, anti-HIF-1 1:50, anti-TNFα 1:50 (generously provided by Dr. Fred Finkelman, Veterans Affairs Medical Center, Cincinnati, OH), and anti-CD68 1:80 (Serotec, Oxford, UK). Following washes with PBS, sections were incubated with biotinylated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for 30 min, washed again with PBS, and incubated 15 min with peroxidase-conjugated streptavidin (Jackson ImmunoResearch). Following washes in PBS, the color was revealed by an incubation of 2 min with 3,3′-diaminobenzidine (Sigma) and the tissues were counterstained with hematoxylin. For TACE and HIF-1 double staining experiments, HIF-1 was stained using the peroxidase technique and TACE with the alkaline phosphatase technique (DakoCytomation K0391) according to the manufacturer’s instructions.

Statistical Analysis—Results were analyzed using unpaired Student’s t test.
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TNFα has a noticeable but nonsignificant increase in TACE mRNA expression at 4 h, a 3.1- and 4.3-fold increase was observed at 8 and 16 h, respectively (Fig. 1A). Similarly, hypoxia-induced stimulation was significantly augmented at 8 and 16 h, reaching up to 3.6-fold induction at 16 h compared with normoxic cells. At this time point, the combination of TNFα and hypoxia resulted in a 9.2-fold induction of TACE mRNA accumulation, indicating an additive effect. To determine whether TACE mRNA expression is regulated at the transcriptional level, synoviocytes were pretreated with actinomycin D, 30 min before stimulation. The addition of this mRNA synthesis inhibitor completely blocked TACE mRNA accumulation induced by hypoxia, TNFα, or their combination (Fig. 1B). TACE transcription is mediated by a 2304-bp promoter located within its 5′-flanking region (39). It presents features of regulated promoters, such as multiple AP2 and Sp1 transcription factors binding sites as well as a CCAAT box. To determine whether TNFα and/or hypoxia regulate TACE promoter activity, the full-length promoter sequence (pTACE-Luc) or the control plasmid (pGL2-Luc) were transiently transfected in synoviocytes. The cells were then incubated overnight with 0, 2, or 20 ng/ml of TNFα under normoxic or hypoxic conditions, and luciferase activity was measured. TNFα-induced TACE promoter activity in a concentration-dependent manner with a 2.6-fold induction observed at 20 ng/ml (Fig. 1C). Also, hypoxia induced TACE promoter activity to similar levels as observed with the highest dose of TNFα (20 ng/ml), with an additive effect upon combination of both stimuli. We therefore concluded that both hypoxia and TNFα enhance TACE mRNA levels through transcriptional activation of its 5′ promoter region.

To define whether the observed changes in TACE mRNA accumulation are reflected at the protein level, Western blot analysis of cell lysates were performed. Results indicated that similarly to mRNA changes, both TNFα- and hypoxia-induced TACE protein accumulation at 16 h with an additive effect observed upon their combination (Fig. 2A). Previous studies have demonstrated that steady-state localization of TACE in unstimulated COS-7 cells is mainly in the perinuclear compartment, with some diffuse localization consistent with surface and endoplasmic reticulum staining (40). To evaluate the cellular localization of TACE in synovial cells, we performed immunofluorescence staining of the cells cultured overnight under normoxic or hypoxic conditions and in the presence or

FIGURE 2. Induction of TACE protein in response to TNFα and hypoxia. Synoviocytes were exposed to hypoxic conditions in the presence or absence of 20 ng/ml of TNFα for 16 h. A, immunoblotting of cell lysates using TACE-specific antibodies. Shown is a representative result; n = 3.8, confocal microscopy. Synoviocytes were prepared for confocal microscopy as indicated under “Experimental Procedures.” Lines numbered 1–4 represent pictures captured within the first, second, third, and fourth cell quarter, respectively. C, pseudocolor representation of pixel intensities recorded for line 2 pictures.
absence of TNFα. Confocal microscopy analysis using TACE-specific antibodies revealed that TACE was mainly localized in the perinuclear compartment with some expression at the cell surface and in endoplasmic reticulum of synovial cells. TACE staining was specific as demonstrated by the lack of fluorescence in cells labeled with isotype-matched IgG (supplementary Fig. S1). Under TNFα or hypoxic stimulation, increasing levels of immunoreactive TACE were observed (normoxia: 3.7 ± 0.1 × 10⁶ SPL; n = 5; TNFα: 5.3 ± 0.4 × 10⁶ SPL (p = 0.01 compared with normoxia; n = 5); hypoxia: 6.2 ± 0.3 × 10⁶ SPL (p < 0.001 compared with normoxia; n = 5) that was further enhanced with the combination of both stimuli (7.7 ± 0.3 × 10⁶ SPL (p = 0.008 compared with normoxia; n = 5)).

TACE is known to be the main enzyme responsible for TNFα shedding from the cell surface (1). To investigate whether the levels of TACE induction by TNFα and/or hypoxia influence TACE proteolytic activity, we evaluated the impact of these stimuli on TNFα released from the cell surface. Transmembrane and soluble TNFα levels, produced by synoviocytes over-expressing human TNFα, were evaluated using an enzyme-linked immunosorbent assay specific to human TNFα. This prevented background contamination with the murine TNFα used for cell stimulation. As observed in Fig. 3, either TNFα or hypoxia stimulations resulted in a significant increase in TNFα release, with an additive effect observed upon co-stimulation. We then concluded that TACE, produced under these stimulations, is proteolytically active.

Synovial Cells Produce Active HIF-1 Upon Hypoxia and TNFα Treatment—Hypoxia is known to induce/activate various transcription factors such as ETS-1 (20), NF-κB (21), and HIF-1 (22). Among these, HIF-1 is of particular interest because it is a critical factor involved in both hypoxic and inflammatory responses (41). In particular, TNFα was shown to induce nuclear accumulation and activity of HIF-1 (28, 42, 43). To determine whether hypoxia and/or TNFα modulate HIF-1 levels in synovial cells, nuclear extracts of cells stimulated for 16 h were subjected to Western blotting using an antibody specific to HIF-1α, the HIF-1 regulatory subunit. Results indicated that both TNFα and hypoxia induced nuclear accumulation of HIF-1α, with an additive effect observed with both stimulations (Fig. 4A). To determine whether HIF-1α produced under these conditions is transcriptionally active, synovial cells were transfected with a luciferase reporter gene driven by three repeats of the HRE retrieved from the erythropoietin gene (HRE)₃-Luc, and luciferase activity was measured. As presented in Fig. 4B, the luciferase activity closely paralleled HIF-1 protein accumulation. Therefore, hypoxia and TNFα induce HIF-1α nuclear accumulation, resulting in an efficient transactivation of HRE cis-acting elements.

HIF-1 Is Required for the Hypoxic/TNFα Responses of the TACE Promoter—Computer-assisted analysis of the TACE promoter sequence revealed the presence of four putative HIF-1 binding sites (HIF-1 binding sequence) and two hypoxia response elements (HRE) composed of one HIF-1 binding sequence and one HIF-1 ancillary sequences (HAS) (Fig. 5A). These binding sites are characteristic of HIF-1-regulated genes (44), suggesting that HIF-1 may regulate TACE promoter activity under hypoxic/inflammatory conditions. To determine whether the TACE promoter bears functional HIF-1 binding sites, the pTACE-Luc construct was co-transfected in synoviocytes together with plasmids encoding HIF-1α and HIF-1β, or a control empty vector. Following oxygen deprivation for 16 h, luciferase activity was assayed. Results presented in Fig. 5B show that exogenous HIF-1 stimulated TACE promoter activity to significantly higher levels, either in normoxic or hypoxic conditions, suggesting the presence of functional binding sites.

To identify promoter regions implicated in the HIF-1/hypoxic responses, 5′ deletion constructs of the pTACE-Luc vector were used in luciferase assays. As shown in Fig. 5C, hypoxia-mediated increased promoter activity was maintained in deletion constructs up to position −991 base pairs, as they demonstrated comparable levels of luciferase activity as the
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A) Sites HRE
H1 5'-CAAGTGGTATGGCCCATGCGATCTCCTA-3'
H2 5'-TGAAGTGTACAGTGAAGTTTATA-3'
H3 5'-AAATGTCAGGAATCGTGAAGAA-3'
H4 5'-CAGCAGACCGATGCGGCGG-3'
H5 5'-CCAGGACTCCCGAAGCTCTT-3'
H6 5'-CAAGTGTGATGGCCCATGCCATCATCCTA-3'

B) Control HIF-1
pTACE-2304
pTACE-1567
pTACE-991
pTACE-903
pTACE-410
pTACE-121

C) Hypoxic Induction

FIGURE 5. Functional HIF-1 binding sites within the TACE promoter sequence. A, schematic representation of putative HIF-1 recognition sequences within TACE promoter constructs shortened in 5' . B, luciferase activity from synovial cells transfected with the full-length TACE-luciferase construct (pTACE-2304) in the presence or absence of pCDNA3 encoding HIF-1α and HIF-1β and exposed to normoxic or hypoxic conditions for 16 h. Data are expressed as the mean ± S.E., n = 3, * p < 0.05, compared with control transfected cells. C, luciferase activity of cells transfected with the various TACE promoter constructs and exposed to normoxia or hypoxia for 16 h. Data are expressed as -fold hypoxic induction relative to the respective TACE promoter fragments exposed to normoxia. Mean ± S.E., n = 4, ** p < 0.005 compared with the hypoxic induction obtained with the full-length promoter.

intact pTACE-Luc vector. However, deletion of the −991 to −903 region (that contains the H3 site) reduced the hypoxic response to 57% and further deletion to position −410 (H4 site) inhibited the activity to 19%. These results indicate the presence of important hypoxia/HIF-1-sensitive sites within the −991 to −410 region of the TACE promoter.

To define the importance of the H3 and H4 sites for hypoxia and TNFα responses of the −991 to −410 TACE promoter region, site-directed mutagenesis was performed on the two putative HIF-1 binding sites, resulting in three distinct mutants, TACE-H3mut, TACE-H4mut, and TACE-H3/H4mut. As illustrated in Fig. 6A, TACE promoter activity, under hypoxic conditions was reduced to 38 and 5% of the induction obtained with the wild-type promoter when the H3 and H4 sites were mutated, respectively. Mutation of both H3 and H4 sites reduced the hypoxia-induced promoter activity to similar levels as observed with the H4 mutant levels. Similarly, the activity of the TACE-H3 and TACE-H4 mutants was mostly abolished in response to TNFα (Fig. 6B). These results indicate that the H4 and to a lesser extent the H3 HIF-1 binding sites are critical for the hypoxic/inflammatory response of the TACE promoter.

To confirm that HIF-1 directly binds to H3,H4 sites in a chromatin environment, chromatin immunoprecipitation assays were performed using synoviocytes incubated overnight under normoxic or hypoxic conditions. Cross-linked synovial cell lysates were immunoprecipitated with anti-HIF-1α or control antibodies and PCR was used to amplify 180- or a 194-bp fragment of TACE promoter using antibodies that flank the H3 and H4 sites, respectively. The results revealed strong HIF-1 binding activity at both of these sites in hypoxic cells with more moderate binding observed in TNFα-treated cells (Fig. 6C). Also, only faint binding was observed in the normoxic chromatin environment, which is consistent with the low levels of HIF-1 protein observed under this condition (Fig. 4A).

To determine the necessity of endogenous HIF-1 for TACE gene regulation by hypoxia and TNFα, we used two HIF-1-deficient cell models, the mouse hepatoma cell lines Hepa-1 c1c7 and its HIF-1β mutant counterpart, Hepa-1 c4, as well as the wild-type and HIF-1α knock-out mouse embryonic fibroblasts. Results in Fig. 7A indicate that transfection of the pTACE-Luc in Hepa-1 c1c7 cells transactivates the reporter gene in response to hypoxia, TNFα, or their combination to similar levels as the ones observed in synovial cells (Fig. 1C). In contrast, the hypoxic induction was completely inhibited in HIF-1β-deficient Hepa-1 c4 cells, whereas TNFα stimulation was partly abrogated. Complementation of Hepa-1 c4 cells with functional HIF-1β restored both hypoxic and TNFα induction of the TACE promoter activity, confirming that the impaired responsiveness of HIF-1β-deficient Hepa-1 c4 cells was specifically due to the lack of HIF-1 molecules. Similar findings were observed for TACE mRNA induction in wild-type and HIF-1α-deficient mouse embryonic fibroblasts cells (Fig. 7B), confirming the complete and partial requirement of endogenous HIF-1 for hypoxia and TNFα TACE gene regulation, respectively.
NF-κB Is Involved in TNFα-mediated TACE Promoter Regulation—One of the main TNFα signaling pathways is through the activation of NF-κB (45), a transcription factor that was also reported to be induced by hypoxia under specific conditions (21, 46, 47). Consistent with previous studies (48) and results shown in Fig. 4B, TNFα induces a 2–3-fold increase in both NF-κB and HIF-1 transcriptional activity in synovial cells as measured by a NF-κB- and HIF-1-dependent luciferase reporter assay, respectively (Fig. 8, A and B). In contrast to the strong HIF-1 induction (Fig. 8B), hypoxic conditions repeatedly failed to activate NF-κB in these cells (Fig. 8A). To define whether NF-κB was involved in TNFα-mediated regulation of the TACE gene, we used both SC-514, a highly selective inhibitor of IKK-2 (49) and an IκB super repressor (IκBSR). For the IκBSR, the IκBα gene was mutated so that the resulting protein cannot be phosphorylated and degraded, thereby keeping NF-κB in an inactive form (50). As shown in Fig. 8C, TACE reporter gene activity was increased following TNFα treatment, in cells co-transfected with the control vector, but strongly inhibited when IκBSR was overexpressed or when cells where exposed to SC-514, using conditions that prevented NF-κB activation (Fig. 8A). Also, using the same NF-κB pathway blockers, the hypoxic stimulation of TACE was mostly spared (Fig. 8C). This indicates the requirement of the NF-κB transduction pathway in TNFα-induced, but not hypoxia-induced TACE regulation.

Immunohistochemical Analysis of TACE and HIF-1 Expression in Rheumatoid Arthritic and Osteoarthritic Synovium—To explore the in vivo relevance of our findings, we next investigated the immunohistochemical expression of HIF-1α, TACE, and TNFα in synovium from representative RA and OA patients. Consistent with previous studies (51, 52), we observed, in a representative staining, a strong expression of HIF-1α in the synovial lining layer of the RA sample, compared with OA, whereas more sparse expression was detected in the synovial sublining of both RA and OA samples (Fig. 9A). Likewise, the staining intensity for TACE and TNFα expression was also strong in the lining layer of the RA sample with a more intense expression of the TACE protease compared with HIF-1 or TNFα. Also, the overall expression of all three proteins was increased in RA when compared with OA tissues (Fig. 9B). Because preliminary data indicating that both HIF-1 and TACE are regulated in
Regulation of the TACE Gene by TNFα and Hypoxia

In this report, we provide evidence that TACE is induced upon exposure to hypoxia and TNFα, and we identified a region within its promoter that induces TACE transcriptional regulation, through enhanced HIF-1 binding. Of significance, we demonstrate that such hypoxic/TNFα regulation of TACE correlates with an increase in the shedding rate of TNFα, a cytokine deeply involved in RA development. Our study reveals a mechanism by which TACE could be regulated in vivo and suggests an intimate cross-talk between the inflammatory microenvironment of the joint and the HIF-1 system.

To elucidate the molecular mechanisms involved in the hypoxic and TNFα induction, we cloned and analyzed the promoter region of the TACE gene. This allowed us to identify two functional hypoxia-responsive elements, H3 and H4, located within the −991 to −410 region of the promoter. Mutation of one or both of these elements resulted in an impaired response to hypoxia or TNFα, demonstrating that the H3 and H4 HRE sites are required for the transcriptional regulation of TACE gene expression. We also observed that the proximal −410 to −121 region of the TACE promoter was necessary for basal promoter activity in synovial cells. These results are consistent with previous findings indicating that the −290 to −1 proximal region of the TACE promoter has strong promoter activity in mouse macrophage cells and appeared to be essential for constitutive mRNA expression (39). They also provide original mechanistic information on the inducible nature of the TACE gene, which is the implication of the −991 to −410 untranslated 5’ region for the hypoxic regulation of this promoter.

To confirm the implication of endogenous HIF-1 in hypoxic induction of the TACE promoter, we used two cellular systems lacking functional HIF-1, the hepatoma cell line Hepa-1 and mouse embryonic fibroblasts. In these systems, we showed that HIF-1 is required by hypoxia to induce TACE expression because its absence resulted in a loss of hypoxia-induced TACE mRNA expression and promoter activity. However, HIF-1 is partly required by TNFα to regulate TACE expression.

Our results also indicate an important role for NF-κB in TACE regulation by TNFα. The pivotal role of NF-κB in inflammatory responses is well established, and NF-κB activation is considered a hallmark of RA pathology. For instance, it has been demonstrated that NF-κB activation is

![FIGURE 8. NF-κB is implicated in TNFα but not in hypoxia, mediated induction of TACE promoter activity.](image)

Luciferase activity from synoviocytes co-transfected with: A, pNFκB-Luc in the presence or absence of plxbBSR; B, a (HRE)3-Luciferase construct; or C, pTACE, and stimulated overnight with 20 ng/ml of TNFα and/or hypoxia. Data are expressed as the mean ± S.E., n = 3, *p < 0.05; **p < 0.005, compared with non-stimulated cells.

murine and human macrophage cell lines in vitro (see supplementary material Fig. S2), we next used a macrophage marker, CD68, to identify which cells of the synovial membrane are expressing TACE. As observed in Fig. 9B, CD68 positive macrophages were more abundant in RA than in OA samples and are present in the lining layer of the tissues, where they localize with TACE at the same areas of adjacent tissue sections. Together, this indicates that TACE is expressed in macrophage-like synoviocytes and presumably in fibroblast-like synoviocytes, the two predominant cell types within the synovial lining layer of RA tissues. The results also suggest that both HIF-1 and TNFα localize at similar tissue areas as the TACE enzyme, suggesting their potential participation in the in vivo TACE regulation.

DISCUSSION

In this report, we provide evidence that TACE is induced upon exposure to hypoxia and TNFα, and we identified a region
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significantly higher in RA synovium that in osteoarthritis synovium (53, 54). Furthermore, immunohistochemical analysis has demonstrated nuclear translocation of the p50 and p65 NF-κB proteins in the synovial lining layer (54). In cultured synovial cells, results from this study as well as work from others (55, 56), have shown that NF-κB is activated upon TNFα stimulation. In these cells, NF-κB was shown to induce the production of various metalloproteases as well as many cytokines, such as interleukin-6 and interleukin-8, through binding to consensus NF-κB elements within their promoter regions (56, 57). Computer-assisted analysis of the TACE promoter sequence failed to detect conventional consensus DNA-binding sites for NF-κB but 3 inverted (3′ to 5′ direction) motifs were observed within the distal as well as the proximal regions of the promoter. Preliminary data indicates that oligonucleotides encompassing the more distal putative NF-κB displays TNFα-inducible complex formation in the electrophoretic mobility shift assay that migrates at the same position as complexes from control oligonucleotides (NF-κB from the human immunodeficiency virus promoter) (data not shown) suggesting that NF-κB directly binds to this DNA site.

Several studies indicate that TACE levels are elevated in RA joints compared with osteoarthritis or normal articulations, suggesting that abnormal TACE activity contributes to TNFα action in RA pathogenesis (16, 58, 59). However, the mechanisms underlying TACE regulation remain unknown. We demonstrate herein that hypoxia and TNFα clearly act through a common HIF-1 pathway together with the NF-κB pathway (TNFα) to increase the expression of TACE, leading to increased TNFα shedding rates. However, future work will be required to characterize the exact mechanism through which HIF-1 and NF-κB signaling pathways interact. Because of the importance of TNFα in the cascade of events leading to inflammation and joint destruction, these findings have important implications for the development of RA where elevated levels of proinflammatory cytokines coexist with hypoxia in the microenvironment of the joint. They also provide additional insight into the interplay between the oxygen-sensing and inflammatory pathways.

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