TNF-α Convertase Enzyme from Human Arthritis-Affected Cartilage: Isolation of cDNA by Differential Display, Expression of the Active Enzyme, and Regulation of TNF-α[1]

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A snake venom-like protease isolated by a differential display screen between normal and osteoarthritis (OA)-affected cartilage (designated as cSVP) has a cDNA sequence identical to TNF-α convertase enzyme (TACE). TACE shows the presence of an unknown prodomain, a cysteine switch, a catalytic domain, a zinc binding region, a disintegrin region, an EGF-like domain, a transmembrane domain, and a unique cytoplasmic region. A TACE construct harboring the signal + prodomain + catalytic region (TACE-SPCΔDETCy), expressed in baculovirus could cleave preferentially (~12-fold) the TNF-specific peptide over the matrix metalloproteases peptide in vitro. This recombinant protein also cleaved the natural substrate GST-ProTNF-α to TNF-α (17 kDa) in vitro. The mRNA for TACE, which is broadly distributed and differentially expressed in a variety of human tissues, is up-regulated in arthritis-affected cartilage, but not normal cartilage. OA-affected cartilage also expressed TNF-α mRNA that was not detected in normal cartilage. The OA-affected cartilage (in explant assays) spontaneously released TNF-α and IL-8 in ex vivo conditions. Addition of TNF-αR fused to IgG Fc fragment (TNF-αR:Fc) in the presence or absence of soluble IL-1R (with which it acted additively) significantly attenuated the spontaneous/autocrine release of articular IL-8 in this assay. These experiments demonstrate a functional paracrine/autocrine role of TNF-α in OA-affected cartilage that may depend, in part, on up-regulated levels of chondrocyte-derived TACE. *The Journal of Immunology*, 1998, 160: 4570–4579.

Tumor necrosis factor α, a pleiotropic cytokine, produces a broad spectrum of injurious effects, which makes it an important target for therapeutic intervention. TNF-α is involved in the pathophysiology of arthritis, AIDS, cancer, autoimmune diseases (immune complex diseases), lung fibrosis, multiple sclerosis, skin delayed-type hypersensitivity reactions, and bacterial and parasitic infections (1–6).

The gene for human TNF-α encodes a prohormone that is inserted into the cell membrane as a polypeptide with a molecular mass of 26 kDa (5). This membrane-bound form of TNF-α is bioactive as assayed by cell cytotoxicity and has been implicated in the paracrine activities of TNF-α in various tissues (6). In response to LPS and other stimuli, the 26-kDa form of proTNF-α is proteolytically cleaved (by a metalloprotease referred to as TNF-α convertase) into a soluble 17-kDa polypeptide (6). TNF-α binds its cognate receptors (p55 and p75) as a bioactive trimer and triggers complex intracellular signaling pathways (6, 7).

Studies in animal models of arthritis indicate that TNF-α may be a pivotal cytokine involved in these disease processes. Injection of anti-TNF-α Abs (8) or soluble TNF-αR (9) has proven to be highly effective in reducing clinical score, paw swelling, and histologic severity of the disease. Extension of these studies in patients with rheumatoid arthritis in phase II and III clinical trials has yielded very encouraging results. These early clinical results indicate that neutralizing the effects of TNF-α may have profound effects on clinical symptoms and signs (10, 11).

The matrix metalloproteases (MMPs)[4] have long been implicated in cartilage degradation in both rheumatoid arthritis (RA) and osteoarthritis (OA), although their precise role in disease progression remains to be determined (12). The two main families of MMPs believed to be responsible for cartilage degradation are collagenases and proteoglycanases (12).

The synthesis of TNF is known to be regulated at transcriptional, translational, and posttranslational levels (6). Three independent groups have demonstrated that broad-spectrum inhibitors of MMPs can specifically inhibit the release of membrane proTNF-α (but not IL-1β or IL-6) from various cell surfaces, including RA synovial cell cultures (13–17). This inhibitor of proTNF-α processing could protect mice against a lethal dose of endotoxin administered to them (14). The "TNF-α convertase" activity was isolated using these inhibitors as ligands by affinity purification, which resulted in identification of an 80-kDa protein with the capacity to cleave the Gln-Ala-↓-Val-Arg sequence of proTNF-α (15, 16).

In the present study we report the following: a) the full-length cDNA sequence of a snake venom-like protease from human arthritis-affected cartilage (cSVP) is identical to TACE cDNA (15,

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[4] Abbreviations used in this paper: MMP, matrix metalloprotease; cSVP, cartilage-derived snake venom-like protease; OA, osteoarthritis; RA, rheumatoid arthritis; sIL-1R, soluble IL-1R; GST-proTNF-α, glutathione-*S*-transferase-proTNF-α; TNF-αR: Fc, TNF-α receptor fused to IgG Fc fragment; TACE, TNF-α convertase enzyme; EGF, epidermal growth facor; RACE, rapid amplification of cDNA ends.

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16); b) the functional expression of a truncated recombinant TACE showing TNF-α convertase activity; c) the up-regulation of both TACE and proTNF-α mRNA in OA- and RA-affected, but not in normal cartilage; and d) the intraarticular TNF-α expressed in OA-affected cartilage is sufficient to modulate the autocrine cartilage-derived IL-8 production. We conclude from these data that TNF-α, which is induced in arthritis-affected cartilage, may be influenced by the up-regulated TNF-α convertase to promote cartilage catabolic activities.

Materials and Methods

Procurement of human cartilage

Cartilage slices were taken from the knees of patients with the diagnosis of advanced OA or RA who were undergoing knee replacement surgery, and from nonarthritic knees. The OA/RA patients were free of nonsteroidal anti-inflammatory drugs for at least 2 wk before surgery. Nonarthritic knee cartilage was obtained within 12 h from accident victims who were undergoing knee amputation. Some of the samples were provided by the National Disease Research Interchange (Philadelphia, PA).

OA cartilage organ culture

Organ culture was conducted as previously described (18). Briefly, knee articular cartilage, from patients with OA or RA undergoing knee replacement surgery, was obtained and cut into 3-mm discs; four to six discs ($\sim$100 mg) were placed in organ culture in 2 ml of medium for 24 to 72 h, with or without modulators. The medium was analyzed for nitrite (18), PGE$_2$ by RIA (Sigma Kit, Sigma, St. Louis, MO), and IL-8 and TNF-α by ELISA (R&D Systems, Minneapolis, MN).

RNA extraction from articular cartilage

Total RNA from OA/RA-affected cartilage and normal cartilage was extracted by guanidium thiocyanate-phenol/chloroform extraction method (19), with minor modifications. The cartilage was milled into fine powder and extracted with 4 M guanidium thiocyanate, 25 mM sodium citrate, 0.5% sodium dodecyl sarcosine, and 0.1 M 2-ME. The aqueous phase was layered onto cesium trifluoroacetate gradient for ultracentrifugation (24,000 rpm/24 h) and the RNA was extracted. The RNA obtained with this method is pure enough for RT-PCR analysis.

PCR amplification of OA/RA-affected and normal cartilage RNA

Reverse transcription from 1 μg of total RNA from OA, RA, and normal cartilage was conducted according to manufacturer's recommendation at 42°C for 1 h with Superscript II and oligo(dT) primers (Life Technologies, Gaithersburg, MD). The first strand cDNA was used for PCR with the following degenerate primers: A) forward primers as a mixture of two cysteine switch domain primers, (i) classical MMP's cysteine switch degenerate 5'-CCN (A/C)GN TG(C/T) GGN GTN CCN, where N = A, G, C or, T, and (ii) snake venom protease cysteine switch degenerate 5'-CCN AA(A/G) ATG TG(C/T) GGN GTN, where N = A, G, C, or T; and B) reverse primer, a degenerate zinc binding domain, 5'-(G/A)TG NCC (G/A)A(G/A) (C/T)TC (G/A)TG NG(C/T). An amount equal to 100 pmol of each primer was used (forward, a 50-pmol mixture of two cysteine switch domain degenerate primers and 100 pmol of zinc binding domain primers). Three PCR reactions for each RNA species (OA/RA-affected and normal) were conducted in a Perkin-Elmer/Cetus DNA Thermal Cycler (Perkin-Elmer/Cetus, Norwalk, CT) for 40 cycles of denaturation (94°C, 1 min), annealing (40°C/45°C/50°C, 1 min), and extension (72°C, 2 min). The PCR products were excised from low melting agarose gel and purified using Promega PCR Cleaning Kit and ligated in pGEM-T vector (Promega, Madison, WI). The collagenase$^+$/stromelysin$^+$ clones were excluded by colony hybridization as described below. DNA isolated from the rest of the colonies were analyzed. Computer analysis of DNA and protein sequences was performed using the GCG software package of the University of Wisconsin (Madison, WI), Genetics Computer Group (20).

Southern hybridization

The amplified RT-PCR fragments (on the filter) were probed with stromelysin (341-bp to 1477-bp fragment) (21) and collagenase (396-bp to 883-bp fragment) (22). Clone A (cSVP) (which represents 768 bp to 1433 bp of the sequence deposited to GenBank (accession number U92649)) and the 500-bp fragment(s) were also used as probes. All fragments were labeled using[^{32}P]dATP (Amersham Life Sciences, Arlington Heights, IL) and Random Primers DNA labeling system (Life Technologies). The mem-branes were hybridized with ^{32}P-labeled probes overnight at 65°C. After hybridization, the membranes were washed at high stringency twice for 10 min at 65°C. The blots were exposed to Kodak x-ray films (Kodak, Rochester, NY) at −70°C. All methods were conducted according to Sambrook, et al. (23).

Colony hybridization

White colonies, including one positive control each for collagenase and stromelysin, were taken on grids on 150-mm plates ($\sim$400 colonies per plate) and incubated overnight at 37°C. Colonies were transferred onto nitrocellulose membrane. The filters were then fixed with a UV Crosslinker (Stratagene, La Jolla, CA). Treatment of membranes, prehybridization, hybridization, and washing conditions used were according to published protocols (22). Membranes were exposed to an autoradiographic film at −70°C. The collagenase$^+$/stromelysin$^+$ clones were excluded from the grids before sequencing the inserts of some of the other clones.

Cloning of full-length cSVP (TACE) by 3' and 5' rapid amplification of cDNA ends (RACE)

The 3' and 5' extensions of cSVP were cloned using the 3'RACE and 5'RACE methods according to manufacturer's instructions (Life Technologies). Briefly, total RNA was extracted from a pool of three OA-affected cartilage samples. For 3'RACE, 1 μg of this pooled RNA was synthesized into first strand cDNA using SuperScript Reverse Transcriptase and adaptor primer 5'-GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT T (Life Technologies). This first strand cDNA was used to amplify the 3' end of cSVP; a sense gene-specific primer called primer MMP.A (5'-TAG TGG TTT GAC GAG CC) and an abridged universal amplification primer, AUAP (5'-GGC CAC GCG TCG ACT AGT AC; Life Technologies), were used for 30 cycles in a Perkin-Elmer thermal cycler (94°C for 1 min, 55°C for 1 min, and 72°C for 2 min). The PCR products were purified using Promega PCR Purification Kit and quantitated. Ten nanograms of PCR product was taken and reamplified, using a gene-specific sense-nested primer called MMP.B (5'-AAG GAA GCT GAC CTG GTT) and AUAP for 20 cycles at the same PCR conditions mentioned earlier. PCR products were electrophoresed on 1% agarose gel, and 1.6- to 2.0-kb bands were excised, purified by PCR Cleaning Kit, ligated into pGEM-T (Promega), and transformed into XL-1 Blue MRF'-competent *Escherichia coli* cells (Stratagene). DNA was prepared from isolated white colonies and digested with *Pst*I restriction enzyme (unique site identified in cSVP Clone 8). The products were run on 1% agarose gel, blotted on nitrocellulose, and probed with ^{32}P-labeled cSVP Clone 8 (*Sal*I-*Sph*I, $\sim$900 bp) fragment. Four positive clones were picked and DNA were sequenced on an ABI 373 sequencer (Perkin-Elmer).

For 5' RACE, the first strand was synthesized using a gene-specific primer, MMP.C primer (5'-GCG CGA AGC TTA CTC TTC TCC TTC ATC CAC), and SuperScript reverse transcriptase (Life Technologies). The first strand was dC-tailed at the 5' end and amplified using MMP.C (shown above) and anchor primer (5'-CUA CUA CUA CUA GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG (Life Technologies), using the above mentioned conditions. The PCR products were run on 1% agarose gel, and bands of 1.0 to 1.6 kb were excised and ligated in pGEM-T and transformed in XL-1 Blue MRF'. White colonies were selected, and DNA was prepared and digested with *Nco*I restriction enzyme (another unique site identified in cSVP Clone 8) and blotted as mentioned above. Four positive clones were selected, and DNA was prepared and sequenced as above.

Northern blot analysis

The filters for the human multiple Northern (MTN) blot were purchased from Clontech (Palo Alto, CA). These blots contained 2 μg of poly(A)$^+$ RNA per lane. Equal loading of the RNA was confirmed by probing representative filters with β-actin cDNA probe. The presence of a single 2.0-kb band (for β-actin) in all lanes was confirmed, except heart and skeletal muscle, which have two isoforms of β-actin: 2.0 kb and 1.8 kb. These filters were probed with TACE catalytic region (spanning the cysteine switch and zinc binding region) as recommended by the manufacturer. Northern blot analysis was performed using the same probe but with different established cell lines, such as THP-1 (American Type Culture Collection, Rockville, MD (ATCC) 202-TIB), human chondrosarcoma (ATCC 94-HTB), Jurkat (ATCC 152), HEK 293 (ATCC 1573), and C20A4 (human immortalized chondrocyte, a kind gift from Dr. Mary Goldring (24). Total RNA was isolated using the TRI REAGENT kit (Molecular Research Center, Cincinnati, OH).

Construction and expression of recombinant truncated TACE in baculovirus

The TACE cDNA containing the signal region, the prodomain, and the catalytic domain (TACE-SPCΔDETCy), which represents amino acids 1–474, was constructed using specific PCR primers and the 5′ RACE No. 1-pGEMT clone as template. The following PCR primers were used: sense primer Met 1, 5′-CGG GAT CCA CC ATG AGG CAG TCT CTC CTA TTC 3′ (to create a *Bam*HI site 5′ to ATG); and antisense primer S474, 5′-GCT CTA GAT TAG CTG CGT TCT TGA AAA CAC TC 3′ (to create stop + *Xba*I site at serine 474). A typical PCR reaction contained 25 pmols of each primer, 100 ng of template DNA, 5 mM of each dNTP, 2 U of Vent DNA polymerase (NEB, Beverly, MA), and water to a final volume of 50 μl. PCR was performed at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, for 20 cycles. The amplified DNA was digested with *Bam*HI and *Xba*I and subcloned into pFast Bac-1 transfer vector (Life Technologies) to generate pFast Bac/TACE-SPCΔDETCy. All other recombinant techniques to generate the recombinant virus and the recombinant protein were conducted as described (25). The recombinant protein was generated in serum-free insect cell culture medium: (Express Five SFM) by infecting the host BTI-TN-5B1–4 (high five) insect cells. Infected cells were harvested 72 h post infection, and the recombinant enzyme recovered from the medium was used in the assays.

Enzyme assay for recombinant TACE-SPCΔDETCy with the TNF-α-specific and MMP-specific peptides

The TNF-α-specific peptide substrate (7-methoxy coumarin-4yb) Acetyl (MCA)-PLAQ AV-(3-[2,4-dinitrophany)]-2,3-diamino-propianyl-RSSSR-NH$_2$) and MMP-specific peptide MCA-PLGL-DPA-A R-NH$_2$ were diluted to a final concentration of 10 μM in a buffer containing 50 mM Tricine pH 7.5, 100 mM NaCl, 10 mM CaCl$_2$, and 1 mM ZnCl$_2$. The enzyme reaction contains 150 μl of a recombinant baculovirus supernatant plus the diluted peptide in 300 μl final volume. This was incubated for 1 h on orbital shaker at 27°C. Reactions were quenched by adding 30 μl EDTA (500 mM), and plates were read at 320 nM excitation.

Enzyme assay for recombinant TACE-SPCΔDETCy with proTNF-α as a substrate

Assay of baculovirus-expressed recombinant protein was performed with [^{3}H]GST-proTNF-α as a substrate prepared by in vitro translation using a rabbit reticulocyte system (Promega). The acrylamide gel assays were essentially conducted as described by Gearing, et al. (13). GST-proTNF-α was first cloned in pET-3b expression vector (Promega). The reaction contained GST-proTNF-α-pET3b (0.5 μg/μl) 2 μl; RNAsin 1 μl, amino acid mix (-cys) reaction buffer 2 μl, T7 RNA polymerase 1 μl, [^{3}H]leucine (5 μci/μl) 12.5 μl, TNT rabbit reticulolysate 25 μl, and water to a final volume of 50 μl. The reaction mixture was incubated at 30°C for 90 min followed by addition of 800 μl of buffer containing 50 mM Tricine, 150 mM NaCl, 0.1% *N*-octyl glucoside, and 1 mM β-mercaptoethanol. The substrate was purified and concentrated using Micro Con 50 (Amicon), diluted to 1 ml, and stored at 20°C in 100-μl aliquots. Twenty microliters of baculovirus-expressed TACE-SPCΔDETCy was mixed with 5 μl of [^{3}H]proTNF-α substrate in a 4× buffer mixture containing 50 mM Tricine, pH 8, 150 mM NaCl, 0.25% Tween-20 (6.25 μl), 10 mM CaCl$_2$, 15% glycerol, and 2.75 μl of a mixture of protease inhibitors. The reaction was incubated at 37°C for 30 to 90 min. After incubation, the reaction was quenched by adding an equal volume of 4× reducing SDS sample buffer. Samples were run on 14% acrylamide gels and stained with Coomassie blue.

RT-PCR analysis of TACE and TNF-α

RT-PCR analysis of TACE and TNF-α was conducted according to the manufacturer's instructions (Life Technologies). The primers used for TACE were (sense) 5′-CGG GAT CCA TGT TAA AAG TGG ATA ATG AAG and (antisense) 5′-GCG CGA AGC TTA CTC TTC TCC TTC ATC CAC, which generated an ~900-bp fragment. The primers for proTNF-α mRNA amplification were (sense) 5′-GGA ATT CAT ATG AGC ACT GAA AGC and (antisense) 5′-CGG CGC CTC GAG TCA CAG GGC AAT GAT, which generated an ~700-bp fragment. The primers to amplify the β-actin were (sense) 5′-GTG GGG CGC CCC AGG CAC CA and (antisense) 5′-CTC CTT AAT GTC ACG CAC GAT TTC, which generated an ~500-bp fragment.

Sequence analysis

The sequence search for the cDNAs was performed with the BLAST program, and the modeling was conducted by using the ICM program as recently described (26, 27).

Results

Differential display and analysis of MMPs and TACE in arthritis-affected cartilage

To identify new members of the metalloprotease family produced by human OA- or RA-affected cartilage, two degenerate oligonucleotides were made. One was from the conserved zinc binding region and the other was from the conserved cysteine switch domain from two families of proteases, the matrixins and the snake venom protease, as described in *Materials and Methods*. The degenerate oligos were used to amplify the first strand cDNA by RT-PCR from RNA obtained directly (without releasing the cells) from normal, OA- and RA-affected cartilage. Using this method, we have identified short half-life (<4 h) mRNAs that are induced in OA-affected cartilage in vivo. These include mRNA for COX-2 (18) and IL-1β (28).

The annealing temperatures used (for OA, RA, and normal cartilage RNA) for the RT-PCR (in 3 separate experiments), using equal amount of RNA, were 40°C, 45°C, and 50°C. The PCR fragments amplified at three different temperatures for each tissue (i.e., OA, RA, and normal cartilage) were pooled and analyzed on agarose gels as shown in Figure 1*A*. The data showed amplification of PCR fragments ranging from 200 bp to 1.2 kb. The PCR products were analyzed for the presence of MMP-1 and MMP-3 by Southern blot analysis as shown in Figure 1, *B* and *C*. As expected, MMP-1 and MMP-3 fragments (matrixins) could be detected in the 300- to 400-bp region, which, upon cloning and sequencing, showed sequence characteristics similar to MMP-1 and -3, respectively (data not shown). These experiments demonstrated that MMPs could be isolated using this technique under conditions used in this study.

The signal on the Southern blot hybridization was arbitrarily normalized during the screening process by randomly selecting and cutting out a band(s) (e.g., "500-bp band(s)" marked by open arrow in Fig. 1*A*) that appeared at equal intensity in normal, OA-affected, and RA-affected cartilage RT-PCR samples after staining with ethidium bromide (Fig. 1*A*). This "500-bp band" has been identified as fibronectin, based on its cDNA sequence homology (data not shown). This fibronectin cDNA was excised from the RA lane, purified, radiolabeled, and used as a probe in the Southern blot hybridization. The filters previously used for probing MMP-1 and -3 were again utilized (Fig. 1*D*). The fibronectin signal was found to be at similar intensity in all 3 lanes. The data therefore show both identical loading of PCR (on gels) and equivalent amplification of mRNA (by RT-PCR) of the fibronectin band in normal, OA-affected, and RA-affected cartilage.

The normal, OA-affected, and RA-affected cartilage RT-PCR bands were grouped into four regions (A, 200- to 400-bp region; B, 500-bp region; C, 500- to 800-bp region; and D, above 800-bp region) and cloned in pGEM-T vector. Colonies were grown on grids and screened as described in *Materials and Methods*. Among these, one clone (from region C of OA-affected cartilage) designated as clone A (668 bp) showed the presence of a partial zinc binding region (HELGH) at the end of the 3′ region of the insert. On subsequent screening, another clone was isolated, which was designated as Clone 8 (which also represented sequences of Clone A). Clone 8 had a complete functional zinc binding region (HEL GHN) and a cysteine switch-like motif (PKVCGY) similar to that seen in snake venom proteases from Jararhagin (PKMCGV, GenBank Accession No. 68251) (29). The distance between the
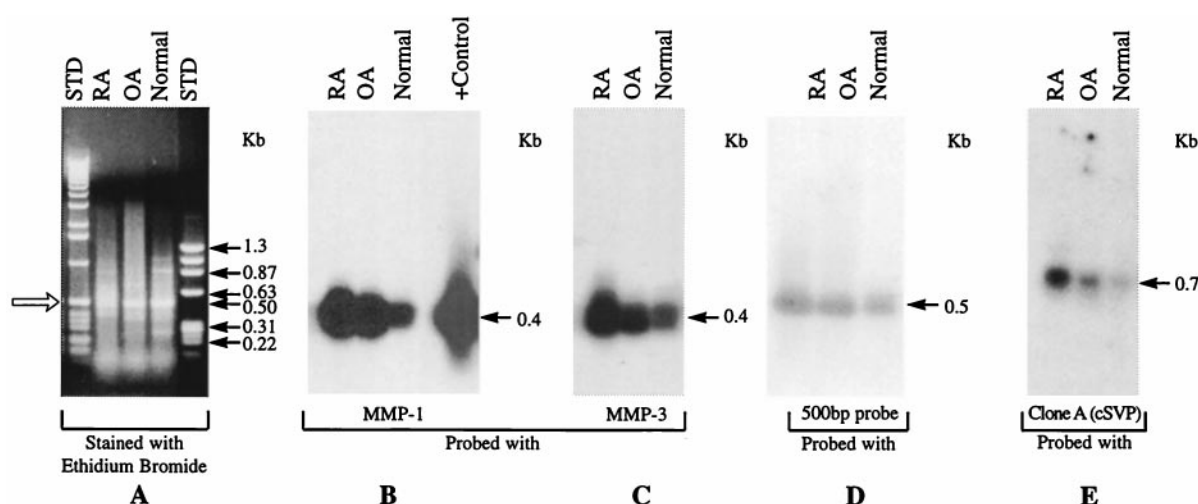
FIGURE 1. *A*, RT-PCR analysis of RNA obtained from OA/RA-affected and normal cartilage. Two micrograms of RNA (pooled from at least three individuals) was used for RT-PCR using various degenerative cysteine switch-like zinc binding region oligonucleotides as described in *Materials and Methods*. An equal amount (100 ng) of cDNA from a pool of samples that were amplified at 40°C, 45°C, and 50°C was run on 1% agarose gels containing 0.05 μg/ml of ethidium bromide. This protocol was partly adopted from Freije et al. (55). *B* and *C*, Southern blot analysis of RT-PCR amplified fragments using MMP-1 (*B*) and MMP-3 (*C*). The cDNAs (100 ng total) shown in *A* were run in duplicate gels, blotted onto the membrane, and then probed with radiolabeled probe for MMP-1 and MMP-3, respectively, as described in *Materials and Methods*. The positive control in *B* represents a restriction-digested fragment (cysteine switch/zinc binding region) of MMP-1. *D*, Southern blot of RT-PCR-amplified fragments using 500-bp fragment(s). The 500-bp region band seen as an open arrow in *A* was cut from the gel, eluted, purified, and labeled with [^{32}P]dATP using the same mixture of degenerative oligonucleotides used to amplify the original mRNA from cartilage. Both the blots shown in *B* and *C* were stripped of their previous signals and reprobed with the labeled 500-bp cDNA fragment(s). One of two similar blots is shown. *E*, Southern blot of RT-PCR-amplified fragments using (Clone A) cSVP. Clone A, which represents part of the catalytic region of cSVP, was labeled with [^{32}P]dATP. The blots *B* and *C* were stripped of their previous signals and reprobed with Clone A. The data show one of two representative blots.

PKVCGY and HELGHN was ~690 bp, further excluding it from the matrixins family of MMPs. To confirm the presence of this "PCR-amplified" cDNA in the arthritis-affected cartilage, the original blots (shown in Fig. 1, *B* and *C*) that had been probed with MMP-1, MMP-3, and fibronectin cDNA were stripped again of their previously used probes and reprobed with Clone A (Fig. 1*E*). Similar to MMP-1 and -3, this unknown cDNA (in the RT-PCR mixture), which hybridizes to Clone A, was also found to be enhanced in the arthritis-affected cartilage, but the positive signal was seen at ~700-bp region, distinct from the bands observed with the collagenase- and stromelysin-probed fragments.

Sequence analysis of Clone 8

The sequence analysis of Clone 8 was performed by examining the characteristic sequence patterns of the PROSITE database. The partial cDNA sequence of Clone 8 showed only ~40% to 50% similarity and 20% to 30% identity to snake venom proteases. The 3-D ribbon structure of Clone 8 was compared with that of the catalytic region of atrolysin and adamalysin (30, 31) as the x-ray crystallography structures of these proteins are solved (pdb code: liag). The peptidase domain of Clone 8 aligns with only 27% amino acid sequence identity with the liag sequences. One of the striking features of this alignment (between Clone 8 and atrolysin/adamalysin) was that all three histidines important for the catalytic function of atrolysin/adamalysin were conserved in Clone 8 (32). Furthermore, the key hydrophobic side chains supporting 3-D folds are also conserved in these three proteins in the catalytic region. Although the overall sequence identity was not very high, the alignment score (20.4 for liag) was found to be several sigmas above the noise level (26). Therefore we concluded (based on the similarity of the PROSITE signature) that there was a structural and functional similarity between the Clone 8 peptidase region and that present in adamalysin (EC:3.4.24.46)/atrolysin (C/D), which

belong to the EC:3.4.24.42 M12B snake venom protease family (32) (Fig. 2*A*). This partial cDNA sequence showed >99% homology to the recently described TNF-α convertase (15, 16). These experiments indicated that Clone 8 may be a fragment of the TNF-α convertase, which seems to be up-regulated in arthritis-affected cartilage.

Full-Length cDNA cloning of TACE and sequence analysis

The full-length cDNA was cloned from an OA-affected cartilage mRNA using both 5′ and 3′ RACE protocols as described in *Materials and Methods*. There was >99% homology in amino acid sequence between cSVP and TACE, thus indicating that cSVP from OA-affected cartilage was TACE (15, 16). The sequence analysis for the full-length TACE was further performed with the new method for sensitive protein fold recognition (27). Characteristic sequence patterns of the PROSITE database were searched. The 3-D ribbon structure based on the amino acid sequence and domain structure for TACE is shown in Figure 2, *B* and *C*. The full-length TACE showed the presence of a signal peptide (aa 1 to 17), a potential prodomain (aa 18 to 216), a cysteine switch-like region (aa 181 to 188), a catalytic region (aa 217 to 474), a zinc binding region (aa 405 to 417), a disintegrin cysteine-rich domain (aa 480 to 559), an EGF-like region (aa 571 to 602). a transmembrane domain (aa 672 to 694), and a cytoplasmic tail (aa 694 to 824).

The prodomain does not show any significant homology to any known protein. The endopeptidase region, as described above, was similar to that seen with the snake venom proteases of family M12B (33). The disintegrin/cysteine-rich region of TACE (29) shows homology to the TNF-αR/nerve growth factor receptor family, which also has a similar cysteine-rich signature (34, 35). TNF-αR has been reported to form dimers in crystal structures without the ligand, and, therefore, the potential of TACE to form
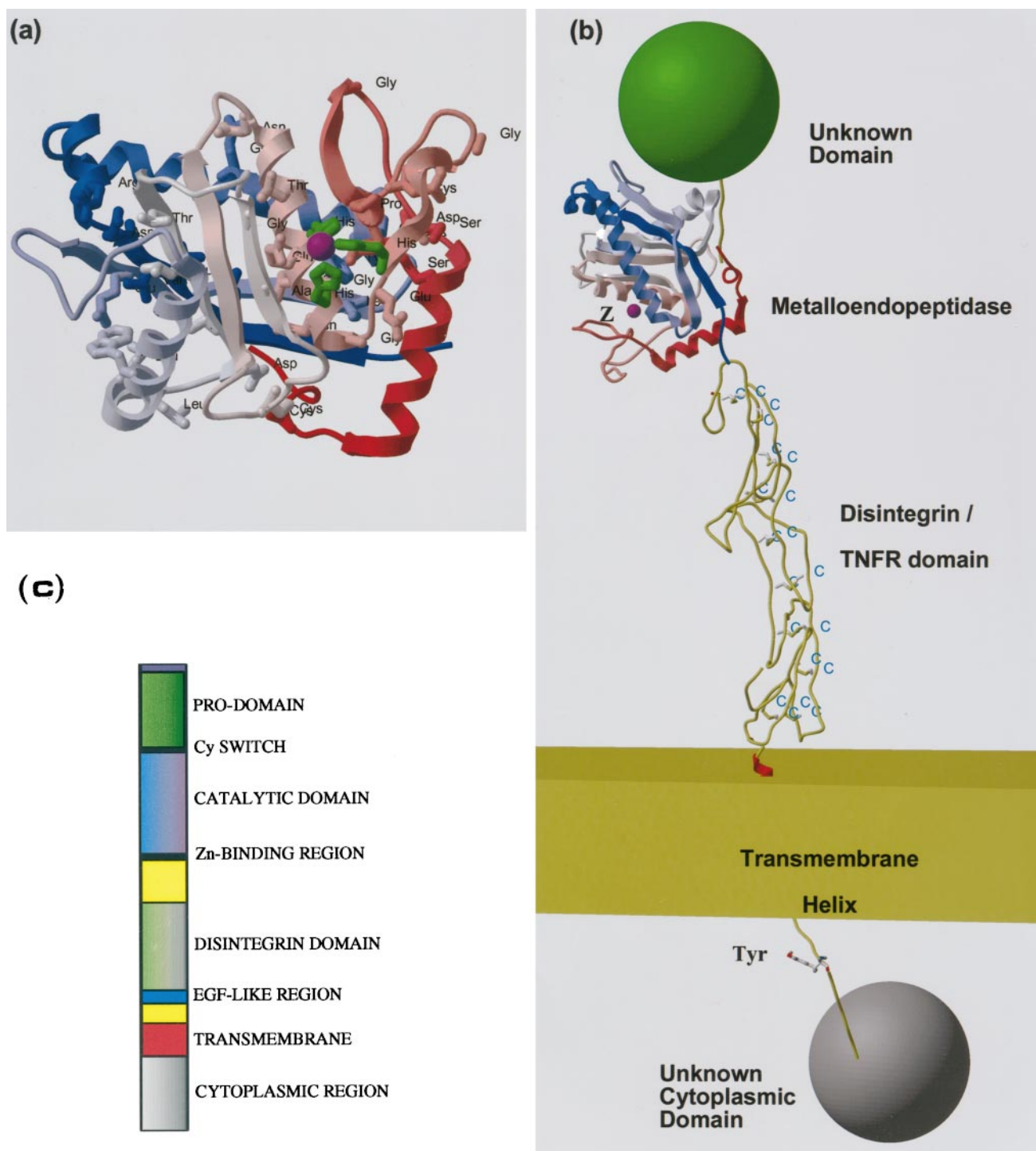
FIGURE 2. Comparison of the catalytic metallopeptidase region of atrolysin (C/D)/adamalysin with Clone 8 (*a*). The 3-dimensional structure of atrolysin was used as a template. Only the residues conserved between atrolysin, adamalysin, and Clone 8 are shown by balls-and-sticks. Three conserved catalytic histidines are shown in green. Zinc ion is shown in magenta. The ribbon is colored from N to C terminus. Topologic structure and domain structure of TACE (*b* and *c*). The putative signal peptide sequence is followed by an unknown prodomain (green sphere). The catalytic/metallopeptidase domain is modeled on the basis of known structure of adamalysin II and atrolysin E. The ribbon is colored blue to red from N to C terminus. Zinc ion is shown in magenta. The disintegrin/TNF-αR cysteine-rich domain is modeled on the basis of the known structure of the TNF-αR dimer (pdb entry 1ncf). Immediately after the transmembrane helix there is a potential tyrosine phosphorylation site. The cytoplasmic domain, which shows no resemblance to any known protein, is represented by a gray sphere. *c* shows the bar diagram of TACE (cSVP)

a dimer cannot be ruled out (35). The TACE also has an EGF-like domain, a transmembrane domain, and a cytoplasmic region. The cytoplasmic region, like the prodomain, does not show any significant homology to any known protein in the public database. The cytoplasmic region has a putative tyrosine phosphorylation site

(KKLDKQYESL). Substrates of tyrosine protein kinases are generally characterized by a lysine or an arginine, seven residues to the N-terminal side of phosphorylated tyrosine. Two lysine residues at positions 5 and 6 and an aspartic acid residue at position 3 to N-terminal of the tyrosine (Y) are found in TACE, which is a
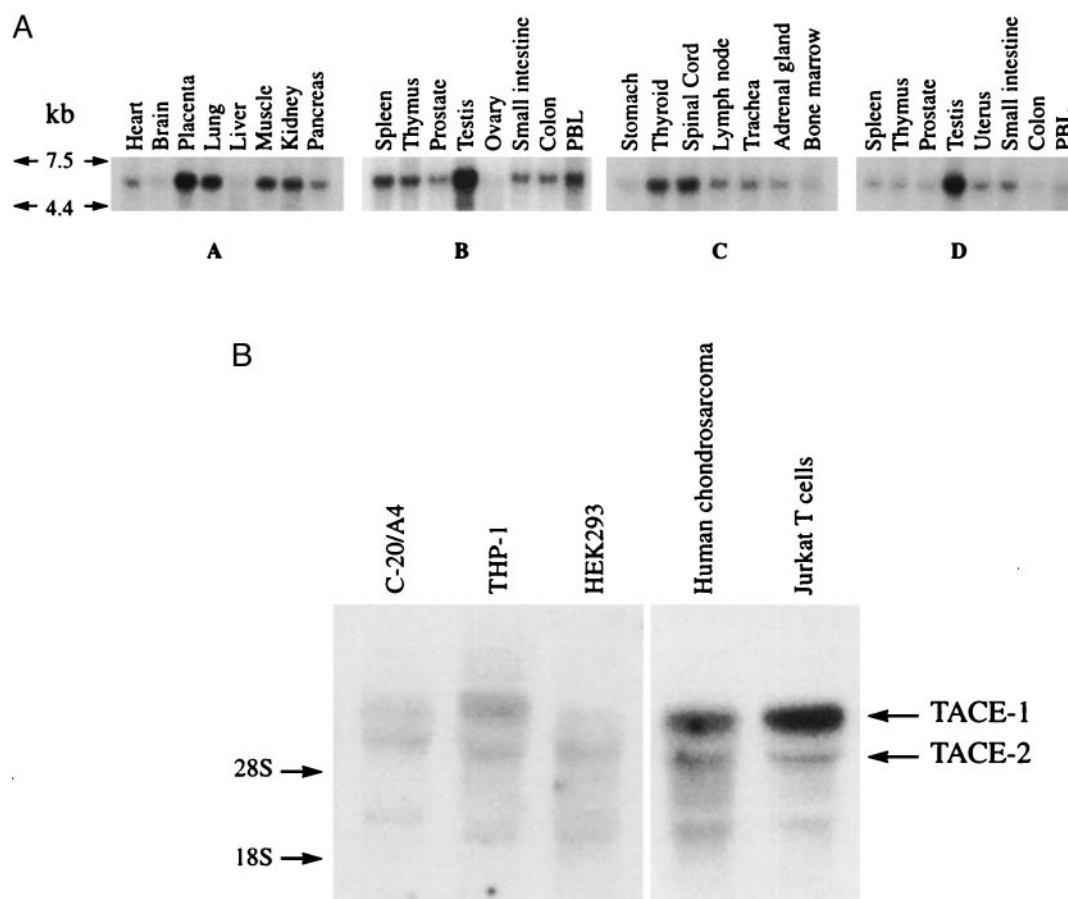
FIGURE 3. Northern blot analysis of TACE from different human tissues. *A*, The RNA blots were purchased from ClonTech. Two micrograms of poly(A)$^+$ RNA were blotted onto a nylon membrane. Equal loading of the RNA was confirmed by probing the blots with β-actin (conducted as part of the quality control data sheet by ClonTech) *B*, The Northern blot from total RNA isolated from different cell lines. Thirty micrograms of total RNA obtained from C20A4 (a human immortalized chondrocyte cell line), THP-1, HEK 293 (human chondrosoma), and Jurkat T cell was loaded onto the gels. The filters were probed with the cDNA representing the catalytic domain of TACE.

classical motif for tyrosine phosphorylation (36). Furthermore, the proline rich cytoplasmic region of TACE has at least two potential Src-homology 3 domains: PAPQTPGR and PAPVIPSA.

Northern blot analysis of TACE

We examined the distribution of TACE mRNA (using the catalytic site of TACE as the probe) in various tissues using a commercial Northern blot (ClonTech) as a source of Poly(A)$^+$ RNA. We found a 5.0-kb mRNA that was differentially distributed in various tissues. The salient features of the blot were the high expression in testis, placenta, lung, and kidney and low expression in ovary, colon, liver, and brain (Fig. 3*A*). Northern blot analysis of 30 μg of total RNA obtained from the various cell lines, including two chondrocyte-derived cell lines as described in Figure 3*B*, showed the presence of a dominant $\sim$ 5.0-kb TACE-1 mRNA and a minor $\sim$4.0-kb TACE-2 mRNA. Thirty micrograms of total RNA (pooled from five to ten samples of cartilage) obtained from OA- or RA-affected cartilage and normal cartilage independently was also examined by Northern blot analysis. A positive detectable signal could not be seen in the cartilage-derived mRNA, although TACE mRNA could be seen in the adjacent lane with equivalent amounts of THP-1 mRNA (data not shown). This is not surprising, since we have observed that the poly(A)$^+$ content of human cartilage RNA is approximately 0.1% of the total RNA (data not shown). However, TACE mRNA in cartilage could be detected by RT-PCR as described below.

Expression of TACE in baculovirus insect cell culture system

The TACE-SPCΔDETCy containing a prodomain (with a putative signal sequence) and the catalytic region containing a cysteine switch-like motif and zinc binding domain were cloned in a baculovirus expression system. This protein was truncated just before the start of disintegrin domain. The recombinant virus made in Sf9 cells were further amplified in BTI-TN-5B1–4 cells using the serum-free medium. The recombinant protein was Western blotted (Fig. 4) using the polyclonal Abs raised against the catalytic region of TACE (expressed in *E. coli*; data not shown) in rabbits. The Western blot shows an $\sim$55-kDa band that corresponds to the unprocessed recombinant protein containing the prodomain and the catalytic region. This blot also shows an $\sim$32-kDa band that seemed to be the processed form of TACE. The processing of TACE seems to occur at the furin-like cleavage site (RVKRR) at residue 211 in the prodomain region. Furin and related enzymes of subtilin-like serine proteases such as PC1/PC3, PC2, PC4, PC5, PC7, and PACE 4 have been described to utilize the consensus R-X-K/R-R site for processing of diverse proteins, especially proenzymes such as factor IX (37), pro-Von Willebrand factor (38), proalbumin (39), complement pro-C3 (39), and proTGF-β1 (40). These results show a) that baculovirus insect cells (Sf9 cells) contain enzymes that have furin-like activity, and b) that the baculovirus insect cell cultures can process and secrete the truncated form of the recombinant TACE. Since TACE contain a cysteine
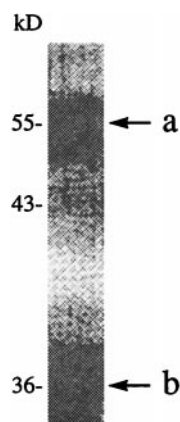
FIGURE 4. Western blot analysis of recombinant baculovirus-expressed TACE-SPCΔDETCy. The prodomain and catalytic region of TACE were expressed in baculovirus as described in *Materials and Methods*. The protein was generated in a serum-free medium and analyzed by SDS-gel electrophoresis and Western blotting using a polyclonal Ab generated in rabbits using the *E. coli*-expressed catalytic site of the TACE. *A* shows the native form of the TACE-SPCΔDETCy; *B* shows the truncated/processed form of TACE-SPCΔDETCy.

FIGURE 5. Determination of the specificity of TACE-SPCΔDETCy using the native proTNF-α on the substrate. [³H]GST-proTNF-α (∼46 kDa) was prepared in vitro using rabbit reticulocyte lysate. The substrate (*lane 2*) was incubated with the recombinant TACE for 30 min (*lane 3*), 60 min (*lane 4*), 90 min (*lane 5*), and analyzed by SDS-PAGE. *Lane 1* represents the m.w. markers. *Lane 6* represents the standard purified 17-kDa TNF-α. The 24-kDa band represents free GST.

switch-like motif PKVCGY characteristic of snake venom-like proteases and a zinc binding domain characteristic of metzincins (41), it is tempting to propose that the TACE could be activated by cysteine switch mechanism as it was determined for enzymes of matrixins family such as collagenase 1 and stromelysin 1 (42).

Specificity of recombinant TACE

The baculovirus expressed TACE (clone TACE-SPCΔDETCy) that harbors the prodomain, and the catalytic site was tested for its specificity in a fluorogenic assay, using an MCA-labeled peptide containing the amino acid sequence around the native site, with proTNF-α (MCA-PLAQA ↓ V-DPA-RSSSR-NH₂) and that of MMPs. (MCA-PLG ↓ L-DPA-AR-NH₂) as the substrate. Table I shows the *Km* and *Vmax* of the enzyme in the presence of the two substrates. The ratio V Ala-Val/V Gly-Leu was 11.94 indicating ∼12-fold preferential cleavage of the TNF-α peptide over the MMP peptide by the truncated recombinant enzyme.

To further ascertain the specificity of the recombinant enzyme, we tested its ability to cleave the native proTNF-α in vitro. ³H-labeled GST-proTNF-α substrate was prepared by in vitro translation using rabbit reticulocyte system. The substrate was incubated with the recombinant enzyme for 30 to 90 min, and the cleavage product (17 kDa TNF-α) was examined by SDS-PAGE analysis as shown in Figure 5. These experiments demonstrate that the recombinant enzyme cleaved the native proTNF-α to TNF-α.

Regulation of TACE and TNF-α in arthritis-affected cartilage

Our recent observations indicate that mRNA for COX-2 and IL-1β are up-regulated in OA-affected cartilage but not in normal cartilage (19, 28). In view of the up-regulation of TACE mRNA in our initial screen in arthritis-affected cartilage, as seen in Figure 1, total RNA was directly isolated from OA- and RA-affected cartilage immediately after surgery. Normal cartilage was used as control for RT-PCR analysis using specific primers for TACE. Figure 6 showed up-regulation of TACE mRNA in five arthritis-affected cartilage samples tested. There was no detectable signal observed in the two normal cartilage samples tested, even at ∼35 cycles. It should be noted that a faint positive TACE signal (after staining
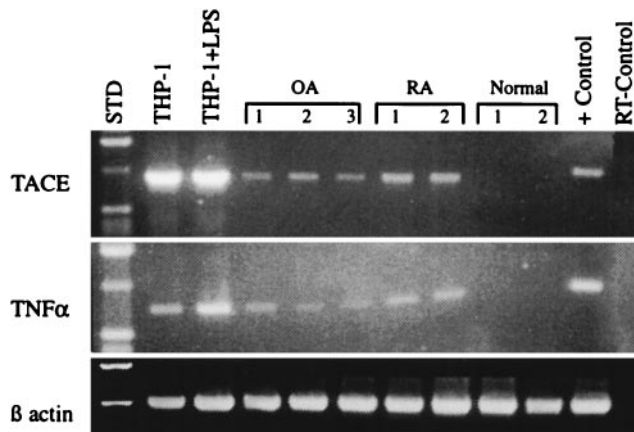
FIGURE 6. Expression of TACE, TNF-α, and β-actin mRNA in normal and OA/RA-affected cartilage. Normal or OA/RA-affected cartilage from different individuals was taken and extracted for total RNA, immediately upon receiving the samples without releasing the cells, as described in *Materials and Methods*. Equal amounts (100 ng) of total RNA were subjected to RT-PCR analysis (30–35 cycles) using specific TACE and TNF-α primers, as described in *Materials and Methods*, which generated ∼900-bp and ∼700-bp fragments, respectively. The same RNA preparation (100 ng) was also subjected to RT-PCR analysis of β-actin, which generated a 475-bp fragment. One hundred nanograms of total RNA from THP-1 cells unstimulated and stimulated with LPS were used as positive and negative controls. In this experiment, RT-PCR analysis of RNA from OA patient No. 3 was conducted without the reverse transcriptase enzyme (RT:Control), thus indicating the lack of potential DNA contamination. "+ Control" designates amplification from standard TACE, TNF-α, and β-actin cDNA. STD designates m.w. standards.

Table I.   *Specificity of recombinant TACE[a]*

	TACE Hydrolysis of	
	MCA-Gly-Leu MMP peptide	MCA-Ala-Val TNF-α peptide
Vmax	1.18 ± 0.5	14.21 ± 2.2
Km (μM)	4.4 ± 0.6	19.0 ± 4.8

[a] The recombinant TACE was incubated with the MCA-labeled peptide containing the amino acid sequence around the native cleavage site within pro TNF (Ala-Val) and MMP (Gly-Leu) in a fluorogenic assay as described in *Materials and Methods*. The *Km* and *Vmax* of the enzyme was determined as described in *Materials and Methods*. The data represent one of the two similar experiments.
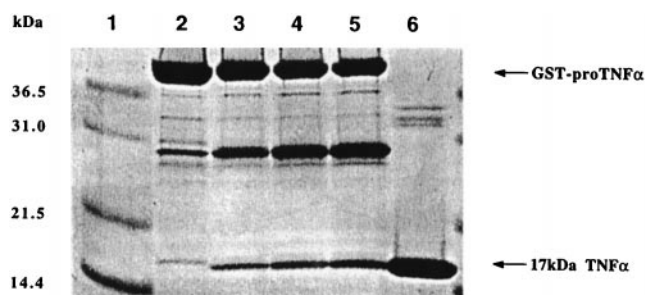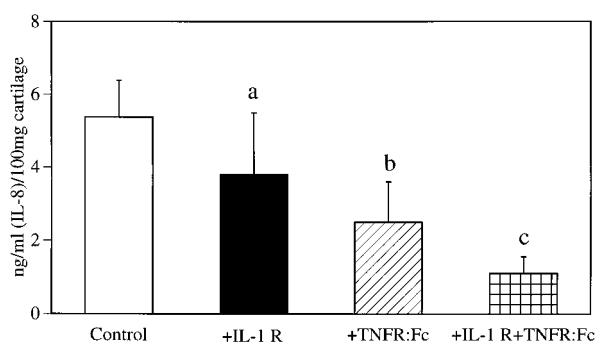
FIGURE 7. Down-regulation of endogenous TNF-α $\pm$ IL-1β in OA-affected cartilage leads to a decreased release of IL-8 in ex vivo conditions. The OA-affected cartilage was set up in organ culture as described in *Materials and Methods*. An amount equal to 10 μg/ml of human soluble recombinant type I IL-1R and TNF-αR:Fc (human TNF-αR (p80) linked to the Fc portion of human IgG1) were added to the OA organ culture. The specific activity of IL-1R and TNF-αR:Fc was 3.9×10^4 U/mg and 1.2×10^6 U/mg, respectively. The cultures were harvested after 48 h, and the IL-8 levels were examined by ELISA. "Control" designates spontaneous release of IL-8 in ex vivo conditions. Data are expressed as ng/ml of IL-8 released by 100 mg of cartilage (wet weight) $\pm$ SD ($n = 3$). The p values are as follows: between control and a, $p < 0.12$; between control and b, $p < 0.015$; between control and c, $p < 0.0015$.

the gel with ethidium bromide) in normal cartilage could be obtained after 40 cycles of PCR amplification (data not shown) or by probing the RT-PCR-amplified products with a ^{32}P-labeled TACE probe by Southern blot analysis, as shown in Figure 1. This may be due to the constitutive expression of TACE, as reported by Black et al. (16) and this paper (Fig. 3, *A* and *B*).

We therefore also examined the expression of TNF-α mRNA in OA-affected and normal cartilage using the same RNA preparations used for amplifying TACE (Fig. 6). RNA from all the arthritis-affected patient material showed the presence of TNF-α mRNA expression that was not detectable in the two normal cartilage samples tested. These experiments indicate that there is an up-regulation of both TACE and TNF-α mRNA in arthritis-affected cartilage.

We have recently observed that OA-affected cartilage explants, when incubated in ex vivo conditions in F-12 medium (in the absence of any exogenous supplements including FBS), spontaneously released nitric oxide, PGE$_2$, and IL-1β in quantities sufficient to cause cartilage damage, and these mediators can be down-regulated by sIL-1R but not by TNF-αR:Fc (19, 28). Therefore, we also examined the spontaneous release of TNF-α from cartilage explants obtained from twelve different patients utilizing the same experimental system. We could detect TNF-α (12.4 ± 11.0 pg/ml per 100 mg OA cartilage, at 72 h, $n = 12$) in the serum-free medium when assayed by ELISA.

We also assayed the spontaneous release of IL-8, which is known to be stimulated by TNF-α in chondrocytes (43), and found it to be present in significant quantities ($\sim$5–6 ng/ml) together with nitric oxide and PGE$_2$ (19, 28). Interestingly, although TNF-α was detected in low concentrations by ELISA in ex vivo conditions, treatment of explants with TNF-αR:Fc significantly inhibited the spontaneous secretion of IL-8 by OA-affected cartilage (Fig. 7). sIL-1R only marginally inhibited the spontaneous release of IL-8. However, sIL-1R acted additively with TNF-αR:Fc to markedly inhibit IL-8 release. These experiments indicate that the OA-affected cartilage spontaneously produces low levels of TNF-α that are sufficient to promote IL-8 production. In these experiments, TNF-αR:Fc + IL-1R could also inhibit the sponta-

neous release of nitric oxide and PGE$_2$ production by more than 50%, as previously reported (28). Thus, chondrocyte-derived TNF-α, like IL-1β (28), acts in a paracrine/autocrine fashion to modulate cartilage function.

Discussion

We have identified a snake venom-like protease (TACE) from human arthritis-affected cartilage that has unique structural properties suggestive of a hybrid between matrixins and adamalysins, similar to other related proteases, such as ADAM-10, -12, and -15 recently observed in chondrocytes (44). The prodomain of TACE shows the closest resemblance (homology) to phospholipase C pleckstrin domain, which was reported to be important for membrane association of proteins involved in intracellular signaling and the cytoskeleton interaction (45). The cysteine switch-like region resembles that of snake venom protease Jararhagin (29) and the functional zinc-binding motif (HELGHNFGAEHDPD), which is distinct from that seen in the matrixins (41).

Moss et al. (15) have shown that the TACE construct harboring signal region + prodomain + catalytic region + disintegrin region expressed in baculovirus is functional. Our studies show that a TACE construct (TACE-SPCΔDETCy) harboring a signal sequence + prodomain + catalytic site (devoid of disintegrin domain) and expressed in baculovirus is sufficient for enzyme activity and specificity for the TNF-α peptide over the MMP peptide. Furthermore, the truncated recombinant TACE could also cleave the natural proTNF-α substrate to 17-kDa TNF-α. Therefore, the C-terminal region of TACE, unlike the matrixins (where the hemopexin/vitronectin repeats are implicated in triple helix recognition) (46), has a disintegrin-like domain that does not seem to have any significant role in the activity or specificity of the enzyme in in vitro assays. Furthermore, human TNF-α convertase could not cleave mouse proTNF-α in vitro (47). Thus, it seems that human TACE is specific for human proTNF-α. Furthermore, Lunn et al. (48) have shown that ADAM-10 isolated from bovine spleen also has TNF-α convertase activity. This is not surprising, since several isolated MMPs have also been demonstrated to cleave proTNF-α in vitro (13, 49). It should be noted that T cells derived from TACE$^{-/-}$ (homozygous mouse) showed >80% decrease in TNF-α release and subsequent enhancement of cell surface pro-TNF-α accumulation, indicating that TACE is the key enzyme in proTNF-α processing in cell-based assays (16). It is quite possible that cysteine-rich region may be involved in dimer formation, based on its sequence homology with TNF-αR/NGF-R (35) and/or may act as an integrin. The disintegrin-like domain-containing proteases derived from snake venom are responsible for inhibiting platelet aggregation by interacting with $\alpha_{IIb}\beta_3$ on platelets (50), and recent studies have also shown that a binding site in the disintegrin domain of guinea pig fertilin (PH-30) is required for sperm-egg fusion (51, 52). These observations further emphasize the importance of disintegrin domain in cell-cell interactions (51).

One difference between the soluble snake venom-like proteases and their mammalian homologues is the presence of a cytoplasmic region and the transmembrane region (29, 52). The mammalian homologues are membrane bound and may show entirely different function. This may include the inside-out/outside-in signaling of the molecules, in view of a potential tyrosine phosphorylation site and Src-homology 3 binding motifs in the cytoplasmic region of TACE.

Our experiments also suggest that at least two forms of TACE (1 and 2) may exist based on the molecular weights of the two mRNAs. The significance of the shorter form is not clear. This is

the first observation that indicates that TACE mRNA is up-regulated in a pathophysiologic condition where TNF-α plays a significant role (4). Although the role of TNF-α in RA is well-documented, it has not been implicated in the pathophysiology of OA, in view of the comparatively low levels seen in the synovial fluids of OA patients (53). IL-β and TNF-α have been shown to independently induce IL-8 in human chondrocytes (43). Our studies demonstrate that IL-8 produced by chondrocytes of OA cartilage explants can be inhibited by TNF-αR:Fc. Furthermore, soluble IL-1R, together with TNF-αR:Fc, demonstrates an additive inhibitory effect on the IL-8 production that also highlights the importance of TNF-α in the pathophysiology of OA, not previously appreciated. These experiments demonstrate that the endogenous TNF-α produced by OA-affected cartilage is functionally active and is present in quantities sufficient to act in an autocrine or paracrine fashion to modulate other inflammatory/chemotactic mediators, such as IL-8. Although OA has been described as a "noninflammatory disease," (54) we have recently shown superinduction of inflammatory mediators, such as nitric oxide (18), PGE$_2$ (19), IL-8, IL-6, IL-1 (28), and MCP-1 (unpublished data) in OA-affected cartilage in ex vivo conditions. The present studies highlight the role of another proinflammatory cytokine, TNF-α, and its regulator, TACE, along with other inflammatory mediators described above in the pathophysiology of OA.

In summary, using a differential display screen between normal and arthritis-affected cartilage, we have cloned the cDNA for a snake venom-like protease that has >99% amino acid sequence homology to the recently described human TNF-α convertase (15, 16). We provide evidence that functional TNF-α is secreted in an active form by OA-affected cartilage, consistent with an up-regulated TNF-α convertase mRNA. These data thus identify two inducible molecules, TNF-α and its regulator, TNF-α convertase, that are produced by OA-affected cartilage and that could serve as future targets for pharmacologic intervention.

Acknowledgments

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