Increased Expression of a Novel Osteoclast-Stimulating Factor, ADAM8, in Interface Tissue Around Loosened Hip Prostheses

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ABSTRACT. ***Objective.*** ADAM8 is a protein of a disintegrin and a metalloproteinase family that can induce osteoclast fusion and activity, perhaps via interactions involving integrin receptors and their cysteine-rich/disintegrin domains. Because loosening of hip replacement implants is characterized by foreign body giant cells and peri-implant osteoclasts, it was speculated that this molecule might be (over)expressed in the synovial membrane-like interface tissues.

Methods. *In situ* hybridization; immunohistochemical staining with or without tartrate-resistant acid phosphatase (TRAP) staining; image analysis/morphometry; isolation, amplification, and cloning of ADAM8; nucleotide sequencing; quantitative reverse transcriptase-polymerase chain reaction (RT-PCR); and Western blot.

Results. *In situ* hybridization disclosed ADAM8 mRNA in mono- and multinuclear cells in both interface and control synovial samples. Quantitative RT-PCR revealed high ADAM8 mRNA copy numbers in interface tissue ($p < 0.01$). Accordingly, extensive ADAM8 immunoreactivity was observed in the lining-like layers and sublining areas of interface tissue ($p < 0.001$). A 65 kDa ADAM8 band in Western blot of tissue extracts confirmed these findings. ADAM8/TRAP double staining showed close spatial relationships of ADAM8 positive precursor cells with other precursors and/or TRAP-positive multinuclear cells.

Conclusion. ADAM8 is (over)expressed in tissues around aseptically loosened total hip implants, which are characterized by chronic foreign body inflammation and peri-implant bone loss. This is compatible with a role for ADAM8 in the formation of foreign body giant cells and osteoclasts. (J Rheumatol 2003;30:2033–8)

Key Indexing Terms:
ADAM8 POLYKARYON SYNOVIAL MEMBRANE OSTEOCLAST
GIANT CELL TOTAL HIP REPLACEMENT

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Total hip replacement (THR) is the most successful and cost-effective treatment for the disabling endstage hip diseases. Aseptic loosening of prosthetic components remains the major complication compromising the functional life of THR, accounting for more than 70% of revision operations[1]. A synovial membrane-like interface tissue can always be seen around aseptically loosened prostheses in the areas close to osteolytic lesions[2,3]. Such tissue is thought to play a central role in periprosthetic osteolysis and implant loosening[4-6].

ADAM form a family of proteins characterized by a disintegrin and a metalloprotease domain. The basic structure of the protein is phylogenetically well conserved. ADAM proteins have a role in cell fusion and adhesion (ADAM 1–3, 12, and 15) and in protein processing (ADAM 10 and 17)[7-11]. Recently, ADAM8 has been suggested to play a role in the fusion and induction of foreign body giant cells and osteoclasts[12]. Based on the function of the deletion mutant form of ADAM8 this effect has been thought to occur via its disintegrin/cysteine-rich domain. Interactions between the disintegrin domain of ADAM8 and integrin receptors on osteoclast precursors may be involved in the

induction of fusion. Since a soluble form of ADAM8 also works[12], the effect of binding may perhaps be mediated via activation of the precursor cells to facilitate fusion, rather than directly mediating the cell-to-cell contact in the way that ADAM 1 and 2 do in sperm-egg adhesion and fusion[7].

We investigated the expression of ADAM8 in synovial membrane-like interface tissue around loosened THR prostheses. Interface tissue is attached to host bone, which undergoes osteolysis and leads to characteristic linear and/or polycyclic lytic bone lesions around loosening implants. Synovial membrane samples from patients with hip osteoarthritis (OA) were used as controls to synovial membrane-like interface tissues.

MATERIALS AND METHODS

Patients and samples. Synovial membrane-like interface tissue samples (n = 18) were collected from areas adjacent to osteolytic lesions between prostheses/cement and bone from patients undergoing revision operations due to aseptic loosening of THR. Of these patients, 10 were men and 8 women, with a mean age of 69.3 years (range 64–82). The indication for primary THR was OA in all cases. The mean interval from primary THR to revision was 12.1 years (range 9–23). There were no clinical or laboratory signs of infection. Synovial membrane samples (n = 18) were obtained from patients with hip OA undergoing primary THR (11 women and 7 men, mean age 62.7 yrs, range 41–84). All samples were snap-frozen in isopentane precooled by dry ice and stored at –70°C.

Immunohistochemistry. Tissue sections (6 µm) were fixed in cold acetone at –20°C for 15 min. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in absolute methanol for 30 min. The sections were then incubated with the following reagents at room temperature: (1) normal goat serum 1:50 in Tris buffered saline (TBS) containing 0.1% bovine serum albumin (BSA) (Vector Laboratories, Burlingame, CA, USA) for 20 min; (2) polyclonal rabbit antiserum to ADAM8 (a kind gift from Dr. Sun Jin Choi[12]; 1:1000 in TBS containing 0.1% BSA) overnight; (3) biotinylated goat anti-rabbit IgG (1:100 in TBS containing 0.1% BSA; Vector) for 30 min; (4) avidin-biotin-peroxidase complex (1:100 in TBS; Vector) for 30 min; (5) a combination of 0.023% 3,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) and 0.006% H_2O_2 for 5 min. Between the steps, the sections were washed for 3 × 5 min in TBS. Finally, the slides were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and mounted using Diatex. Nonimmune rabbit IgG was used at the same concentration as and instead of the primary antibody as a negative staining control.

The ADAM8-stained areas were measured using a computer-assisted image analysis system (SensiCam, Kelheim, Germany). Under ×200 magnification, the sections without counterstaining were inspected with Leitz Diaplan microscope (Wetzlar, Germany) coupled to a 12-bit PC digital image camera (SensiCam). Images were analyzed using a semiautomatic Analysis Pro 3.0 image analysis and processing system (Soft Analysis System, GmbH, Germany). The whole section area and ADAM8-stained area were measured. Scores were reported as the percentage of positively stained areas. The data were expressed as mean and standard error of mean (SEM). After verification of the normality of the data, a 2-sided unpaired t test was used for statistical analysis.

ADAM8 and tartrate-resistant acid phosphatase (TRAP) double staining. After ADAM8 immunostaining, the slides were washed 3 × 10 min with PBS, and 3 × 10 min with distilled water. TRAP staining was carried out with a commercial kit (Leukocyte Acid Phosphatase kit; Sigma) according to the manufacturer's instructions. After a slight hematoxylin counterstaining, the slides were air-dried, mounted with glycergel (Dako, Glostrup, Denmark), and kept in the dark at 4°C.

Western blot. Tissue samples were minced into small pieces and homogenized in RIPA buffer (RIPA buffer set, Boehringer Mannheim GmbH, Germany) in an ice bath. Homogenates were centrifuged at 15,000 g at 4°C for 40 min. The supernatant was collected and stored at –70°C. Electrophoresis was performed with 10% polyacrylamide slab gel. The samples were boiled 5 min, then applied to the gel. After electrophoresis, the gel was blotted onto nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA, USA). The membrane was blocked overnight using 3% BSA in TBS. After washes, the membrane was incubated with a polyclonal rabbit antiserum to ADAM8 (1:10,000) for 60 min. This was followed by incubation with alkaline phosphatase-conjugated sheep anti-rabbit IgG (1:5000 in washing buffer containing 2% BSA; Silenus Laboratories, Hawthorn, Australia) for 60 min. The alkaline phosphatase-binding sites were revealed using color development solution (alkaline phosphatase conjugate substrate kit, Bio-Rad) for about 15 min. The membrane was washed in water for 30 min to stop the color reaction.

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Since osteoclastoma tissue is a rich source of osteoclasts, it was used as a source of ADAM8 mRNA. To clone ADAM8 PCR fragment, total RNA from 2 osteoclastoma samples was isolated using TRIzol reagent (Gibco BRL, Rockville, MD, USA). Messenger RNA (mRNA) was isolated from total RNA using magnetic $(dT)_{25}$-polystyrene beads (Dynal, Oslo, Norway). Extraction was controlled using spectrophotometric measurement and ß-actin amplification. 100 ng mRNA of each sample was used for first-strand cDNA synthesis (SuperScript preamplification system, Gibco BRL). PCR amplification was performed using 0.2 mM of target-specific primers for ADAM8: sense 5'-CAC AGA GGA TGG CAC TGC GTA TGA-3' and antisense 5'-CGT GCA CCT CAG TCA GCA GCT T-3', producing a 221 bp band; for ß-actin: sense 5'-TCA CCC ACA CTG TGC CCA TCT ACG A-3' and antisense 5'-CAG CGG AAC CGC TCA TTG CCA ATG G-3', producing a 295 bp band; and for ADAM17/TACE: sense 5'-AGA CCA TTG AAA GTA AGG CCC AGG A-3' and antisense 5'-TGC AGC AGG TGT CGT TGT TCA GAT-3'. The primers were mixed with 100 µM of dATP, dCTP, dGTP, and dTTP and 1 unit of the AmpliTaq Gold7 DNA Polymerase (Applied Biosystems, Foster City, CA, USA) in 50 µl of PCR buffer (15 mM Tris-HCl, pH 8.0, 1.5 mM $MgCl_2$, 50 mM KCl). For primers, corresponding sequences (ADAM8 accession XM 005675 and ß-actin accession X00351) were searched from the National Center for Biotechnology Information (NCBI) Entrez search system and sequence similarity search was done using NCBI blastn program. The reaction was run in a thermal cycler (RoboCycler 40 Temperature Cycler; Stratagene, La Jolla, CA, USA) for 40 cycles of 1 min denaturation at 95°C, 1 min annealing at 60°C, 1 min extension at 72°C, with 10 min extra extension used for the last cycle. The amplified PCR fragment was cloned with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) into a pCRII-TOPO vector. Plasmid DNA was isolated with a High Pure Plasmid Isolation Kit (Roche Molecular Biochemicals, Mannheim, Germany). Plasmid concentrations were analyzed spectrophotometrically. The plasmid used for quantitative PCR was sequenced using fluorescein-labeled dye terminator kits and analyzed on an automatic sequencer 373A (Applied Biosystems). The acquired sequence was verified with the NCBI blastn program. The ß-actin and ADAM17/TACE plasmids were cloned in a similar way.

Synovial membrane-like interface tissue (n = 10) and control OA synovial membrane samples (n = 10) were analyzed for the presence of ADAM8 and ADAM17 mRNA. The samples were handled in the same way as osteoclastoma samples. Quantitative PCR amplification was performed from 10 ng cDNA using 0.25 mM of target-specific primers in LightCycler™ SYBR Green I PCR mix with a LightCycler™ PCR machine (Roche). The identity of the product was verified by a melting curve analysis. Serial dilutions of cloned human ADAM8 in plasmid DNA were used to determine the copy number of the amplicon per 10,000 ß-actin mRNA copies. Each individual sample was amplified at least twice in both genes. All data are expressed as mean and SEM. Statistical analysis was performed with GraphPad Prism software using 2-sided unpaired t tests to compare the difference between the 2 groups.

In situ hybridization. The frozen sections (9 µm) from synovial membrane-like interface tissue (n = 6) and control OA synovial membrane samples (n = 6) were processed for *in situ* hybridization as described[13]. RNA probes were produced by *in vitro* transcription from cDNA of ADAM8. Digoxigenin-conjugated UTP (Boehringer-Mannheim) was incorporated into the RNA probes during the synthesis phase. Hybridization (overnight at 58°C) and post-hybridization washes were done under stringent conditions. Digoxigenin was labeled by alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer-Mannheim) and the color was developed in a mixture of nitroblue tetrazolium (NBT), 5-bromo-4-chloro-3-indolylphosphate (BCIP), and levamisole. The slides were counterstained with methylene green for 15 min. After thorough washing, the slides were air-dried, mounted with glycergel, and stored in the dark at 4°C.

RESULTS

Histology of synovial membrane-like interface tissue. The interface tissue samples were characterized by a foreign body-type reaction with histiocytosis and fibrosis. The principal cell types include macrophage-like cells, giant cells, and fibroblast-like cells. A synovial lining-like structure, usually 1–3 cell layers thick, was found in all samples. The stroma consisted of cell-rich areas containing macrophage-like cells and giant cells and the fibrotic areas with dense collagenous fibers and fibroblast-like cells. Under polarized microscopy, there was always a large amount of polyethylene debris. Metal particles were small and black. Bone cement particles usually appeared as irregular voids as they were dissolved during tissue processing. Thus, the samples were representative for aseptic loosening of total hip implants[2,3,14].

ADAM8 immunoreactivity. ADAM8 immunoreactivity was detected in all synovial membrane-like interface tissue samples around loosened THR prostheses. The synovial lining-like layers and sublining areas always exhibited intense staining in interface tissue samples (Figure 1A). The intensity of ADAM8 staining varied in different regions in the stroma of the interface tissue samples. The immunoreactivity was always strong in the cell-rich areas with the macrophage-like cell infiltration. In contrast, it was scarcely detectable in the fibrotic areas (Figures 1B, 1D). Immunoreactivity was always strong in small capillaries (Figure 1C), while large blood vessels often exhibited weak staining. ADAM8/TRAP double staining disclosed the close spatial relationship of ADAM8 immunoreactivity with TRAP-positive cells, with the staining sometimes overlapping. Such a close spatial relationship was found between ADAM8 positive mononuclear cells and between ADAM8 positive mononuclear and TRAP-positive multinucleate cells (Figures 1E, 1F). Synovial lining cell layer of the control OA synovial membrane samples also exhibited immunoreactive ADAM8, although the staining was usually very weak and restricted to certain areas (Figure 1G). Further, ADAM8 immunoreactive cells were found only occasionally in the deep stroma of OA synovial membrane samples (Figure 1H), in clear contrast to the cell-rich areas of interface tissue stroma, which contained high numbers of ADAM8 immunoreactive cells (Figure 1D). As there was some extracellular staining, it was difficult to count the number of positive cells. Instead, the percentage area of ADAM8 staining was calculated. T tests revealed a significant difference of the extent of staining ($p < 0.001$) between the synovial membrane-like interface tissue (56.4 ± 2.1) and OA synovial samples (12.7 ± 1.3). Western blot detected the 65 kDa band of ADAM8 in both interface tissue and control synovial samples (Figure 2).

Quantitative RT-PCR. All samples contained ADAM8 and ADAM17/TACE mRNA. The copy number of ADAM8 mRNA was significantly higher ($p < 0.01$) in synovial membrane-like interface tissue samples (10.3 ± 2.7 copies per 10,000 ß-actin mRNA copies) than in control OA synovial membrane samples (1.7 ± 0.3 copies per 10,000 ß-actin mRNA copies). Similarily, the copy number of ADAM17/TACE mRNA was significantly higher ($p < 0.05$) in interface tissue samples (224.8 ± 37.4 copies per 10,000 ß-actin mRNA copies) than in control samples (101.5 ± 25.3 copies per 10,000 ß-actin mRNA copies).

In situ hybridization. ADAM8 mRNA was detected in all synovial membrane-like interface tissue samples around loosened hip prostheses. The signal was especially strong in the lining-like layers and sublining areas (Figure 3A). The transcripts occasionally appeared in the deep stroma of the interface tissue samples. ADAM8 mRNA was also found in control OA synovial membrane samples (Figure 4), but the intensity of the signal was substantially lower than that observed in the interface tissue samples. The sense RNA probe used for ADAM8 did not show any staining (Figure 3B).

DISCUSSION

Cytokines able to stimulate monocyte recruitment and osteoclast precursor proliferation and differentiation have been described in interface tissue. One critical step in the formation of multinucleate cells, either foreign body giant cells or osteoclasts, is the fusion of mononuclear precursor cells to the maturing multinucleate cell. The docking molecule mediating this fusion, a key step in polykaryon formation, has evaded detection. Recently, ADAM8, a member of the ADAM (a disintegrin and a metalloproteinase) family[7-11], has been linked to this critical step[12]. ADAM are a large group of type I transmembrane proteins containing multiple domains including an N-terminal signal sequence followed by a prodomain, metalloproteinase, disintegrin, cysteine-rich, epidermal growth factor-like, transmembrane and cytoplasmic domain[7,8]. About 30 ADAM family members have been identified, and they have been implicated in adhesion, proteolysis and extracellular matrix-to-cell communication, intracellular signalling, processing of plasma membrane proteins, and procytokine conversion[9-11]. This study describes for the first time the presence of ADAM8 in the synovial membrane-like interface tissue surrounding asepti-

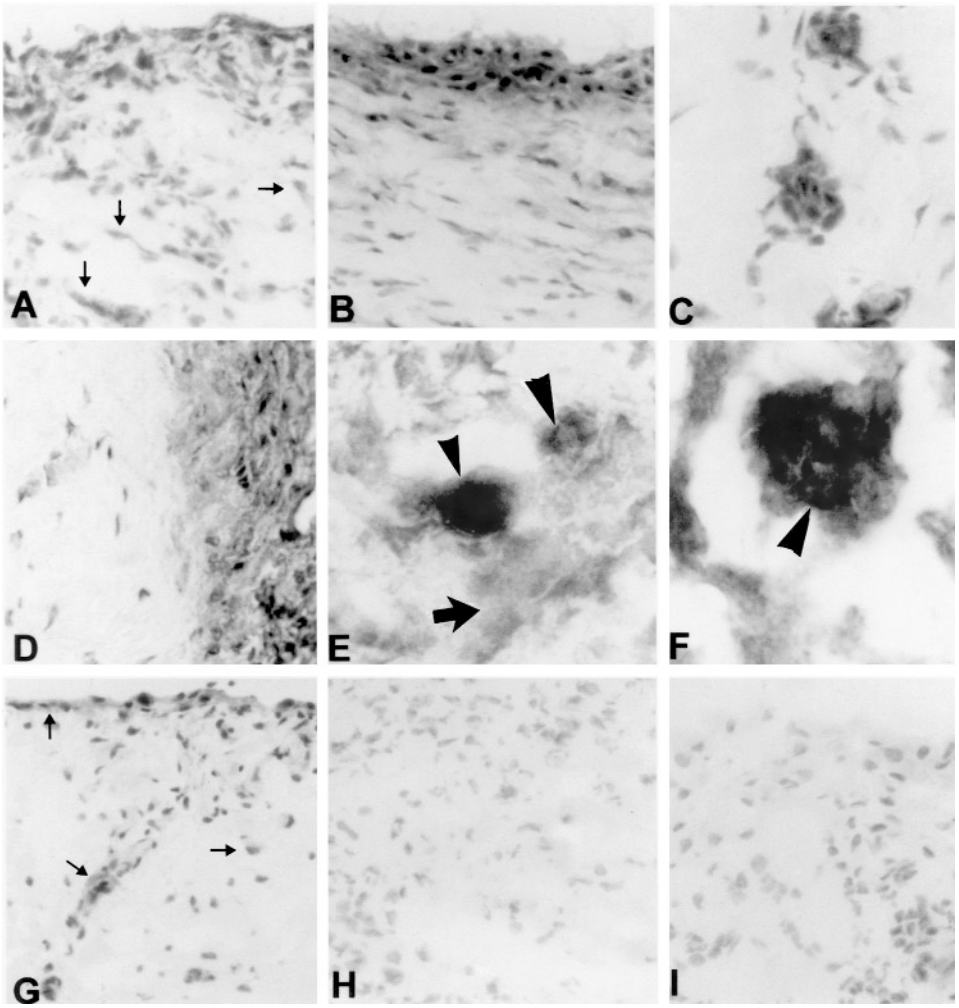

Figure 1. ADAM8 immunoreactivity. (A) Strong staining in the lining-like layers and sublining areas of the synovial membrane-like interface tissue around loosened hip prostheses. Note some pericellular staining in the connective tissue stroma (arrows). (B) Very strong staining in the lining-like layers of an interface tissue sample. (C) Very strong staining in small capillaries. (D) Very strong staining in the cell-rich area, whereas in the neighboring fibrotic area staining is scarcely visible. (E) ADAM8-positive mononuclear cells (arrow) close to TRAP-positive multinucleate cells (arrow heads). (F) ADAM8 immunoreactivity overlapping with TRAP staining (arrow head) in a double-positive multinucleate cell. (G) Relatively weak staining in the synovial lining cell layer and in occasional ADAM8 immunoreactive cells in stroma (arrows) in control OA synovial membrane sample. (H) A positive cell in the deep stroma of a synovial sample. (I) Negative staining control (counterstained with hematoxylin; original magnifications, A: ×156, B: ×250, C: ×400, D: ×156, E: ×400, F: ×625, G: ×156, H: ×250, I: ×250).

cally loosened hip prostheses. ADAM8 expression at the mRNA and protein level was clearly upregulated in the interface tissue[14,15] compared to OA synovial membrane. This would clearly seem to favor the local formation of both foreign body giant cells and osteoclasts. This conclusion is supported by our double staining experiments: ADAM8-positive and TRAP-positive mononuclear cells revealed close spatial relationships to other ADAM8-immunoreactive mononuclear cells and TRAP-positive multinucleate cells in the interface tissue. This strengthens the evidence for a role of ADAM8 in osteoclastogenesis[12].

The mRNA expression of ADAM8 but not ADAM 1, 9, 15, or 17 is increased during osteoclastogenesis[12]. We analyzed the mRNA expression of ADAM17/TACE in interface tissue. ADAM17 mRNA expression was significantly increased in interface tissue when compared to control samples. The increased ADAM8 expression in interface tissue is not necessarily due to monocytes/macrophages; we found that *in vitro* activation of fibroblasts with some cytokines, e.g., tumor necrosis factor-α (TNF-α), induces fibroblasts to express ADAM17/TACE (manuscript in preparation). Although ADAM17/TACE is not involved in osteoclastogenesis, it is naturally involved in inflammation[2] and mediates solubilization of the cell membrane-bound

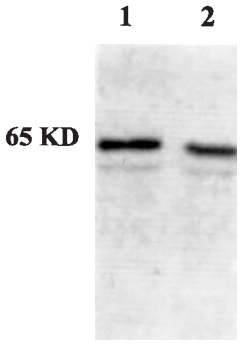

Figure 2. Representative Western blot of ADAM8 showing the 65 kDa bands in both the synovial membrane-like interface tissue (lane 1) and OA synovial membrane sample (lane 2).

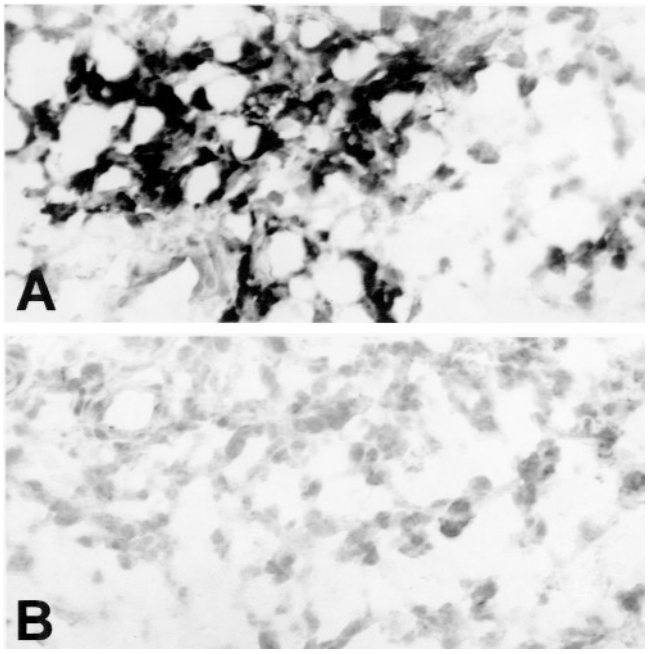

Figure 3. *In situ* hybridization analysis of expression of ADAM8 in the synovial membrane-like interface tissue. (A) ADAM8 mRNA was detected in the lining-like layers and sublining areas. (B) In the same area of the same section, the sense RNA probe used for ADAM8 showed no staining (counterstained with methylene green, original magnification ×250).

TNF-α into a soluble form via proteolysis and ectodomain shedding[10]. This also shows that if osteolysis is related to and probably caused by chronic inflammation, it may be impossible to isolate proinflammatory and osteolytic members of the ADAM family from each other. In this respect, ADAM8 upregulation is not exceptional, but instead reflects the role of the entire ADAM family in this setting.

ADAM8 can occur as a membrane molecule and in soluble form. Soluble ADAM8 was also observed in this study in the form of specific extracellular matrix staining. This implies proteolytic processing of transmembrane

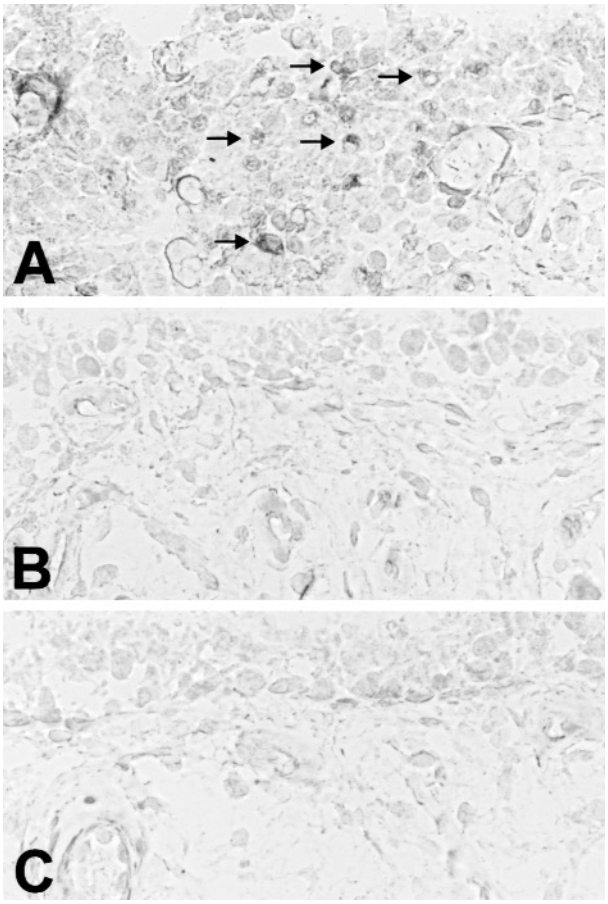

Figure 4. *In situ* hybridization analysis of ADAM8 expression in the synovial membrane of OA joint. (A) Positive cells with weak expression of ADAM8 mRNA were detected in regions of the synovial lining and sublining layers (arrows). (B) In the same area of the same section, the control sense ADAM8 RNA probe showed no staining. (C) Same sample but different region from that shown in panel A showing no ADAM8 mRNA expression in the lining layer or sublining (methylene green counterstain, original magnification ×250).

ADAM8 and diffusion of the soluble proteolytically cleaved fragment from its cellular site of production to surrounding matrix. Choi, *et al* have shown that soluble ADAM8 plays an important role in osteoclast formation[12]. Treatment of bone marrow cultures with soluble ADAM8-conditioned media significantly increased the number of bone resorption pits and the resorption areas in a dose-dependent manner. Their study also shows that ADAM8 acts at a late stage of osteoclastogenesis when osteoclast precursors start to fuse with each other. It has little effect at the proliferation stage. The mutated form, which contained only cysteine-rich and disintegrin domains, was as effective as the soluble full-length ADAM8[12]. Thus, it is likely that the interaction between integrin receptors on osteoclast precursors and the integrin-like domain in ADAM8 molecule mediates cell fusion. There could be 2 ways this interaction might induce the fusion. (1) The binding stimulates the cells to fuse rather

than directly mediating cell-to-cell contact, as soluble ADAM8 can also increase cell fusion. (2) ADAM8 molecules form dimers with each other or then heterodimerize[7] with other members of the ADAM family and finally really acts as a docking molecule between cells.

Intense ADAM8 immunoreaction was also found in small capillaries. Based on the amino acid structure of ADAM8 and the function of other homologous ADAM proteins, it can be speculated that ADAM8 may perhaps be involved in the degradation of the vascular basement membrane and of the collagen fibers in the perivascular extracellular matrix[11,16,17].

TNF-α can mediate the induction of ADAM8[18]. Indeed, expression of TNF-α is enhanced in aseptic loosening of THR[19], and strong ADAM8 staining was observed in the cell-rich areas where TNF-α is also produced, whereas in the neighboring fibrotic area ADAM8 staining was hardly visible. Similarly, ADAM8 expression at both mRNA and protein levels was low in the OA samples, which were characterized only by a low grade inflammation. TNF-α may in part explain the upregulation of ADAM8 in the interface tissue. Locally accumulated ADAM8 protein may contribute to periprosthetic osteolysis and aseptic loosening of THR directly by its proteinase-like effect and via its role in foreign body giant cell and osteoclast formation. ADAM8 inhibitors appear to be attractive therapeutic agents for aseptic loosening of THR prostheses.

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