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A Critical Role for Adrenomedullin-Calcitonin Receptor-Like Receptor in Regulating Rheumatoid Fibroblast-Like Synoviocyte Apoptosis

Benjamin Uzan,* Hang-Korng Ea,* Jean-Marie Launay, †‡ Jean-Michel Garel,* Romuald Champy,* Michèle Cressent,* and Frédéric Liote‡§

Rheumatoid arthritis (RA) is characterized by fibroblast-like synoviocyte (FLS) hyperplasia, which is partly ascribable to decreased apoptosis. In this study, we show that adrenomedullin (ADM), an antiapoptotic peptide, is constitutively secreted in larger amounts by FLS from joints with RA (RA-FLS) than with osteoarthritis (OA-FLS). ADM secretion was regulated by TNF-α. Peptidylglycine α-amidating monoxygenase, the ADM-processing enzyme, was expressed at the mRNA level by both RA-FLS and OA-FLS. Constituents of the ADM heterodimeric receptor calcitonin receptor-like receptor (CRLR)/receptor activity-modifying protein (RAMP)-2 were up-regulated at the mRNA and protein levels in cultured RA-FLS compared with OA-FLS. ADM induced rapid intracellular cAMP production in FLS and reduced caspase-3 activity, DNA fragmentation, and chromatin condensation in RA-FLS exposed to apoptotic conditions, indicating that CRLR/RAMP-2 was fully functional. ADM-induced cAMP production was less marked in OA-FLS than in RA-FLS, suggesting differences in receptor regulation and expression. ADM dose-dependently inhibited RA-FLS apoptosis, and this effect was reversed by the 22–52 ADM antagonist peptide. ADM inhibited RA-FLS apoptosis triggered by extrinsic and intrinsic pathways. Our data suggest that ADM may prevent or reduce RA-FLS apoptosis, via up-regulation of its functional receptor CRLR/RAMP-2. Regulation of ADM secretion and/or CRLR/RAMP-2 activation may constitute new treatment strategies for RA. The Journal of Immunology, 2006, 176: 5548–5558.
Plasma ADM concentrations are higher in patients with RA than in patients with other inflammatory conditions, such as systemic lupus erythematosus or scleroderma, or with osteoarthritis (OA) (20). Recently, ADM was detected in synovial fluid and tissue from patients with RA (21, 22), and some evidence of ADM expression by synovial fibroblasts and endothelial cells was found (15). Whether ADM exerts anabolic effects on synovial tissue is unknown, but ADM in rodents has been shown to increase bone mass and possibly cartilage thickness (23). No data are available on ADM receptor expression in synovial tissues or on the role of ADM in regulating apoptosis of FLS from rheumatoid synovium.

In the present study, we investigated the expression of ADM, its receptor CRLR/RAMP-2/3, and the processing enzyme PAM in FLS isolated from human joints with RA (FLS-RA) or OA (FLS-OA). We also looked for AC-PKA pathway-mediated regulatory effects of ADM in preventing RA-FLS apoptosis induced by serum deprivation, Fas ligand (Fas-L), and TNF-α.

Materials and Methods

Synovial specimens

Synovial tissue specimens were collected under sterile conditions from 12 patients with RA and 9 patients with OA. Specimen collection occurred during total knee or hip arthroplasty in all nine OA patients and in four RA patients; in the other eight RA patients, specimens were taken during wrist synovectomy for active disease. All RA patients fulfilled 1987 American College of Rheumatology criteria for RA (24), and all OA patients had joint pain with radiological evidence of degenerative joint disease at the time of surgery. Medications were not recorded.

Primary cultures of FLS

Synovial tissues were minced in prewarmed Ham’s F-12 culture medium (Aldrich) and 1 mg/ml dispase (Sigma-Aldrich). After dissociation, fibroblasts were pelleted by centrifugation at 1000 rpm for 5 min and plated in a 95% air-5% CO2 atmosphere. All in vitro experiments were conducted using primary synovial cells cultured between passages 3 and 6. FLS were treated with 10 ng/ml, respectively. Human ADM (1–52 aa) and the 22–52 ADM fragment (22–52 ADM) were purchased from Bachem. CRLR, RAMP-2, and ADM polyclonal Abs were purchased from Santa Cruz Biotechnology, for Western blotting. KL, Tris-HCl, MgCl2, dNTP, and the RNase inhibitor (RNAsin) were from Promega. Primers, Superscript II reverse transcriptase, Taq polymerase, and Ham’s F-12 culture medium were obtained from Invitrogen Life Technologies. Kodak Biomax autoradiographic film was purchased from Kodak. The cAMP donor forskolin, the PKA inhibitor H-89, and the nonselective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich.

Semiquantitative RT-PCR

Total RNA was extracted from RA-FLS and OA-FLS. The RNA pellets were dissolved in sterile distilled water, and the RNA was quantified based on OD at 260 nm. Synthesis of cDNA was achieved using 2 μg of total RNA. The reaction mixture had a final volume of 20 μl and contained 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 3 mM MgCl2, 10 mM DTT, 20 μl of RNasin, 200 U of Superscript II reverse transcriptase, 1 mM of each dNTP, and 0.25 μg of oligo(dT). Then, PCR was performed on an aliquot of this mix by adding 22 mM Tris-HCl (pH 8.4), 55 mM KCl, 1.4 mM MgCl2, 10 pmol of each specific primer (Table I), 2 nmol of each dNTP, and 1 U of TaqDNA polymerase. Semiquantitative PCR was performed by determining the exponential phase of the PCR products. Thus, the number of amplification cycles was 20 for GADPH controls, 32 for CRLR, 28 for RAMP-1 and RAMP-2, 34 for RAMP-3, 26 for ADM, and 30 for PAM. Temperatures during each cycle were 94°C for 30 s, 60°C (59°C for RAMP-3) for 30 s, and 72°C for 30 s. During the first cycle, the 94°C step was extended to 3 min, and during the last cycle, the 72°C step was extended to 3 min. Densitometric analysis of RT-PCR gel bands was performed using Bioprint image analysis (Vilber Lourmat) and each value was corrected for GADPH controls.

Western blotting

We evaluated the expression of CRLR and RAMP-2 protein in FLS cell lysates. CRLR protein in FLS cell membranes, and ADM protein in culture supernatants. Cells were lysed in buffer containing 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 10% glycerol, 0.5% Triton X-100, and 1/1000 antiprotease (Sigma-Aldrich).

For culture supernatants, medium was centrifuged at 5000 × g for 15 min, and proteins were precipitated by addition of 10% trichloroacetic acid, with overnight incubation at 4°C and subsequent centrifugation at 11,000 × g for 30 min. The pellet was resuspended in 50 μl of lysis buffer. Cell lysates were centrifuged at 12,000 × g for 30 min at 4°C. For cell membrane protein isolation, cell lysates were first centrifuged at 300 × g for 10 min at 4°C to eliminate cell debris, and lysates were further ultra-centrifuged at 100,000 × g for 10 min at 4°C. Proteins were quantified by protein assay (Pierce). Proteins (20–40 μg) were loaded after dilution in 50 mM Tris, 300 mM SDS, 0.25 M Tris (pH 6.8) and denaturation for 5 min at 95°C. We used 10 or 15% polyacrylamide gels, and we transferred proteins by electrophoresis onto a Hybond polyvinylidene difluoride membrane (Amersham Biosciences). Membranes were saturated for 2 h in TBS-0.05% Tween 205% nonfat milk.

Goat polyclonal Ab against ADM (sc-16496; Santa Cruz Biotechnology) and rabbit polyclonal Ab against RAMP-2 (sc-11380; Santa Cruz Biotechnology) and overnight incubation at 4°C. Western blotting showed that ADM, CRLR, and RAMP-2 were present in FLS cell lysates and in culture supernatants. Western blotting also revealed that ADM, CRLR, and RAMP-2 were present in FLS cell lysates and in culture supernatants.

Table I. Oligonucleotides used for the amplification

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Biotechnology) were used at 1/500 dilution overnight at 4°C. Peroxidase-conjugated anti-rabbit and anti-goat Abs (Sigma–Aldrich) were used at 1/15,000 dilution for 1 h as secondary Abs. For CRLR identification, affinity-purified goat polyclonal Ab raised against the C terminus of human CRLR (sc-18007; Santa Cruz Biotechnology) was used at 1/500 dilution overnight at 4°C. After three washes, the membrane was incubated in chemiluminescence Luminol reagent (SuperSignal West Pico Chemiluminescent Substrate; Pierce). Biomax MR (Kodak) was used to visualize the chemiluminescence. To standardize the results, membranes were reblotted with anti-actin (1/500, A2066; Sigma–Aldrich).

Assay of cAMP

Cells were cultured in Ham’s F-12 supplemented with 10% FCS in 12-well plates for 24 h at a density of 2 × 10⁵ cells/well. Cells were washed twice with Ham’s F-12, starved overnight in 1% FCS, and then pretreated with 1 mM IBMX for 30 min in serum-free culture medium. The effect of ADM was evaluated in the presence of 1 mM IBMX for 30 min. Cells were collected after trypanosinization and centrifugation. Cellular cAMP content was measured by radioimmunoassay (26) and normalized for protein content as determined by the bicinchoninic acid assay (Pierce).

Apoptosis assays

A set of three tests was used to evaluate RA-FLS apoptosis and the effect of ADM. Four apoptotic conditions were used, namely, serum deprivation alone, 10 ng/ml recombinant human TNF-α, a combination of both, and 20 ng/ml Fas-L.

Determination of caspase-3 (Cas-3) activity. FLS were cultured in Ham’s F-12 supplemented with 10% FCS in 6-well plates for 24 h at a density of 3 × 10⁵ cells/well. Each well was washed twice with Ham’s F-12, and the cells were starved overnight with 1% FCS, then treated with TNF-α and/or ADM in quadruplicate for 8, 24, or 48 h in serum-free culture medium. Cells were lysed in 130 μl of lysis buffer (10 mM Tris (pH 7.4), 200 mM NaCl, 5 mM EDTA, 10% glycerol, and 1% Nonidet P-40) for 30 min on ice and stored at −20°C. Lysates were centrifuged (12,000 × g, for 10 min at 4°C), and the supernatant was collected. Cas-3 activity was determined by cleavage of synthetic fluorogenic substrates containing the amino acid sequence recognized by Cas-3. The substrate was DEVD (Asp-Glu-Val-Asp) combined with a fluorophore (7-amino-4 trifluoromethylcoumarin) (BioSource Europe). Upon cleavage of the substrate by Cas-3, free 7-aminomalonamide and fluorophore were released, allowing fluorescence measurement. To determine activity, 120 μl of samples were incubated for 2 h at 37°C with 200 μl of reaction buffer (0.1 mM PMSF, 10 mM DTT, 10 mM HEPES/NaOH (pH 7.4) containing 10 μM of specific substrate (200 μM). Results were expressed as arbitrary units and normalized for protein content.

Hoechst-33258 staining. To visualize nuclear morphology and nucleosomal DNA condensation, FLS were cultured on Labteck, and treated under the same conditions for the Cas-3 activity assay. The cells were fixed in 4% PFA for 20 min at 4°C, stained with 0.2 μM Hoechst-33258 (Sigma–Aldrich), and incubated for 10 min at 37°C in the dark. The cells were washed with PBS, mounted (DakoCytomation), and examined under a epi fluorescence microscope. FLS with nuclei containing condensed chromatin were defined as apoptotic cells. Apoptotic cells were counted in three microscope fields from two different experiments.

Cytoplasmic histone-associated DNA fragments. For detection of apoptotic DNA cleavage, specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates was performed using a photometric enzyme immunoassay (cell death detection ELISA; Roche).

Viability assay

Cells were seeded at 10⁵/well in 96-well plates in 10% FCS culture medium and cultured for 24 h, then starved in 1% FCS overnight before treatment. After 24 h, cell viability in each group (six replicates) was measured using the MIT assay. Ten microliters of 5 mg/ml MITT was added to each cell suspension, and the cells were incubated for 4 h. The supernatants were discarded and the purple formazan product formed by the action of mitochondrial enzymes in living cells was solubilized by the addition of 100 μl of DMSO. The absorbance of each cell suspension was measured using a microplate reader at a test wavelength of 560 nm and a reference wavelength of 655 nm.

ADM assay

ADM protein was measured by ELISA (Phoenix) in conditioned medium from OA-FLS and RA-FLS under basal conditions and after serum deprivation, with or without TNF. RA-FLS (n = 5 patients) and OA-FLS (n = 5 patients) were plated at 10% FCS in 12-well plates at 2.5 × 10⁵ cells/ml overnight and starved in 1% FCS for 24 h before treatment. For each FLS sample, we evaluated four culture conditions (1% FCS, 1% FCS plus 10 ng/ml TNF-α, serum deprivation, and serum deprivation plus 10 ng/ml TNF-α). Samples were stored at −80°C before the assay.

Statistical analysis

Data are expressed as the mean ± SEM for proliferation and apoptosis experiments. Comparisons between groups were performed by ANOVA and post hoc tests (Fisher  Scientific). Unless specified otherwise, all experiments and functional studies were conducted at least three times using different FLS samples. Comparisons between ADM secretion by OA and RA-FLS were performed using the two-dimensional Kolmogorov-Smirnov test.

Results

Expression of ADM and its receptor in isolated FLS

In isolated cultured FLS from joints with RA or OA, RT-PCR consistently detected ADM mRNA (Fig. 1A), without any difference between RA and OA (Fig. 1A). DNA amplification was confirmed by sequencing the RT-PCR products (Genome Express). After overnight serum deprivation, ADM protein was detected in FLS culture supernatants from both RA and OA specimens, by Western blotting (Fig. 1B) and ELISA (Fig. 1C). ADM protein expression was higher in RA-FLS (Fig. 1B) than in OA-FLS supernatants. As shown in Fig. 1C, ELISA confirmed the presence of ADM secreted by OA-FLS and RA-FLS in 24-h-conditioned medium. Under basal conditions, e.g., 1% FCS, ADM was secreted by both cell types and a nonsignificant trend toward higher concentrations with RA-FLS compared with OA-FLS was seen. With serum deprivation, similar to the Western blotting condition, ADM secretion was significantly greater in RA-FLS compared with OA-FLS supernatants (p < 0.05).

We looked for expression of PAM, the amidating enzyme that cleaves immature ADM to the mature peptide. PAM mRNA was expressed with ADM mRNA by both RA-FLS and OA-FLS (Fig. 1A). Under basal culture conditions, without cytokine stimulation, no apparent difference was found between PAM mRNAs produced by RA-FLS and OA-FLS.

CRLR mRNA was expressed in all five RA-FLS samples and in only two of four OA-FLS samples, in tests using up to 400 ng of cDNA (Fig. 2A). However, CRLR mRNAs and protein were expressed at higher levels in RA-FLS lysates, as shown in Fig. 2, A and B, further supporting stronger CRLR protein expression by RA-FLS than by OA-FLS. After cytoplasmic membrane isolation, CRLR protein was detected by Western blot at a higher level in RA-FLS samples (Fig. 2C), suggesting up-regulation of the receptor. The three RAMP mRNAs were expressed in all RA-FLS and OA-FLS samples. RAMP-2 mRNA expression was higher in RA-FLS than in OA-FLS samples (Fig. 3A). The RAMP-2 protein was strongly detected in RA-FLS lysates, with two bands ~40 and 20 kDa corresponding to homodimers and monomers, respectively (Fig. 3B) (27). Protein expression was higher in RA-FLS than in OA-FLS lysates.

In contrast, RAMP-3 mRNA expression was similar in RA-FLS and OA-FLS samples (Fig. 4). RAMP-3 protein was not detected in cell lysates (data not shown). The mRNA for RAMP-1, a protein that yields CGRP by heterodimerization with CRLR, was also present in both RA-FLS and OA-FLS samples (Fig. 4).

Overall, these data suggest that both CRLR and RAMP-2 may be up-regulated in RA-FLS and may act as a complete and possibly active receptor for ADM in FLS.

ADM receptor present in FLS acts via the AC/PKA-dependent pathway

Human ADM induced a dose-dependent increase in cAMP levels in cell lysates (Fig. 5). A significant 2-fold increase in cAMP (10
pmol/mg of protein) was found in RA-FLS samples with an ADM concentration as low as $10^{-10}$ M (Fig. 5A). The cAMP increase was greatest (6-fold) with $10^{-7}$ M (32 pmol/mg of protein); higher ADM concentrations induced a decrease in cAMP, consistent with receptor desensitization (28). In contrast, OA-FLS were sensitive to ADM only in a concentration greater than $10^{-8}$ M (100-fold difference) (Fig. 5B). Both cell types expressed functional ADM receptor, although sensitivity of the RA-FLS receptor to ADM was greater.
ADM inhibits RA FLS apoptosis

To determine whether ADM regulated synovial cell apoptosis, we cultured RA-FLS in serum-free medium and we evaluated RA-FLS apoptosis using four indicators: Cas-3 activity, Hoechst staining, cell viability, and DNA fragmentation. Culturing in serum-free medium induced a large rise in Cas-3 activity normalized for cell lysate protein content (Fig. 6A) and a reduction in cell viability as assessed by the MTT test (Fig. 6B). Cas-3 activation was greater after 24 h of serum deprivation (Fig. 6A). In these preliminary experiments, when serum-free FLS were stimulated by TNF-α, Cas-3 activity showed a further nonsignificant increase that peaked after 24 h of stimulation, and cell viability decreased by up to 50% (Fig. 6B). To confirm that Cas-3 activation was followed by apoptosis, we evaluated the activation of PARP, a downstream protein involved in DNA repair. PARP cleavage was demonstrated by Western blotting after apoptotic conditions (data not shown).

Therefore, we used a combination of serum-free medium and TNF-α stimulation in 24-h cultures to induce apoptosis in additional experiments. Microscopic studies confirmed the reduction in cell viability as assessed both by phase-contrast microscopy, which showed an increased number of detached RA-FLS (Fig. 7, A and C), and by chromatin condensation visualized by Hoechst staining (Fig. 7, B and D). RA-FLS apoptosis was confirmed by DNA fragmentation (Fig. 8C).

We then evaluated the ability of various ADM concentrations to prevent RA-FLS apoptosis as assessed by Hoechst staining (Fig. 7), Cas-3 activity (Fig. 8A), cell viability (Fig. 8B), and DNA fragmentation (Fig. 8C). Apoptotic conditions induced a 3.5- to 5-fold increase in Cas-3 activity. Apoptosis was inhibited by ADM in a dose-dependent fashion. Cas-3 activity in 24-h cultures fell by 50% when cells were treated with 10^{-7} M ADM (Fig. 8A). Chromatin condensation visualized by Hoechst staining (Fig. 7, D and F) decreased significantly in the presence of 10^{-7} M ADM (Fig. 7, lower panel; *p < 0.01). The MTT assay showed a 45–50% reduction in RA-FLS viability under combined apoptotic conditions. When ADM was added in concentrations of 10^{-6}–10^{-9} M, RA-FLS viability improved to 85–90% of the control value. A significant increase in cell viability was seen with an ADM concentration as low as 10^{-9} M (Fig. 8B). Finally, ADM 10^{-7} M significantly reduced DNA fragmentation (Fig. 8C).

When the PKA inhibitor H-89 was added at 10^{-9} M to cultures containing 10^{-7} M ADM, increases occurred in Cas-3 activity (Fig. 9A; *p < 0.01) and in chromatin condensation as assessed by Hoechst staining (Fig. 7I; *p < 0.01). Under control conditions, H-89 also dramatically increased Cas-3 activity, strongly suggesting a protective role for PKA in this system. Indeed, when H-89 at
concentrations ranging from $10^{-6}$ M to $10^{-8}$ M was added, the inhibitory effect of ADM on Cas-3 activity was lost, in a dose-dependent fashion. Conversely, forskolin ($10^{-11}$–$10^{-6}$ M), an AC stimulator, induced a marked reduction in Cas-3 activity that was equal to or greater than the inhibition achieved with ADM (Fig. 9B). These results strongly suggest that ADM may inhibit RA-FLS apoptosis through the CRLR/RAMP-2-AC/PKA pathway.

The effects of other apoptotic conditions such as serum deprivation alone and Fas-L-induced apoptosis were also inhibited by ADM (Fig. 10). ADM dose-dependently inhibited serum deprivation-induced apoptosis (Fig. 10A). These results suggest that different apoptotic pathways may be regulated by ADM.

**TNF-α induces ADM secretion by RA FLS**

Because ADM was secreted in RA-FLS supernatants under control conditions, e.g., 1% FCS and serum deprivation, the effect of TNF-α alone or combined with serum deprivation on ADM secretion were studied. TNF-α has been shown to regulate ADM secretion in various cell types. After TNF-α stimulation alone, ADM secretion over 24 h was significantly greater in RA-FLS than in OA-FLS supernatants ($p < 0.01$; Fig. 1C). Also, serum deprivation alone or in combination with TNF-α significantly increased ADM secretion by RA-FLS compared with OA-FLS (median, 75 vs 25 pg/ml, respectively; $p < 0.05$). Of note, OA-FLS ADM secretion was not modified under any of the culture conditions.

**ADM receptor antagonist 22–52 ADM peptide inhibits the antiapoptotic effects of ADM on RA FLS**

We added 22–52 ADM, an ADM-receptor peptide antagonist (29), to RA-FLS cultures in serum-free medium with TNF-α. 22–52 ADM in concentrations of $10^{-8}$ and $10^{-6}$ M inhibited the antiapoptotic effect of $10^{-7}$ M ADM as assessed by Cas-3 activity (Fig. 11A) and chromatin condensation visualized by Hoechst staining (Fig. 7, upper panel, F and H). Similarly, RA-FLS viability was reduced when $10^{-7}$–$10^{-6}$ M of 22–52 ADM was used in combination with ADM (Fig. 11B). Microscopic examination showed fibroblast cell detachment and chromatin condensation with 22–52 ADM (Fig. 7, F, H, J, and L, respectively) as compared with ADM (Fig. 7, E and F, respectively). Interestingly, Cas-3 activity was increased, 2-fold compared with control apoptotic conditions, when RA-FLS were treated with $10^{-6}$ M 22–52 ADM. ADM-dependent cAMP production was completely inhibited when cells were incubated with 22–52 ADM (data not shown).

**Discussion**

This study provides the first evidence that the two components of ADM receptor CRLR/RAMP-2 coupled to AC/PKA are up-regulated at the mRNA and protein levels in cultured RA synovial fibroblasts. The data also confirm that ADM mRNA is present in FLS and that ADM is secreted into FLS-conditioned medium. In earlier studies, ADM was identified by RT-PCR and immunohistochemistry (15) in RA synovial tissues and fluids (15, 22) and by...
immunoradiometry in RA synovial tissues (22). Interestingly, in the present study, larger amounts of ADM protein were secreted by RA-FLS than by OA-FLS. The regulation of ADM secretion, including posttranscriptional control, remains to be evaluated, but our preliminary results suggest that TNF-α may contribute to increase ADM secretion and, therefore, may represent a regulatory loop. PAM, the processing enzyme that cleaves a glycine residue on immature ADM, was also coexpressed at the mRNA level in both RA-FLS and OA-FLS.

The heterodimeric receptor CRLR/RAMP-2 components were overexpressed at both the mRNA and the protein levels by RA-FLS as compared with noninflammatory OA-FLS. CRLR protein was detected at the cytoplasmic membrane, and the ADM receptor was functional, as shown by the increased cAMP production by both cell types in response to ADM. The marked difference in cAMP production between RA-FLS and OA-FLS may be ascribable to differences in the number and/or affinity of the receptors, and further studies are needed to explore this issue. In RA-FLS samples, ADM reduced apoptosis as assessed by Cas-3 activity, chromatin condensation, and DNA fragmentation.

ADM exerted a strong antiapoptotic effect on RA-FLS under various apoptotic conditions, including serum deprivation with or without TNF-α and Fas-L stimulation. TNF-α induced a slight increase in cell death after serum deprivation. TNF-α can initiate the activation of a proapoptotic pathway, in which recruitment of the death domain, containing protein FADD, is followed by activation of specific caspases. Many cells are resistant to the cytotoxic effects of TNF-α, due to concurrent activation of prosurvival pathways involving the transcription factor NF-κB and TNFR-associated factor-2 (30). TNF-α can also simultaneously activate Cas-8 (an apoptotic inducer) and induce NF-κB (a cell death inhibitor) (31). There is a balance between the apoptotic and antiapoptotic effects of TNF-α. In this study, serum deprivation may have also contributed to the final effect achieved with TNF-α. It has been shown that both serum deprivation and TNF-α increase the sensitivity to Fas-induced death and the expression of Fas mRNA and Fas receptor in murine renal interstitial fibroblasts (32).

Conversely, the 22–52 ADM receptor antagonist peptide induced a dose-dependent decrease in the antiapoptotic effect of ADM. In addition, experiments with the PKA inhibitor H89 and the direct AC activator forskolin showed that ADM exerted its antiapoptotic effects on RA-FLS through a cAMP/PKA-dependent mechanism. Because the ADM receptor CRLR/RAMP-2 is functional and up-regulated in RA-FLS, our results strongly suggest that ADM may prevent or reduce FLS apoptosis in RA.

Growth factors and cytokines other than ADM have been shown to inhibit fibroblast cell apoptosis. They include insulin-like growth factor-1 (11), bone morphogenic protein (10), TGF-β (33), platelet-derived growth factor (34), IL-4, and IL-13 (11). Interestingly TGF-β and platelet-derived growth factor prevent FLS apoptosis through an Akt-dependent mechanism (34), which is up-regulated and activated in RA synovitis.
Until now, ADM protein had been detected only in plasma, synovial fluids, and synovial tissues from RA patients. In these earlier studies, the cell sources of ADM were not determined. However, the present study shows that FLS secrete ADM, with enhanced production by FLS from RA. Synovial capillary endothelial cells (15) or other cell types such as resident macrophages may also produce ADM.

ADM exerts a potent regulatory role in cancer growth, as it promotes angiogenesis, enhances tumor-cell survival, and is upregulated under hypoxic conditions (16, 35). Similar tumor-like features are also achieved by RA tissue. Pro-ADM N-terminal 20 peptide, the ADM gene-related peptide that is cleaved to release ADM, also exhibits potent angiogenic effects at femtomolar concentrations (35). Intratumoral administration of a specific polyclonal anti-ADM Ab resulted in a dramatic reduction in s.c. glioblastoma xenograft tumor, along with a decrease in blood vessel density (36). Conversely, ADM cDNA transfected into endometrial tumor cells increased the tumor-forming potential of the cells in athymic mice (37). This issue of potential therapeutic importance remains to be investigated in RA animal models.

Our preliminary results show that TNF-α treatment can increase ADM secretion in supernatants from RA-FLS. ADM production is increased by oxidative stress and by proinflammatory cytokines, such as IL-1β and TNF-α in skin fibroblasts and smooth muscle cells (38, 39). ADM overproduction occurs constitutively in various human tumors such as ovarian, prostatic, and lung cancers (35). Hypoxia stimulates ADM production in vitro (40), and ADM prevents hypoxia-induced apoptosis of malignant cells (37). Decreased apoptosis has also been found in another cell type isolated from rheumatoid pannus, namely, activated infiltrating T cells (3). Hypoxia-inducing factor 1 (HIF-1) prevented apoptosis of T cells simultaneously exposed to hypoxia and to anti-CD3, two apoptosis-inducing factors (3). HIF-1, through its receptor, may be a factor of importance in regulating synovium homeostasis. Further studies should focus on interactions linking HIF-1, ADM, and CRLR/RAMP-2 during hypoxia, which is the rule in RA synovial tissues (41).

In addition to having antiapoptotic effects, ADM is an autocrine-paracrine regulator of cell proliferation (19). It enhances proliferation of skin fibroblasts and tumor cells by activating a protein G-coupled receptor/AC and PKA signaling pathway (42). However, ADM may also exert opposite effects on cell proliferation and apoptosis as observed in kidney mesangial cells in vitro (43). In the present study, we focused mainly on apoptosis of RA-FLS,
but preliminary experiments using RA-FLS showed a broad range of proliferative responses to ADM depending on FLS origin and activation (B. Uzan H.-K. Ea, and F. Liote, unpublished observations).

The regulation of CRLR expression is still largely unknown. The mechanisms of CRLR/RAMP-2 regulation in cultured RA-FLS are currently under investigation, but some evidence can already be found in the literature. Binding elements for transcription factors such as Sp-1, Pit-1, glucocorticoid receptor, and HIF-1α have been found in the human CRLR gene (41), and glucocorticoids are known to increase CRLR mRNA in vascular smooth muscle cells (VSMC) (44). Hypoxia increases CRLR expression in human VSMC from coronary arteries (45). Nagoshi et al. recently showed that NF-κB, P38 kinase/Akt-1, STAT-3, and MAPK pathways are strongly activated in rheumatoid synovial cells (34, 56). Activation of these signaling pathways contributes not only to the expression of a variety of antiapoptotic molecules, including FLICE inhibitory protein, Bcl-2, and Mcl-1, which protect against apoptosis initiated through death receptor- or mitochondria-dependent pathways. In RA-FLS the specific antiapoptotic molecules involved in ADM inhibition of extrinsic (TNF-α and Fas-L) and intrinsic (serum deprivation) apoptosis pathways are not yet known.

These findings carry hope for the development of new treatments for RA. Anti-TNF agents are now widely used to treat patients with the more severe forms of RA, and their potential role in regulating ADM secretion, CRLR/RAMP-2 expression, or FLS apoptosis deserves investigation. In vitro studies have suggested that an anti-TNF Ab used in RA patients may decrease Fas-mediated apoptosis of cultured RA-FLS (57), but in vivo studies showed no such effect (58). Agents capable of regulating synovial apoptosis are not yet available. A fall in ADM secretion by macrophages in vitro has been noted in response to corticosteroids, estradiol, and TGF-β (16). The receptor complex CRLR/RAMP-2 may be another target, as apoptosis is impaired in rheumatoid synovium. However, we suggest that ADM antagonists or analogues, such as 22–52 ADM, may hold promise as a therapeutic strategy.

In summary, we hypothesize that CRLR/RAMP-2 up-regulation via the AC/PKA-dependent pathway and basal ADM production contribute to inhibit apoptosis of human RA-FLS (Fig. 12), allowing growth and persistence of the rheumatoid pannus. Given the functional characteristics of CRLR/RAMP-2, 22–52 ADM or other ADM peptide antagonists may be useful for treating rheumatoid...
synovitis. In addition, further understanding of the downstream pathways in RA-PLS apoptosis, as well as from CRLR/RAMP-2 regulation in animal models of arthritis, may translate into new treatment approaches for RA.

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Disclosures
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References


