Autocrine induction of gliostatin/platelet-derived endothelial cell growth factor (GLS/PD-ECGF) and GLS-induced expression of matrix metalloproteinases in rheumatoid arthritis synoviocytes


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Abstract

Objective. The purpose of this study was to examine how gliostatin/platelet-derived endothelial cell growth factor (GLS/PD-ECGF) is involved in the molecular mechanism of cartilage degradation in rheumatoid arthritis (RA) with special reference to the GLS-induced gene expression and protein synthesis of matrix metalloproteinase (MMP)-1 (collagenase-1) and MMP-3 (stromelysin-1).

Methods. Fibroblast-like synoviocytes (FLSs) obtained from RA patients were cultured and stimulated by GLS. Changes in the expression levels of GLS, MMP-1 and MMP-3 were assessed by Northern blot analysis and reverse transcription-polymerase chain reaction (RT-PCR) for GLS, and by RT-PCR and enzyme-linked immunosorbent assay for MMPs and tissue inhibitor of metalloproteinase 1.

Results. GLS demonstrated a self-induction of mRNA in cultured RA FLSs. GLS evoked a dose-dependent induction of MMP-1 and MMP-3 mRNAs, and subsequently their extracellular secretion.

Conclusion. These findings suggest that GLS is a plausible pathogenic factor causing the extensive joint destruction in RA mediated via MMPs.

Keywords: Gliostatin, Platelet-derived endothelial cell growth factor, Thymidine phosphorylase, Rheumatoid arthritis, Matrix metalloproteinase.

Rheumatoid arthritis (RA) is a systemic disease whose features include extensive inflammation in regional synovium. The characteristic pathological findings of RA synovitis are aberrant proliferation of synovial lining cells, neovascularization by small vessels, accumulation of inflammatory cells in the synovium, and subsequent degradation of cartilage matrix. In particular, this cartilage degradation has long been explained by the actions of extracellularly secreted matrix metalloproteinases (MMPs) [1–5] released from synoviocytes and chondrocytes stimulated by several cytokines, interleukin (IL)-1β [6–9], tumour necrosis factor alpha (TNF-α) [7], epidermal growth factor (EGF) [10] and basic fibroblast growth factor (bFGF) [11, 12]. Here, we address the action of gliostatin (GLS) as a new member in the process leading to cartilage degradation in RA joints.

GLS was first isolated from neurofibroma as an inhibitor of glial cell growth [13], and successively discovered to promote the survival and neurite outgrowth of cortical neurons [14]. The chemical structure of GLS is identical to that of platelet-derived endothelial cell growth factor (PD-ECGF), and acts as a chemotactic and angiogenic factor on endothelial cells [15–17] and is accompanied by thymidine phosphorylase (dThdPase) activity [18–20]. Separately, we recently documented that GLS existed at a high concentration in synovial fluids and sera from patients with RA [21, 22], and that GLS in synovial fluid and sera of RA has a close correlation with its disease activity [22]. Furthermore, GLS was detected in synovial lining cells and cultured human fibroblast-like synoviocytes (FLSs) submitted 17 March 1999; revised version accepted 4 June 1999.

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in large quantity, and its expression was induced by TNF-α, IL-1β, IL-6 and IL-8 [23]. These findings led us to believe that the pathogenic potential of GLS in RA may not be limited to angiogenesis and could include the induction of cartilage degradation. In this study, we examined the effect of GLS stimulation on FLSs in changing the expression and extracellular secretion of MMP-1 and MMP-3, which are major triggers of cartilage degradation in RA. We have tried to introduce a new pathogenic aspect, cartilage destruction, in addition to the angiogenic action of GLS in RA.

Materials and methods

Reagents

Recombinant human (rHu)IL-1β and rHuTNF-α were purchased from Genzyme (Cambridge, MA, USA).

Isolation of FLSs

Synovial tissues were obtained at total knee arthroplasty. Three samples were from patients who met the American Rheumatism Association 1987 revised criteria for the classification of RA [24]. Informed consent was obtained from all patients. The tissues from the active synovitis region were washed with Ca²⁺/Mg²⁺-free Tyrode’s solution, minced into small pieces and digested with 0.1% trypsin for 10 min. Digested cells were collected by centrifugation (600 g for 10 min) and cultured for 7 days in Ham’s F-10 medium (Gibco, Grand Island, NY, USA) containing 10% fetal calf serum (FCS) with penicillin (100 U/ml) and streptomycin (100 μg/ml).

FLSs in confluence passed from three to nine times were used for experiments. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂/air. FLSs were grown to confluence in 25 cm² flasks and treated with IL-1β or GLS in a total medium volume of 4 ml. The conditioned media were stored at -20°C. The cultures were confirmed to be free of lymphoid and monocytic cells with the precise analysis of morphology and immunocytochemistry as previously described [23].

Construction of the bacterial expression vector

The GLS cDNA containing the open reading frame was produced by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA of A431 cells was extracted by the acid guanidium–phenol–chloroform (AGPC) method [25]. Reverse transcription was conducted in 20 μl of 50 mM Tris–HCl (pH 8.3) containing 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol (DTT; Wako, Osaka, Japan), 4 mM dNTP, 200 U Superscript II RNase H⁻ Reverse Transcriptase (Gibco BRL, Paisley, UK) and 2.5 mM oligo d(T)₁₆. The enzyme reaction mixture was prepared in a 500 μl tube and cDNA was synthesized at 42°C for 60 min. Then, mRNA–cDNA chains were denatured and the RT activity was arrested by heating up to 99°C for 5 min.

PCR was successively carried out in 100 μl of 10 mM Tris–HCl (pH 8.3) containing 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 1.5 mM MgCl₂, 50 mM KCl, 10% dimethyl sulphoxide (DMSO) and 0.025 U/ml AmpliTaq (Perkin Elmer Cetus, NJ, USA). Using the upstream primer (5'-CCGGAGCGATGGCAGCCTTGATGACCC-3') and downstream primer (5'-GGCAAGAGGCTTTATGCGCGCGC-3'), PCR was carried out for 35 cycles under the following conditions: denaturing step: 94°C for 30 s; annealing step: 58°C for 30 s; extension step: 72°C for 1 min. The PCR product was treated with Klenow fragment (Takara, Shiga, Japan) to prepare a complete blunt end, then ligated to the HinclI site of pUC19 vector. Clones were sequenced using Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham, Buckinghamshire, UK) and analysed on an automatic A.L.F. red sequencing apparatus (Pharmacia, Upplands Väsby, Sweden). One of the clones was found to contain a cDNA coding the correct reading frame of GLS/PD-ECGF.

Expression and purification of rHuGLS

The rHuGLS protein was expressed as a His-tag fusion protein in E. coli using a pET-19b vector and purified by Ni²⁺-chelate affinity chromatography (HiTrap-Affinity column, Pharmacia). Analysis of protein was carried out with 10 μg aliquots of each fraction, boiling at 100°C for 5 min in the presence of 0.1 M mercaptoethanol, 2% sodium dodecyl sulphate (SDS) and 15% glycerol. A single band on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) attested to the purity of the recombinant. After dialysis against 10 mM sodium phosphate buffer (NaPB) (pH 7.5) containing 2 mM DTT overnight, the purified protein was shaken gently with A.T.A.F. red sequencing apparatus (Amersham, Buckinghamshire, UK) and its dThdPase activity was determined by the spectrophotometric method as previously described [23].

Northern blot analysis

Full-length fragments of GLS cDNA were labelled with [³²P]dCTP (Amersham) by the Multiprime DNA labelling kit (Amersham) and used as probes for appropriate mRNA detection. Total RNA samples (5 μg) were denatured in 1× MOPS buffer, containing 0.66 M formaldehyde, and 50% (v/v) formamide, run on a 0.9% agarose-formaldehyde gel at 100 V until the xylene cyanol dye had migrated 25 mm, and transferred on to...
a membrane (Hybond-N; Amersham) by capillary transfer, and were hybridized with 32P-labelled GLS-specific probe. The membrane was pre-hybridized for 4 h in buffer containing 50% formamide, 5 × SSC, 0.1% SDS, 1% glycine, 5 × Denhardt solution and 500 µg/ml denatured salmon sperm DNA at 42°C. Hybridization was carried out overnight in buffer containing 50% formamide, 5 × SSC, 0.1% SDS, 1.5 × Denhardt solution, 10% dextran sulphate, 100 µg/ml denatured salmon sperm DNA and the 32P-labelled probe at 42°C. The membrane was washed twice at 65°C for 15 min each with 5 × SSC–0.1% SDS and once at 45°C for 15 min each with 2 × SSC–0.1% SDS prior to autoradiography. Filters were exposed to Kodak (Rochester, NY, USA) X-ray film at –70°C.

**Enzyme-linked immunosorbent assay (ELISA) for MMPs**

Immunoreactive MMP-1 and tissue inhibitor of metalloproteinase 1 (TIMP-1) in conditioned media were measured by the commercially available ELISA kit (Amersham) according to the manufacturer’s instructions included. Immunoreactive MMP-3 concentrations were determined according to the method of Obata et al. [26] with minor modifications.

**RT-PCR assay**

Expression of the MMP-1 and MMP-3 gene was assessed by RT-PCR. Reaction conditions are described in the GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT, USA). PCR was performed using a thermal cycler (TaKaRa, Osaka, Japan) with 26 cycles for MMPs and 35 cycles for GLS, consisting of 30 s at 95°C for denaturing, 30 s at 58°C for annealing and 1 min at 72°C for extension. Five microlitres of reaction product were analysed on 2% agarose gel. The oligonucleotides of specific primers (Table 1) in PCR were synthesized according to published information [23, 27, 28]. In this study, primers detecting β-actin mRNA were used to normalize the amount of cDNA in each reaction. β-Actin primer set 1 (amplified PCR product 218 bp) was used for GLS assay and β-actin primer set 2 (amplified PCR product 542 bp) was used for MMP assays. PCR products were quantified by laser-induced fluorescence-linked capillary-gel electrophoresis (LIF-CGE) using capillary electrophoresis P/Ace® system 5010 (Beckman, Fullerton, CA, USA) as previously described [29].

**Statistical analysis**

Results are expressed as the mean ± s.d. Statistical significance of differences was calculated using the Mann–Whitney U-test (non-parametric).

**Results**

*Change of GLS mRNA in FLSs stimulated by GLS, IL-1β, and TNF-α*

We measured the changes of GLS mRNA in RA FLSs stimulated for 12 h by IL-1β (0.1, 1, and 10 ng/ml) and GLS (30 and 300 ng/ml). In Northern blot analysis, IL-1β strongly augmented the GLS mRNA expression of FLSs in a dose-dependent manner. GLS also dose-dependently caused the self-induction of GLS mRNA in FLSs, whereas the induction rate by GLS itself was lower than that by IL-1β (Fig. 1A). Similar inductions of GLS were obtained from the RT-PCR assay (Fig 1B), whose methodological precision has been previously confirmed by Matsukawa et al. [27].

**Effect of GLS stimulation on the expression of MMP-1 and MMP-3 mRNAs**

RT-PCR detected very low expressions of MMP-1 and MMP-3 mRNAs in control FLSs. Both expressions were markedly induced by GLS (300 ng/ml, lane 2) or IL-1β (1 ng/ml, lane 3) (Fig. 2A). It was proved in advance by PCR lacking the RT step that the RNA fractions used are free from concomitant DNAs (data not shown). Total RNA ranging from 0 to 100 ng gave RT-PCR products for both MMPs and β-actin in a linear manner (Fig. 2B). Accordingly, all RT-PCR examinations were performed within this range. Time-dependent induction of MMP mRNAs by GLS stimulation (300 ng/ml) exhibited a bell-shaped profile giving a maximal value at 12 h for both MMP-1 and MMP-3 (Fig. 3). At 12 h, GLS elicited a dose-dependent induction of both mRNA expressions at concentrations from 3 to 300 ng/ml (Fig. 4).

**Promotion of extracellular secretion of MMP-1, MMP-3 and TIMP-1 by GLS**

In addition to the intracellular induction of MMP mRNA by GLS, we tested the extracellular secretion of

**Table 1. Oligonucleotides of 5’ primers and 3’ primers of GLS, MMPs and β-actin**

<table>
<thead>
<tr>
<th>Primer</th>
<th>PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLS</td>
<td>-GTGGAGGGGCTGTCGCCTCTGTGGTGCGAGGAA-5’</td>
</tr>
<tr>
<td>β-Actin for GLS assay</td>
<td>-TACATGCGGGTTGGTGCGAGGAA-5’</td>
</tr>
<tr>
<td>MMP-1</td>
<td>-AAAGAGGAGCTCCTCCACCCCTG-3’</td>
</tr>
<tr>
<td>-GTGGAGGGGCTGTCGCCTCTGTGGTGCGAGGAA-5’</td>
<td>155</td>
</tr>
<tr>
<td>β-Actin for MMPs assay</td>
<td>-TTCTAGATATTCTCGACTGACTCCTACTG-3’</td>
</tr>
</tbody>
</table>
MMPs into their conditioned media from cultured RA FLSs. Immunoreactive MMP-1, MMP-3 and TIMP-1 were detected by the respective ELISA systems in the conditioned media of cultured FLSs for 3–48 h in the presence of GLS (300 ng/ml). The concentrations of MMP-1 and MMP-3 were cumulatively increased by GLS stimulation in a time-dependent manner (Fig. 5).

Both levels of MMPs were increased by GLS stimulation (300 ng/ml) to two-fold for MMP-1 and to 100-fold for MMP-3 at 48 h, also by IL-1β stimulation (1 ng/ml) to 5-fold for MMP-1 (16.4 ± 7.6 ng/ml) and to 370-fold for MMP-3 (7480.0 ± 435.9 ng/ml). Results represent the mean ± s.d. of four determinations. However, the TIMP-1 level retained a control value at 48 h after stimulation.

Discussion

Recently, it has been reported that GLS is expressed in several tumours [18–20]. GLS is related to angiogenesis and tumour growth. This same angiogenic function is suggested for GLS in RA. However, the high concentration of GLS existing chronically in the joint cavity in RA patients is a cellular environment differing from that of tumours. We determined the effect of high concentrations of GLS mimicking local conditions in the RA joint on FLSs.

We have first demonstrated that GLS made clear a marked induction of GLS itself at the mRNA level as well as MMP-1 and MMP-3 at both mRNA and protein levels. The ELISA system ascertained the production and extracellular secretion of MMPs subsequent to the mRNA inductions by GLS. The effective dose of GLS necessary for this induction (300 ng/ml) corresponded well to the concentration observed in RA synovial fluids (223 ng/ml) previously described [22]. However, GLS concentrations in synovial fluids from patients with osteoarthritis (8.7 ng/ml) are not high enough to induce the expressions of GLS, MMP-1 and MMP-3. MMP-1 and MMP-3 are implicated in the connective tissue degradation in RA. MMP-1 degrades type I, II, III and X collagen. MMP-3 degrades proteoglycans, type III, IV and VIII collagen, laminin, fibronectin and gelatin [30]. These proteolytic actions are the initial step leading to extracellular matrix degradation, and are speculated to be regulated or modulated by a variety of extracellular signals in RA synovia. Indeed, there are numerous reports describing that extracellular secretion of MMP-1 and MMP-3 in gingival and synovial fibroblasts is induced by IL-1 [6–9], TNF-α [7], EGF [10] and bFGF [11,12]. It is of interest that some of these cytokines, such as TNF-α, IL-1, IL-6 and IL-8, are capable of inducing GLS synthesis [23]. Furthermore, TNF-α elicited the extracellular secretion of GLS from RA synoviocytes [23]. Levels of GLS detected clinically in RA synovial fluid are very high, whereas its extracellular secretion by TNF-α in vitro is at a low level. This discrepancy may be explained by the turnover of synoviocytes in vitro releasing proteins, accentuated by mechanical stimulation occurring with joint motion. Taken together with these observations, the present findings provide support for the pathogenic role of GLS in cartilage degradation in RA. GLS rich in RA synovial fluids may act to mediate the signal transduction of those cytokines via augmentation of the synthesis and secretion of GLS itself, and simultaneously to elicit the induction and extracellular secretion of MMP-1 and MMP-3 triggering cartilage degradation. It is conceivable that GLS is also involved in the inflammatory cell infiltration and neovascularization in RA synovial tis-
GLS-induced expression of GLS and metalloproteinases

Fig. 2. (A) Expression of MMP mRNA in GLS-stimulated FLSs. Agarose gels show the products of PCR amplification. Confluent FLSs were incubated in 25 cm² flasks in the presence or absence of IL-1β or GLS for 12 h. Isolated RNA was subjected to reverse transcription and followed by PCR for MMP-1 and MMP-3 at 26 cycles together with β-actin as an internal control. Lane L, 100 bp DNA ladder; lane 1, unstimulated FLSs; lane 2, IL-1β 1 ng/ml; lane 3, GLS 300 ng/ml. (B) Quantitative determination of RT-PCR products for MMP-1 or MMP-3. RT-PCR products from the indicated amounts of total RNA were quantified by LIF-CGE. The vertical axis indicates the amounts of PCR products expressed as each peak area obtained from elution profiles.

sues by its strong chemotactic and angiogenic actions [15–17]. Furthermore, it is worth noting that GLS production is induced by GLS itself, as well as by those cytokines detected in the RA synovial fluids. This auto-
crine induction of GLS, as reported with other cytokines [31–33], may in part explain the ‘vicious circle’ in the chronic process of RA leading to cartilage degradation. It is most likely that there should be a certain kind of
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Time-dependent induction of MMP-1 and MMP-3 mRNA after GLS stimulation. FLSs were cultured at confluence in 25 cm² flasks in F-10 medium containing 10% FCS, and then stimulated by GLS (300 ng/ml). Total RNA of stimulated FLSs by GLS (300 ng/ml) was subjected to RT-PCR at the indicated time points after stimulation. MMP mRNA levels are expressed as a relative RT-PCR product ratio (MMPs/β-actin). Results are the mean ± S.D. of three determinations.

Fig. 3. Time-dependent induction of MMP-1 and MMP-3 mRNA after GLS stimulation. FLSs were cultured at confluence in 25 cm² flasks in F-10 medium containing 10% FCS, and then stimulated by GLS (300 ng/ml). Total RNA of stimulated FLSs by GLS (300 ng/ml) was subjected to RT-PCR at the indicated time points after stimulation. MMP mRNA levels are expressed as a relative RT-PCR product ratio (MMPs/β-actin). Results are the mean ± S.D. of three determinations.

Cell surface receptor for GLS. However, the receptor is not evident. It is necessary to identify the receptor for GLS and thus to examine whether or not the MMP induction is evoked by a receptor-mediated feedback regulation through GLS as well as other cytokines.

MMP activity is also regulated by endogenous inhibitors such as α2-macroglobulin and TIMP-1 and TIMP-2. Synovial lining cells from RA have also been found to be capable of producing TIMP-1 [34]. Therefore, we have attempted to quantify TIMP-1 in the conditioned medium of FLSs, and demonstrated that TIMP-1 does exist at a low level, but is not induced by GLS in FLSs. These results suggest that this unbalanced expression between MMPs and their inhibitors in RA synovium, a high induction rate of MMPs and a low expression of TIMP-1, may accelerate the degradation of cartilage in the RA joint cavity.

Fig. 4. GLS-mediated upregulation of MMP-1 and MMP-3 mRNA in FLSs. FLSs were cultured at confluence in 25 cm² flasks in F-10 medium containing 10% FCS, and treated with various concentrations of GLS (3–300 ng/ml) for 12 h. Levels of MMP mRNA are expressed as a relative RT-PCR product ratio (MMPs/β-actin). IL-1β was used as a positive control. P < 0.05 as compared with control. Results represent the mean ± S.D. of three determinations.
Fig. 5. Induction of MMP-1, MMP-3 and TIMP-1 secretion by GLS. FLSs were grown to confluency in 25 cm² flasks in F-10 medium containing 10% FCS and simultaneously were changed to fresh medium with FCS in all flasks. GLS (300 ng/ml) was added to the medium at intervals after medium change, and conditioned media were obtained at the end of 48 h incubation, giving results of stimulation with GLS from 0 to 48 h. Immunoreactive human MMP-1, MMP-3 and TIMP-1 were determined by ELISA. *P < 0.05 and **P < 0.01 as compared with control (0 h). Results represent the mean ± S.D. of four determinations.

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References

15. Ishikawa F, Miyazono K, Hellman U, Drexler D, Wernstedt C, Hagiwara K et al. Identification of angio-


