GLIOSTATIN/PLATELET-DERIVED ENDOTHELIAL CELL GROWTH FACTOR AS A CLINICAL MARKER OF RHEUMATOID ARTHRITIS AND ITS REGULATION IN FIBROBLAST-LIKE SYNOVIOCYTES

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SUMMARY

The objective was to assess the congruity of gliostatin/platelet-derived endothelial cell growth factor (GLS/PD-ECGF) with other clinical markers of rheumatoid arthritis (RA) and to define its molecular mechanism of action in the complicated cytokine network during RA pathogenesis. Immunoassay systems were used to quantify GLS or cytokine levels in laboratory and clinical samples. Expression levels of GLS were determined by reverse transcription-polymerase chain reaction methods. The GLS levels in synovial fluid were correlated with interleukin-1 (IL-1) and IL-8. The serial data of serum GLS levels reflected well changes in the disease activity during the clinical course of four representative patients with RA. In cultured fibroblast-like synoviocytes, tumour necrosis factor-α (TNF-α), IL-1, IL-6 and IL-8 induced GLS expression. In conclusion, our results suggest that the serum GLS level, mostly derived from cytokine-stimulated synoviocytes, was a useful clinical marker of RA.

KEY WORDS: Gliostatin, Platelet-derived endothelial cell growth factor, Thymidine phosphorylase, Rheumatoid arthritis, TNF-α, IL-1, IL-6, IL-8, Fibroblast-like synoviocytes, Reverse transcription-polymerase chain reaction.

Gliostatin/platelet-derived endothelial cell growth factor (GLS/PD-ECGF) is a protein factor, which induces angiogenesis by a mechanism including the proliferation and chemotactic migration of endothelial cells [1–3], inhibits growth of glial cells [4, 5], promotes glial differentiation and has neurotrophic actions on cortical neurons [6]. Since the high degree of chemical homology between PD-ECGF and thymidine phosphorylase became evident, and the enzymatic action of PD-ECGF has been reported [2, 3, 7–9], it has become controversial as to whether or not the biological actions depend on the catalytic activity of the factor metabolizing pyrimidine from thymidine to deoxyribose-1-phosphate and thymine. A variety of biological functions, such as angiogenic [2, 3], neurotrophic [4–6] and glial differentiative actions [6], cannot be explained by this enzymatic action alone. Although Haraguchi et al. [10] speculate that the angiogenic action of PD-ECGF is not due to direct stimulation of endothelial cell proliferation, but to the degradation product (deoxyribose) derived from its enzymatic product (deoxyribose-1-phosphate), differences in thymidine levels of culture medium did not affect the neurotrophic and glial differentiative actions of GLS in our experiments [11]. Thus, we believe that there is another unknown function of GLS, other than its enzymatic action, to evoke these neurotrophic and angiogenic actions.

Rheumatoid synovitis is known to be characterized by neovascularization inducing mononuclear cell emigration and proliferation of synovial tissues. We have previously measured the concentration of the angiogenic factor, GLS, in sera and synovial fluids of rheumatoid arthritis (RA) patients, and demonstrated for the first time an enormously high concentration in RA synovial fluids as well as in RA sera [11, 12]. This fact suggests that the GLS in synovial fluid might be produced by synovial tissue inducing neovascularization in rheumatoid synovial tissues. In this study, we primarily confirmed the usefulness of GLS as a clinical marker to evaluate systemic disease activity and therapeutic efficacy in RA. Secondly, we investigated the induction mechanism of GLS expression in synovial cells by tumour necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6 and IL-8 which are reported as pathogens in RA.

MATERIALS AND METHODS

Reagents

Recombinant human tumour necrosis factor-α (rHuTNF-α), recombinant human interleukin (rHuIL)-1α, rHuIL-6 and rHuIL-8 were purchased from Genzyme (Cambridge, MA, USA).

Patients

Serum and synovial fluid (SF) samples were obtained from 38 patients in an out-patient clinic at the Department of Orthopedics, Nagoya City University Medical School. The details of the patients are shown in Table I. All patients met the American Rheumatology Association (ARA) 1987 revised criteria for the classification of RA [13]. All were under treatment with varying dosages of non-steroidal anti-inflammatory drugs (NSAIDs). Of these, 29 also received disease-modifying anti-rheumatic drugs (DMARDs) and four received low-dose oral steroids. The Activities of Daily Living (ADL) score [14] of the Japanese Rheumatism Association, C-reactive protein (CRP) and erythrocyte

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sensation rate (ESR) were used as clinical assessments. The ADL score is a self-assessment score evaluating upper and lower extremity function on a five-step scale for various actual activities, e.g. turning the tap, combing or brushing the hair, dressing oneself, etc. for the upper extremities, and getting out of bed, walking up and down stairs, crouching down, etc. for the lower extremities. The scores were graded: 0, without limitation; 1, adequate for normal activities with some difficulty; 2, inadequate without support; 3, limited; 4, wholly incapacitated. In patients presenting with joint hydrops, SFs were collected under sterile conditions with EDTA (5 mg). After centrifugation at 1800 g for 30 min at 4°C, supernatants were aliquotted and stored at −80°C until analysis. All patients also had blood samples taken at the same time as joint aspiration with sera being stored at −20°C before testing for the presence of immunoreactive GLS.

**Enzyme immunoassay (EIA) for GLS**

GLS was measured using an EIA system, as described by Hirano et al. [15]. The polyclonal antibodies on the solid phase were obtained by immunizing New Zealand albino rabbits with 40 mg of purified natural human GLS, and the monoclonal antibody was used for the secondary antibody labelled with β-galactosidase. The detection limit of this assay was 150 pg/ml. No significant cross-reactivity or interference were observed.

**Cytokine immunoassays**

All cytokine measurements were performed in duplicate on each sample of SF. Immunoreactive human IL-1β, IL-8 and TNF-α were measured using commercially available ELISA kits (Quantikine; Research and Diagnostic Systems, MN, USA) according to the published protocol. IL-6 was also tested with an ELISA system (Fujirebio, Tokyo, Japan). Before measurement, SFs were incubated for 30 min at 37°C with 500 U/ml hyaluronidase (Sprase; Mochida, Tokyo, Japan). Specimens were centrifuged at 500 g for 10 min and then supernatants were used for analysis.

**Synoviocyte isolation and culture**

Synovial tissues were obtained at the time of total knee joint replacement surgery. Three samples were from patients who met the ARA 1987 revised criteria for the classification of RA [13] and who had active synovitis. The tissue were washed with Ca2+ - and Mg2+-free Tyrode’s solution, minced and treated with 0.1% trypsin for 10 min. Dissociated cells were collected by centrifugation (600 g for 10 min) and the cells were washed three times and cultured as primary synoviocytes. All media were routinely supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO2/air. Cells from passages 3–9 were used for experiments, during which time the cell population was homogeneous with fibroblast-like synoviocytes (FLSs), as assessed by morphology (Wright–Giemsa staining) and immunostaining with anti-SB5 (anti-human fibroblast), KP-1 (anti-human macrophage), D33 (anti-human desmin), 1A4 (anti-human z-smooth muscle actin) and F8/86 (anti-human von Willebrand factor) monoclonal antibodies (Dako, Glostrup, Denmark). Immunoreactivity was strongly expressed by SB5 and equivalvocally by KP-1. D33, 1A4 and F8/86 were negative. The cultures were found to be completely free of lymphoid and monocyteic cells. For GLS production studies, FLSs were grown to confluence in 25 cm2 flasks (~ 1 x 106 cells) and treated with cytokines in a total medium volume of 4 ml. Ham’s F-10 medium (Gibco, Grand Island, NY, USA) with 10% fetal calf serum (FCS) was used when FLSs were stimulated with rHuTNF-α, rHuIL-1, rHuIL-6 and rHuIL-8. Supernatants and cell pellets were harvested and frozen. Before measurement, cell pellets were sonicated.

**Measurement of lactate dehydrogenase (LDH)**

Measurement of LDH in conditioned media and cell pellets was carried out using the lactate substrate tetrazolium method. Kits were purchased from Wako (Osaka, Japan). The release of LDH from FLSs induced by cytokines was estimated using the equation:

\[
\% \text{LDH release} = \left( \frac{B}{A + B} \right) \times 100
\]

where \( A \) is the mean (LDH) in cell pellets/flask and \( B \) is the mean (LDH) in conditioned medium/flask.

**Thymidine phosphorylase assay**

Thymidine phosphorylase activity was determined by the modified spectrophotometric method [3]. A 78 μl volume of conditioned medium was incubated with 80 μl of 366 mM potassium phosphate buffer (pH 7.4), 2 μl of 10 mM thymidine at 37°C for 1 h, and then the reaction was stopped by the addition of 480 μl methanol. After centrifugation at 10 000 r.p.m. for 10 min, 30 μl supernatant added together with 270 μl 50 mM potassium phosphate buffer (pH 7.4) were applied to the high-performance liquid chromatography (HPLC) column (ODS A-5; Nomura Chemical, Aich, Japan). The amount of thymine produced was measured with an ultraviolet monitor (254 nm). Enzyme activity is expressed as the amount of thymine (μmol thymine formed/mg protein/h).

**Reverse transcription-polymerase chain reaction (RT-PCR)**

All RNA extraction and RT-PCR steps were performed in a neighbouring laboratory to eliminate
PCR contamination by expression constructs. Total RNA was isolated from stimulated and unstimulated FLSs using the acid guanidinium thiocyanate method, precipitated with ethanol, and stored at −70°C [16]. An optical density reading was taken at 260 nm to assess the purity and the quantity of each RNA sample. Reaction conditions for the RT and PCR amplification were as described in the GeneAmp RNA PCR kit (Perkin Elmer Cetus, NJ, USA). Oligonucleotides were synthesized on an automated DNA synthesizer (Perkin Elmer, Applied Biosystems) and used for PCR. The GLS-specific downstream primer was 5’-GT-GGAGGGGCTGCTCGTGGTGTTGAC-3’ and the upstream primer was 5’-CAGCATCCGCTC-GAAGCGGCAAAGGGCGA-3’ (amplified PCR product: 378 bp). Primers detecting β-actin mRNA were used to normalize for the amount of cDNA in each reaction. The sequences of primers for β-actin were 5’-TACATGGCTGGGGTGTTGAA-3’ and 5’-AAGAGGGCATCCTCACCT-3’ (amplified PCR product: 218 bp). The reaction was carried out for 30 cycles, using a 95°C, 30 s denaturing step; a 58°C, 30 s annealing step; and a 72°C, 1 min extension step. PCR products were visualized by ethidium bromide staining following resolution on 1% agarose gel and detected by capillary electrophoresis (ABI, Model 270A-HT, Perkin Elmer, Applied Biosystems) on the basis of absorbance at 260 nm of DNA products.

**Statistical analysis**

All data were entered into a computer database and analysed using the Stat View II program (Brain Power, Catabasas, CA, USA). Data were expressed as mean (S.E.M. or S.D.). Regression and correlation analysis were also performed using the same program. The statistical significance of the differences in PCR product among cytokine-stimulation groups and a control group was examined using non-parametric Mann–Whitney U-test.

**RESULTS**

**Concentrations of immunoreactive GLS and four other cytokines in SFs obtained from patients with RA**

The mean GLS concentration (S.E.M.) in synovial fluids of 38 samples from patients with RA was 384.5 (42.9) ng/ml. Its level (ng/ml) was 2–3 orders of magnitude higher than those of the other cytokines (pg/ml) which have been reported to have a close relevance to RA aetiology (Table II).

**Correlation between levels of GLS in sera and SF, and levels of cytokines in SF**

TNF-α, IL-1α, IL-6 and IL-8 in SF were examined for correlation with levels of GLS in serum and SF. As shown in Table III, levels of immunoreactive GLS in SFs were highly correlated with IL-1α and IL-8, but not with TNF-α and IL-6. There was no correlation between serum immunoreactive GLS levels and the levels of these synovial cytokines.

**Time course of serum GLS and markers of inflammation in patients with RA**

Serial data of GLS were obtained in 22 patients and assessed for congruence with routinely checked clinical figures of CRP, ESR and ADL score. Figure 1A–D shows the time course of four patients that yielded follow-up periods of over 18 months. Shown in Figure 1A is a patient with seronegative RA, stage III by the classification of rheumatoid progression [17], who was refractory to treatment with NSAID (loxoprofen), DMARDs (bucillamine, aurothiomalate and methotrexate) and oral steroid. GLS, CRP, ESR levels and ADL scores followed a similar course with clinical activity of RA. Figure 1B shows the course of a patient with seropositive RA, stage IV, who was treated with NSAID (loxoprofen) and DMARD (bucillamine). GLS, CRP, ESR levels and ADL score followed a similar course. Figure 1C shows a patient with seropositive RA, stage IV, who was treated with NSAID (nabumetone) and DMARD (auranofin). Despite normal CRP levels throughout the entire observation period, GLS, ESR levels and ADL score were significantly elevated in correlation with the clinical activity of RA. Figure 1D shows the course of a patient with seropositive RA, stage III, who was treated with DMARDs (bucillamine and aurothiomalate). CRP levels did not appear to be a monitor of disease activity in Cases C and D. In 16 of 22 patients (73%), serum levels of GLS, CRP and ESR followed similar profiles as observed in Case A and B, although in 6/22 patients (27%) either the CRP or ESR profile was incongruous with the GLS profile as in Case C and D. Furthermore, we have divided patients into those who did and did not respond to DMARDs and carried out analysis to determine the effects of various treatments on changes in GLS and other disease.

**TABLE II**

Cytokine concentration in synovial fluid from knee joints of patients with rheumatoid arthritis

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Concentration mean (S.E.M.)</th>
<th>Range</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLS</td>
<td>384.5 (42.9) ng/ml</td>
<td>44–760</td>
<td>38</td>
</tr>
<tr>
<td>TNF-α</td>
<td>237.6 (39.6) pg/ml</td>
<td>28–1318</td>
<td>38</td>
</tr>
<tr>
<td>IL-1α</td>
<td>1.437 (0.12) pg/ml</td>
<td>0.7–4.9</td>
<td>38</td>
</tr>
<tr>
<td>IL-6</td>
<td>8303.6 (659.5) pg/ml</td>
<td>429–13191</td>
<td>38</td>
</tr>
<tr>
<td>IL-8</td>
<td>1319.6 (76.9) pg/ml</td>
<td>755–2563</td>
<td>38</td>
</tr>
</tbody>
</table>

**TABLE III**

Correlation of serum and synovial fluid (SF) levels of immunoreactive gliostatin (GLS) and other cytokines in rheumatoid arthritis

<table>
<thead>
<tr>
<th>SF</th>
<th>Serum GLS level</th>
<th>n</th>
<th>r</th>
<th>P</th>
<th>SF GLS level</th>
<th>n</th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>38</td>
<td>0.106</td>
<td>NS</td>
<td>38</td>
<td>0.099</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1α</td>
<td>38</td>
<td>0.094</td>
<td>NS</td>
<td>38</td>
<td>0.342</td>
<td>&lt; 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>38</td>
<td>0.128</td>
<td>NS</td>
<td>38</td>
<td>0.106</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>38</td>
<td>0.044</td>
<td>NS</td>
<td>38</td>
<td>0.409</td>
<td>&lt; 0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P determined by Pearson’s correlation coefficient. NS, not statistically significant. The concentration of serum GLS (mean and S.E.M.) was 3.92 (0.80) ng/ml.
Fig. 1.—Serially determined levels of serum GLS and commonly used clinical markers of inflammation: CRP, ESR and ADL score. Four cases are shown with follow-up periods over 18 months. All four were under treatment with NSAIDs and DMARDs. Case A also received low-dose oral steroids. (A) 69-yr-old female; disease duration 9 yr. (B) 75-yr-old female; disease duration 12 yr. (C) 60-yr-old female; disease duration 17 yr. (D) 65-yr-old female; disease duration 10 yr. GLS (open squares), CRP (open triangle), ESR (open circles).

markers. Serum levels of patients (n = 25) who responded to DMARDs were 2.61 ± 0.93 ng/ml (mean ± s.e.m.) for GLS, 55.9 ± 4.9 mm/1 h for ESR and 2.77 ± 0.56 mg/dl for CRP, and those of patients (n = 13) who did not were 3.85 ± 1.00 ng/ml for GLS, 61.7 ± 9.5 mm/1 h for ESR and 3.96 ± 0.7 mg/dl for CRP, respectively. Generally, patients who responded showed lower values than non-responding patients, but there was no statistically significant difference between the two groups in either set of analyses.

Induction of GLS by cytokines

We measured the immunoreactive GLS produced by RA FLSS treated for 24 h with the cytokines rHuTNF-α (1 ng/ml), rHuIL-1α (1 ng/ml), rHuIL-6 (5 ng/ml) and rHuIL-8 (5 ng/ml). While GLS in control RA FLSSs was not detected, the exogenously added cytokines augmented the levels at 24 h after stimulations (Table IV). In particular, rHuTNF-α increased GLS production in a concentration-dependent manner (Fig. 2). The time-course experiment of GLS production by rHuTNF-α exhibited a bell-shaped profile (Fig. 3). A similar profile was observed on levels of GLS in the conditioned medium, indicating an extracellular secretion of GLS by rHuTNF-α. The immunoreactive GLS was only detected in the conditioned medium of treated RA FLSSs with rHuTNF-α but not other cytokines. The extracellular secretion of GLS was further evidenced by a reduction of GLS production at 24 h after stimulations in the presence of anti-IL-1α and anti-TNF-α antibodies (Fig. 4).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>GLS (ng/ml)</th>
<th>β-actin mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>14.13 ± 3.26</td>
<td>8.33 ± 1.08*</td>
</tr>
<tr>
<td>IL-6</td>
<td>12.80 ± 6.44</td>
<td>2.65 (n = 2)</td>
</tr>
<tr>
<td>IL-8</td>
<td>14.67 ± 5.35</td>
<td>2.26 (n = 2)</td>
</tr>
</tbody>
</table>

Table IV: Induction of gliostatin (GLS) by IL-1α and TNF-α in FLSSs

*Effects of various cytokines on GLS production. Fibroblast-like synoviocytes (FLSSs) were incubated in the presence or absence of cytokines for 24 h (for protein detection) or 12 h (for mRNA detection) and cells were harvested. The amounts of immunoreactive GLS were determined by EIA. Values are the mean and s.e.m. of quadruplicate measurements. ND, not detected.

Total RNA was extracted and 1 μg of total RNA from FLSSs was analysed for GLS and β-actin mRNA levels by RT-PCR. PCR products were detected by high-performance capillary electrophoresis. Values are the mean ± s.e.m. (n = 4–6).

*P < 0.01 vs control.
secretion of GLS was also confirmed by the thymidine phosphorylase activity (11.9 μmol thymine formed/mg protein/h) in the conditioned medium at 24 h after rHuTNF-z stimulation, but no activity in the control F-10 medium containing 10% FCS. That the GLS secretion until 30 h after stimulation was not due to cytotoxicity is evident by no change in LDH activity in the conditioned medium. The induction of GLS mRNA in FLSs by four cytokines was also examined by an RT-PCR (Table IV). Using capillary electrophoresis, the proportion of absorbance, reflecting copies of GLS mRNA to that of β-actin mRNA, was estimated. It was proved in advance by PCR without the reverse transcription step that the PCR products are not derived from concomitant DNA fractions. The accuracy of the PCR product (372 bp) was confirmed by the restriction map of NcoI digests comprising two enzymatic fragments (145 and 227 bp), and further by a direct sequence analysis (data not shown). Control FLSs expressed GLS mRNA at a GLS/β-actin ratio of 1.91, and FLSs stimulated with cytokines for 12 h showed a higher ratio of 8.33 for rHuIL-1z and 5.03 for rHuTNF-z compared with the control ratio with a statistically significant difference ($P < 0.01$). In contrast, either rHuIL-6 or rHuIL-8, which evoked a comparable induction of GLS to the former two cytokines, failed to show an increase in GLS mRNA expression, mostly due to the instability of mRNA within 12 h incubation.

**DISCUSSION**

We have reported previously that sera and SFs in RA patients contained a high concentration of GLS mostly produced by synovial lining cells [11, 12]. These findings led us to believe that besides its potential as an index for the disease activity and therapeutic efficacy of drugs in RA, GLS could have a strong pathogenic potential as an angiogenic factor. Results reported in this study have further confirmed the clinical usefulness of GLS as a much better clinical indicator of RA than other cytokines known to be related to RA pathogenesis (TNF-z, IL-1, IL-6 and IL-8) which were also a potent inducer of GLS synthesis in synovial cells. Various cytokines, including IL-1 [18, 19], IL-6 [19–22], IL-8 [23, 24] and TNF-z [19, 20], have been identified in RA SFs and tissues as mediators of typical pathological features of RA, such as extensive infiltration of mononuclear cells and hyperplasia of synovial lining cells involving neovascularization. First of all, we attempted to compare the GLS levels with the levels of these cytokines in sera or SFs of RA patients. Most cytokines exist in a trace amount in the serum of RA patients, so that it is not usually easy to detect them in patient serum by ordinary EIA or radioimmunoassay. In contrast, the serum GLS level is $0.67 ± 1.90$ ng/ml for normal adults and $6.05 ± 5.63$ ng/ml for RA patients [11]. The levels of GLS in RA SF (ng/ml) were two or three orders higher than those of the cytokines (pg/ml), suggesting easier quantification by a simple laboratory method such as EIA. There was no correlation between serum or synovial GLS levels and the levels of the above cytokines, except for the correlation between synovial GLS and IL-1 ($r = 0.342$, $P < 0.05$, $n = 38$) or IL-8 ($r = 0.409$, $P < 0.05$, $n = 36$). In confirmation of our previous result that serum GLS levels were highly correlated with CRP and ESR [12], we have found that alterations in serum GLS levels correlated well with the clinical severity of RA (Fig. 1A–D).

It is speculated that cytokines produced at the inflammatory region of synovia play a crucial role in the perpetuation of RA synovitis and the resulting bone destruction [25]. To elucidate which is more closely related to the aetiology of RA—GLS or the...
other above cytokines—we firstly examined the actions of cytokines to induce GLS synthesis in cultured synovial cells and to release GLS into culture medium. rHuTNF-α, rHuIL-1β, rHuIL-6 and rHuIL-8 induced GLS expression at the protein level (Table IV). rHuTNF-α increased GLS production in a concentration-dependent manner (Fig. 2). The newly synthesized GLS induced by rHuTNF-α was released into the culture medium by synovial cells, which was not the case in GLS induced by rHuIL-1β (data not shown). That the observed secretion was not due to cytotoxicity is evident by the undetectable LDH activity in the experimental periods from 0 to 36 h after TNF-α stimulation (Fig. 3). Although the lack of a signal peptide [1] conflicts with the physiological release of GLS from synovial cells, there may be alternative mechanisms for release of the protein factor from the producing cells, as speculated in acidic fibroblast growth factor (FGF), basic FGF [26–29], ciliary neurotrophic factor [30, 31], IL-1β [32] and glia maturation factor [33]. In fact, Usuki et al. [8] demonstrated that PD-ECGF lacking a hydrophobic signal sequence was actually released, but not in a large quantity, from A431, human epithelial carcinoma cells [34], and proposed a plausible mechanism of nucleotidylation of PD-ECGF.

In RA, the GLS expression may primarily be induced in fibroblast-like synoviocytes by cytokines such as TNF-α and IL-1β which bring about the synovial GLS secretion as well as synthesis through a cytokine network. Our findings suggest a clinical potential of GLS measurement to monitor the disease activity as an alternative or adjunct to the conventional RA indicators. In particular, upon the discrepancy of clinical data between CRP and ESR levels, it is more useful to measure serum GLS levels.

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