Enoxaparin but not unfractionated heparin causes a dose-dependent increase in plasma TGF-β1 during haemodialysis: a cross-over study

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Abstract

Background. Transforming growth factor-β1 (TGF-β1) is a multi-functional cytokine that presents as a mediator of the heparin’s pleiotropic action. In this cross-over study, we compared the effects of enoxaparin and unfractionated heparin (UFH) used as anticoagulants during haemodialysis (HD) on plasma TGF-β1 levels and some platelet activation markers: platelet-derived growth factor–AB (PDGF-AB), β-thromboglobulin (β-TG) and platelet factor-4 (PF-4).

Methods. Plasma immunoreactive markers (in 22 chronically HD patients) were quantified at the start, at 10 and 180 min of HD session. Enoxaparin was administered as a single dose of 0.67 ± 0.14 mg/kg at the onset of HD, while UFH was given as a bolus of 1500 (500–3500) IU followed by an infusion of 2750 (1500–6500) IU. The time of evaluation for each heparin was 3 months.

Results. Pre-dialysis, TGF-β1 levels tended to be lower in patients anticoagulated with enoxaparin compared with UFH [6.9 (3.3–21.9) ng/ml vs 8.4 (3.8–30.2) ng/ml, respectively; P = 0.05]. Overdialysis, TGF-β1 levels showed a significant 44.8% increase to 10.0 (2.9–28.0) ng/ml after 10 min (P = 0.002) and to 9.32 (5.3–23.7) ng/ml after 180 min (P = 0.016) of enoxaparin–anticoagulated HD and remained stable during UFH administration [9.4 (3.9–25.3) ng/ml after 10 min, 8.1 (4.1–21.9) ng/ml after 180 min; P = 0.385]. The 35% increase in plasma TGF-β1 after 180 min of HD positively correlated with the enoxaparin dose/kg (r = 0.553, P = 0.008) and, interestingly, negatively with the baseline level of the cytokine (r = –0.544, P = 0.009). Despite a positive correlation between TGF-β1 and PDGF-AB during HD, there were no associations between TGF-β1 and β-TG or PF-4 regardless of the type of anticoagulation.

Conclusion. Enoxaparin, compared with UFH, induces a rapid overdialytic but not sustained increase in plasma TGF-β1 levels. The effect is closely dose-dependent and may reflect systemic activation of this multi-potential cytokine.

Keywords: enoxaparin; haemodialysis; transforming growth factor-β1; unfractionated heparin

Introduction

Transforming growth factor-β1 (TGF-β1) is a pleiotropic cytokine involved in diverse biological processes from axis specification and tissue differentiation in early organogenesis to regulation of a range of immune functions in the adult organism [1,2]. However, there are three fundamental activities of TGF-β1: (i) modulation of cell proliferation, generally its suppression; (ii) enhancing deposition of extracellular matrix through promotion of its synthesis and inhibition of degradation; (iii) immunosuppression through a variety of mechanisms [1,2]. Thus, misregulation of TGF-β1 has been proposed to play a key role in the development of numerous diseases in which the normal tissue architecture is progressively lost, including scarring during wound repair, carcinogenesis, osteoporosis and neurodegenerative diseases [1–3]. The biological role of TGF-β1 in human diseases encompasses two major issues: one involving increased TGF-β1 activity, as occurs in patients with fibrosis and progressive cancers and a second involving decreased TGF-β1 activity, as occurs in early tumourigenesis, developmental defects and atherosclerosis [1–3].

TGF-β1 is produced by most cell types in an acid-activatable latent form; in platelets it exists in a latent complex with a putative precursor polypeptide [4]. While the precise activational mechanism is uncertain, it seems likely that the complex is proteolytically activated by plasmin-like enzymes to form a 26 kDa homodimeric active molecule [4]. Active TGF-β1 is rapidly bound to α2-macroglobulin (α2-M), presumably serving as a clearance mechanism. Thus, TGF-β1 may exist in two distinct, biologically inactive forms:
the original latent complex with its precursor, and a second clearance complex with α2-M [3–5]. The active TGF-β1 has a high affinity to types I and II TGF-β receptors. The ligand-receptor complexes signal through SMAD proteins that exhibit regulatory activity on TGF-β1 function [4]. In pathological conditions, TGF-β1 is overexpressed whereas type II receptors are strikingly decreased, which can explain the development of cells resistance to TGF-β1 regulatory activity.

TGF-β1 is a heparin-binding protein. Its associations with heparin prevent TGF-β1 from the binding to the activated form of α2-M, thus protecting the cytokine from proteolytic degradation [4,6–8]. In vitro, heparin tripled the half-life of TGF-β1 and doubled its cell-associated amount [7]. Consistent with this protective effect, heparins potentiate TGF-β1 biological activity [7,8]. This action of heparin on TGF-β1 likely contributes to the anti-proliferative effect of heparin on vascular smooth muscle cells. Unquestionably, beside inhibition of blood coagulation, heparins interfere with many physiological and pathological processes involved in inflammation and multiple chronic diseases [9–12]. It is also well known, that heparins constitute a heterogenous group and each of them should be regarded as a distinct medication having peculiar anticoagulant and, importantly, extra-anticoagulant properties [10,13,14].

We aimed to compare the effect of unfractionated heparin (UFH) vs low-molecular-weight heparin (LMWH), enoxaparin, on circulating and active TGF-β1 levels during the haemodialysis (HD) procedure. Platelet activation markers such as platelet-derived growth factor-AB (PDGF-AB), β-thromboglobulin (β-TG), and platelet factor-4 (PF-4) were also measured to account for this potential source of plasma TGF-β1.

Subjects and methods

Patients

We enrolled 22 patients (11 men, 11 women; mean age 68.7 ± 9.2 years) who were undergoing maintenance HD for a median period of 38.5 (range 4.0–111) months. Primary renal diseases were chronic glomerulonephritis (n = 6), interstitial nephritis (n = 5), diabetic nephropathy (n = 3), polycystic kidney disease (n = 1), hypertensive nephropathy (n = 1), secondary amyloidosis (n = 2), acute renal failure (n = 1) and unknown (n = 3). None of the patients received steroids, other immunosuppressive agents, non-steroidal anti-inflammatory drugs, oral anticoagulants, contraceptive drugs, was positive for HIV 1/2 and hepatitis C/B with an evidence of severe liver injury. Patients with a hereditary deficiency of coagulation factors, lupus anticoagulant or antiphospholipid syndrome were also excluded. Thirteen (59.1%) patients were treated with aspirin because of cardiac ischaemia. At the time of evaluation, 21 (95.5%) patients had hypertension and 15 (68.2%) of them were treated with angiotensin converting enzyme (ACE) inhibitors. Eleven (50%) patients were receiving calcium channel blockers, 15 (68.2%) beta-blockers and 13 (59.1%) had symptoms of moderate heart failure. All the patients received erythropoetin (62.9 ± 43.1 IU/kg/week). None of them had suffered from any inflammatory or infectious disease prior and during the study. Dry body weight was 73 ± 19 kg, haematocrit 33.6 ± 3.09%, Kt/V was 1.1 ± 0.3, platelet count 185 ± 59 x 10³/μl, white blood cell count 5.8 ± 1.7 x 10³/μl, and the routine haemostatic tests were normal. Plasma lipids were as follows: total cholesterol 199 (104–276) mg/dl, HDL-cholesterol 47.5 ± 9.7 mg/dl, LDL-cholesterol 108 ± 37 mg/dl, and triglycerides 137 ± 66 mg/dl. Serum albumin was 3.6 ± 0.4 g/dl.

The patients were dialysed for 4–5 h thrice weekly using the double-needle technique, native arteriovenous fistulas, standard bicarbonate buffer, low-flux modified cellulose dialysers, and machines with controlled ultrafiltration. Before each HD session, the extracorporeal circuit was rinsed with 1000 ml of 0.9% saline containing 2.0 IU/ml of UFH (Heparin; Biochemie, Kundl, Austria). Prior to the study all the patients had been anticoagulated with enoxaparin (Clexane®, Bellon Rhône-Poulenc Rorer, Montrouge, France) during the HD session.

Thirty healthy, non-smoking, age- and gender-matched volunteers served as controls for determination of plasma TGF-β1 levels.

Study protocol

The study was performed in conformity with the Helsinki Declaration. Ethics committee approval was obtained and informed consent was sought from each patient and control subject.

The study lasted 6 months. Twenty-two patients were randomly assigned to UFH (n = 11) or LMWH (n = 11) anticoagulated HD for three months; then the patients were switched to another heparin for the next 3 months. The anticoagulation regimens were as follows: UFH—a bolus of 1500 (500–3500) IU into the arterial line followed by an infusion of 2750 (1500–6500) IU via a syringe pump; LMWH enoxaparin—a single dose of 40 (20–60) mg via the first-access needle at the onset of HD.

The total dose of UFH was 4840 ± 1880 IU (66 ± 19.2 IU/kg) per HD session. The infusion was started just after the bolus, and stopped 1 h before the end of HD. The UFH dosage had been individually titrated on the basis of whole blood activated partial thromboplastin time (WBAPTT) and established during the first three sessions after randomization. The goal was to obtain a 2-fold prolongation of WBAPTT at both 30 and 120 min of HD compared with the baseline value.

The effective dose of enoxaparin was 0.67 ± 0.14 mg/kg (1 mg = 100 IU of antifactor Xa). It was established on the basis of the following clinical guidelines: no visible fibrin clots in the arterial and venous bubble traps during HD, no clotted filters after HD and no bleeding from the fistula puncture sites after compression [15].

After three months since the randomization procedure, plasma TGF-β1, PDGF-AB, β-TG and PF-4 levels were measured predialysis (T₀), at 10 min (T₁₀) and 180 min (T₁₈₀) after the beginning of the HD session in the patients anticoagulated with either UFH or LMWH. The measurements were next repeated after a further 3 months since the heparin switch. Each patient was tested against him/herself.
The study was designed to keep the heparin doses, other HD prescriptions and pharmacological treatment stable during the follow-up. All the patients uneventfully completed the study.

**Laboratory procedures**

Fasting blood was drawn without use of a tourniquet through a wide-gauge butterfly needle into DiaTubes™H (BD Vacutainer Systems, Plymouth, UK) containing a 0.5 ml solution consisting of citrate (0.109 M), adenosine (3.7 mM), theophylline (15 mM), and dipyridamol (0.198 mM) to minimize platelet degranulation [16]. In the patients, blood was obtained during a mid-week morning HD session: at $T_0$ from the arterial needle (before heparinization), and at $T_{10}$ and $T_{180}$ from the pre-dialysr port after slowing the blood flow to 100 ml/min for 1 min. In healthy controls, fasting morning blood was obtained without stasis from the antecubital vein punctured with a 19-gauge needle. The first 1 ml of blood was discarded; then 4.5 ml of blood was collected. Tubes were placed in melting ice and centrifuged 15 min later at 2000 g for 30 min. One-third of the plasma was collected from middle region of the supernatant, aliquoted, and immediately frozen at $-80^\circ$C. Batch analyses were performed within 8 weeks.

Plasma TGF-$\beta$1 (activated forms) and PDGF-AB antigen levels were determined using solid-phase enzyme-linked immunosorbent assays (ELISA) kits purchased from R&D Systems, Inc., Minneapolis, MN, USA (Quantikine®); the assays quantify human-specific factors; their mean minimum detectable levels were 7 pg/ml and 1.7 pg/ml, respectively. For detection of plasma $\beta$-TG and PF-4 antigens ELISA kits from Diagnostica Stago, Asnieres, France were used; their detection limits were 11 IU/ml.

All the measurements were performed in duplicate using a 400 SFC photometer (SLT-Labinstruments, Gröding/Salzburg, Austria), and calibrated using provided recombinant human reference samples and standards. For calculation of the results, a computer and a curve-fitting program were used. The within- and between-assay coefficients of variations were <8%. The $T_{180}$ values were corrected for haemoconcentration according to serum albumin levels. WBAPTT was measured using an automated coagulation system and reagents from BioMérieux (Marcy-l’Étoile, France).

**Table 1.** Overdialysis plasma transforming growth factor-$\beta$1 (TGF-$\beta$1), platelet derived growth factor-AB (PDGF-AB), $\beta$-thromboglobulin ($\beta$-TG) and platelet factor-4 (PF-4) levels in patients anticoagulated with enoxaparin and unfractionated heparin (cross-over study) during haemodialysis procedure

<table>
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<tr>
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<th>Enoxaparin (n = 22)</th>
<th>Unfractionated heparin (n = 22)</th>
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<tr>
<td></td>
<td>0 min</td>
<td>10 min</td>
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<tr>
<td><strong>TGF-$\beta$1, ng/ml</strong></td>
<td>6.9 (3.3 – 21.9)**</td>
<td>10.0 (2.9 – 28.0)**</td>
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<tr>
<td><strong>PDGF-AB, pg/ml</strong></td>
<td>14.0 (1.0 – 44.5)</td>
<td>26.1 ± 17.6*</td>
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<tr>
<td><strong>PF-4, IU/ml</strong></td>
<td>26.2 (10.6 – 88.6)</td>
<td>59.2 (41.1 – 105.2)**</td>
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$n$, number of patients; min, minutes.

Data are shown as mean ± SD and median (range) depending on their normal or skewed distribution.

aPredialysis TGF-$\beta$1 levels under enoxaparin anticoagulation compared with UFH treatment: $P = 0.050$.

Predialysis cytokine levels vs those at 10 or 180 min of haemodialysis session: *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$.

**Statistical analysis**

Shapiro–Wilk’s $W$ test of normality was used for data distribution analysis. The normally distributed data were presented as mean ± SD, and the skewed data as median (full range). The baseline plasma TGF-$\beta$1 levels comparison between patients and controls was tested with non-parametric Mann–Whitney’s U-test. Intragroup comparisons were performed with non-parametric Friedman’s ANOVA by ranks and Wilcoxon’s single-rank tests. Bivariate correlations were assessed using non-parametric Spearman’s regression analysis. All statistical tests were two-sided, and $P < 0.05$ was considered significant. Statistica software (version 6.0 PL, StatSoft, Tulsa, OK, USA) was used.

**Results**

**Baseline TGF-$\beta$1 levels in HD patients and healthy controls**

Predialysis plasma TGF-$\beta$1 levels were markedly higher in HD patients receiving both UFH and enoxaparin (Table 1) than in healthy controls (3.57 ± 1.72 ng/ml; all $P < 0.0001$).

Baseline TGF-$\beta$1 levels in HD patients tended to be lower during enoxaparin anticoagulation compared with UFH treatment [6.92 (3.32–21.9) ng/ml vs 8.39 (3.80–30.2) ng/ml; $P = 0.050$] (Table 1, Figure 1).

**TGF-$\beta$1 during LMWH and UFH haemodialysis**

Plasma TGF-$\beta$1 levels significantly changed over enoxaparin HD ($\chi^2$ ANOVA = 11.5, $P = 0.003$; Table 1, Figure 1A). They increased by a mean of 44.8% after 10 min ($P = 0.002$) and by a mean of 35% after 180 min ($P = 0.016$) of HD compared with predialysis levels. The increment in plasma TGF-$\beta$1 after 180 min of HD positively correlated with the enoxaparin dose/kg ($r = 0.553$, $P = 0.008$; Figure 2A), and negatively with the baseline level of the cytokine ($r = -0.544$, $P = 0.009$; Figure 2B). We did not observe any associations between TGF-$\beta$1 levels after both 10 and 180 min of HD and appropriate white blood cell...
count (5.5 ± 1.9 × 10^3/μl at T_10, 5.7 ± 1.7 × 10^3/μl at T_180; all P > 0.05).

Plasma TGF-β1 levels did not show any significant changes during UFH anticoagulated HD (χ² ANOVA = 1.91, P = 0.385; Table 1, Figure 1B).

**PDGF-AB during LMWH and UFH haemodialysis**

Plasma PDGF-AB levels tended to change during enoxaparin HD (χ² ANOVA = 5.18, P = 0.075; Table 1, Figure 3A). They increased by a mean of 50.8% (P = 0.01) at T_10 and returned to the predialysis levels after 180 min of the session (P = 0.135). The percentage increments of PDGF-AB and TGF-β1 at T_10 (r = 0.440, P = 0.040) as well as their levels at T_180 (r = 0.789, P = 0.00001) were closely associated with each other.

Plasma PDGF-AB levels over UFH HD followed the same pattern (χ² ANOVA = 4.73, P = 0.094; Table 1, Figure 3B). They increased by a mean of 59.6% (P = 0.009) at T_10 and returned to the baseline values at T_180 (P = 0.055). Similarly, the PDGF-AB levels after 180 min of UFH anticoagulated HD positively correlated with the relevant TGF-β1 changes (r = 0.478, P = 0.024).

Predialysis plasma PDGF-AB levels and those at T_10 and T_180 were comparable during enoxaparin and UFH treatments and were not associated with the heparin dosage.

**β-TG during LMWH and UFH haemodialysis**

Plasma β-TG levels did not show overt-dialytic changes during enoxaparin anticoagulation
PF-4 during LMWH and UFH haemodialysis

Plasma PF-4 levels significantly changed over enoxaparin HD ($\chi^2$ ANOVA = 30.1, $P < 0.0001$; Table 1, Figure 3D). They increased by a median of 109% compared with $T_0$ and returned to the predialysis values at $T_{180}$ ($P = 0.708$). The percentage increment in PF-4 and PDGF-AB at $T_{10}$ directly correlated with each other ($r = 0.490$, $P = 0.020$). No associations between overdialytic PF-4 changes and those of TGF-β1 as well as β-TG were found.

Plasma PF-4 levels followed the same pattern over UFH anticoagulated HD ($\chi^2$ ANOVA = 33.8, $P < 0.0001$; Table 1, Figure 3F). At $T_{10}$ they increased by a median of 109% compared with $T_0$ ($P < 0.0001$) and returned to the predialysis levels at $T_{180}$ ($P = 0.871$). There were no correlations between overdialytic PF-4 changes and those of TGF-β1, PDGF-AB as well as β-TG.

Predialysis plasma PF-4 levels and those at $T_{10}$ and $T_{180}$ were comparable during enoxaparin and UFH treatments and were not associated with the heparin dosage.

Discussion

The novel finding of this study is that TGF-β1 levels in maintenance HD patients are under the influence of the type and dose of heparin administered during blood purification procedures. In the respect that TGF-β1 is a multi-functional cytokine playing an important role in a wide range of physiological and pathological processes, including wound healing, development, oncogenesis, immunomodulation and atherosclerosis, the issue seems to be of special significance [3]. Moreover, HD patients are unique in their exposure to heparin. Over the course of a year, the patient receives on average of about 600 000 IU of the drug [17].

This prospective study shows that HD with enoxaparin causes a rapid and dose-dependent increase in plasma TGF-β1 level, while the UFH anticoagulated procedures are devoid of such effect. To our knowledge, previous studies concerning TGF-β1 changes during HD procedures are contradictory and were carried out mainly during UFH anticoagulation. Some of them have mentioned that TGF-β1 levels significantly decrease at 120 min of UFH anticoagulated HD [12,13]. On the other hand, Jiang et al. [18] have reported a high TGF-β1 increment after HD procedure, while Fujisawa et al. [19] have shown no differences between TGF-β1 values before and after the HD session; unfortunately, the anticoagulation regiment was not precisely described in these two studies.

The reason for the intriguing TGF-β1 increase over enoxaparin HD and its biological relevance are not clear. It has been postulated, that in vivo heparin releases TGF-β1 from its binding site to z2-M and forms a stable, electronegative complex that is receptor-competent [4]. The large molar excess of standard heparin necessary to achieve this dissociation suggests that either a small molecular derivative of heparin is effective or that UFH has a lower affinity for TGF-β1 than z2-M [4]. However, no studies have addressed the respective effects of LMWH so far.

Puzzling are also the other potential sources of plasma TGF-β1. We are aware of and have considered the possibility of ex vivo thrombocyte activation; firstly—because it inevitably occurs during the HD procedure, secondly—due to the hardly avoidable platelet degranulation during blood sampling [3,20].

Measuring plasma TGF-β1 levels requires the use of specific protocols [3]. Accordingly, in our study, we have optimized blood sampling procedure to minimize ex vivo platelet degranulation to less than 0.1% [3]. As known, during HD sessions, blood- artificial material contact causes the release of the content of both platelet dense granules (adenosine diphosphate and serotonin), and alpha-granules, which contain PDGF-AB, PF-4 and β-TG [20,21]. With regard to this release reaction, there is a general agreement that PDGF-AB, PF-4 and β-TG are sensitive markers of platelet activation [20]. Even though we found a positive correlation between the increments of TGF-β1 and PDGF-AB at $T_{10}$ and $T_{180}$, which could indicate that the TGF-β1 overdialytic increase depends on platelet degranulation, there were, however, no associations between TGF-β1 values and those of β-TG or PF-4.

This finding indirectly supports the fact that platelets are not a major source of plasma TGF-β1 during enoxaparin anticoagulated HD. Moreover, it has been known, that LMWH causes reduced platelet activation compared with UFH [17]. On the other hand, according to chromatography studies (comparison of the column retention times of the plasma TGF-β1 complexes), performed by Grainger et al. [3], there is a suggestion, that the plasma TGF-β1 complexes have a composition distinct from those of the platelets' origin.
Thus, if TGF-β1 is released from platelets in vivo, the composition of the complexes must be modified (by acquisition of additional binding protein(s) or loss of a protein component) once in the plasma. Nevertheless, the almost complete absence of platelet large latent TGF-β1 complex from plasma during chromatography demonstrates that either this modification occurs very rapidly after release from the platelets relative to the half-life of plasma active TGF-β1 (~2 min), or supports the hypothesis that platelets are not a major source of plasma TGF-β1 [3].

Aside from platelets, there are a number of potential sources of TGF-β1 present in human plasma. Leucocytes contain even more TGF-β1 per cell than platelets. However, we did not observe any correlation between TGF-β1 levels and leucocyte count in our patients. Almost every tissue of the body synthesizes TGF-β1, for example bone and muscle contain TGF-β1 antigen associated both with the cell and the extracellular matrix. At present, it is unknown whether there is any flux of TGF-β1 across the vascular endothelium (in either direction) that could lead to equilibration between tissue and blood levels of this cytokine [3].

Another issue of our study is that maintenance HD patients receiving UFH present with higher plasma TGF-β1 levels between consecutive HD procedures than those under enoxaparin treatment. Referring to the statement that low baseline TGF-β1 levels are independent contributors to atherosclerosis risk in dialysis patients [11], it would appear that patients permanently anticoagulated with UFH might benefit from it. However, the biological importance of this finding is unclear and seems to be limited by the increased baseline TGF-β1 levels in HD patients. All the patients in our study, regardless of the type of anticoagulation, have higher predialysis TGF-β1 levels than healthy controls. On the other hand, this extended release of TGF-β1 and certain changes induced by it, namely activation of endothelium and fibroblasts with concomitant reorganization in the vascular system and connective tissue would nicely fit with the observation of vascular complications and amyloidosis in some chronically HD patients [22]. Chronic renal failure, dialysis and long-term heparin (particularly UFH) administration lead to systemically increased levels of TGF-β1 that may have other important consequences. First, impairment of the immune system, a fact that indeed has been observed in HD patients; second, a tendency toward increased fibroblast/stroma remodeling with a more or less latent trend to excessive proliferation of TGF-β1-responsive cells. This certainly describes a condition wherein additional tumourigenic events could be more effective. Thus, systematically elevated TGF-β1 could be one of the factors accounting for the increased risk of certain tumours observed in these patients. However, these and other under-appreciated effects of UFH and LMWH on numerous pleiotropic heparin-binding growth factors deserve further detailed studies in the maintenance HD patients.

In conclusion, heparin used for blood anticoagulation during HD procedures seems to be an important modulator of the pluripotent TGF-β1. Type and dose of heparin may thus have a profound impact on vital body function and progression of critical disease in chronic HD patients.

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Conflict of interest statement. None declared.

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