Heat shock protein 90 maintains the tumour-like character of rheumatoid synovial cells by stabilizing integrin-linked kinase, extracellular signal-regulated kinase and protein kinase B

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Abstract

Objective. To clarify the contribution of heat shock protein 90 (HSP90) to the pathogenesis of RA, we studied the effects of geldanamycin (GA), an inhibitor of HSP90, on excessive cellular extension and resistance to apoptosis induction of rheumatoid synovial cells.

Methods. Expression of integrin-α5β1 and integrin-linked kinase (ILK) in synovial cells was determined by western blot. The peripheral localization of ILK, reorganization of F-actin, complex formation of ILK with particularly interesting new cysteine-histidine protein (PINCH) and α-parvin, and activation of Rac/cdc42 in synovial cells were examined by using immunohistochemistry and immunoprecipitation. Apoptosis induction by GA treatment was analysed by nuclear staining, cell proliferation assay and western blot of caspase. Effects of GA on mitogen-activated protein kinase (MAPK), PI-3K/protein kinase B (Akt) pathway, mitochondrial Bcl-2 pathway and activation of nuclear factor-κB (NF-κB) were examined by western blot and ELISA.

Results. HSP90 was overexpressed in synovial cells while GA decreased the expression of integrin-α5β1 and ILK. The peripheral localization of ILK, reorganization of F-actin, complex formation of ILK with PINCH and α-parvin, and activation of Rac/cdc42 in synovial cells were all inhibited by GA treatment. We found that HSP90 stabilized and regulated the MAPK and PI-3K/Akt pathway, thereby inhibiting HSP90-potentiated synovial apoptosis by stimulating caspases and the mitochondrial Bcl-2 pathway on the one hand and inhibiting the activation of NF-κB on the other.

Conclusion. The contribution of HSP90 is important in the pathogenesis of RA that potentiates a tumour-like synovial overgrowth by stabilizing ILK, extracellular signal-regulated kinase and Akt.

Key words: Heat shock protein 90, Synovial cell, Integrin-linked kinase, Protein kinase B, Mitogen-activated protein kinase.

Introduction

A tumour-like synovial hyperplasia is characteristic of RA [1, 2]. Upon pursuing the contribution of c-Fos to arthritis and arthritic joint destruction [3, 4], we have found that c-Fos directly transactivates wee-1 kinase and thus the mitotic cell division of rheumatoid synovial cells is halted in the face of increased proliferation driven by c-Fos [5]. We have studied heat shock protein 90 (HSP90) in relation to the overgrowth of rheumatoid synovial cells, because HSP90 is one of the important target proteins of wee-1 [6]. Molecular chaperone, Hsp, maintains the structure of its target proteins by protein folding, transportation and complex formation [6]. Among various Hsp members, a 90-kDa protein HSP90 is highly conserved in eukaryotes [7] and expression of HSP90 is specifically inhibited by geldanamycin (GA), a benzoquinone ansamycin antibiotic.
integrin-specific inhibition of HSP90. The function of synovial cells. The contribution of HSP90 to playing all together in concert, are equally important for and PI3-K/Akt pathways. Importantly, ILK, ERK and Akt, via ILK and potentiates survival of synovial cells via MAPK contribution of HSP90 to synovial overgrowth, and show rat in vivo in vitro [18]. GA inhibits the production of inflammatory cytokines destruction.

In this study, we aim at clarifying the entire picture of the HSP90 [6]. Phosphoinositide-dependent protein kinase (PDK) and protein kinase B (Akt), major components of another Ras/phosphatidylinositol 3-kinase (PI3-K)/PDK/ Akt pathway, are also targets of HSP90 [6]. Recently, integrin-linked kinase (ILK), a β1-integrin subunit interactor, has been identified as a novel chaperonic part- ner of HSP90 [13]. Since cell detachment and decrease in ILK expression when cells were treated with HSP90 inhibi- tors, ILK has been determined as one of the HSP90 target proteins that binds to HSP90 through its middle kinase domain. In the cartilage–pannus junction of RA synovium, integrin–β5α1 and its ligand fibronectin (Fn) are expressed abundantly and induce pannus invasion in rheumatoid synovium [14–16] and mediate rearrangements of actin filaments [17], thereby aggravating rheumatoid joint destruction.

Previous studies show that anti-HSP90 antibody is increased in patients with long-standing erosive arthritis [18]. GA inhibits the production of inflammatory cytokines in vitro [19], and ameliorates adjuvant-induced arthritis in rat in vivo [20]. Further, AP-1/DNA binding activity was inhibited functionally by inactivating HSP90 [21]. In this study, we aim at clarifying the entire picture of the contribution of HSP90 to synovial overgrowth, and show that HSP90 potentiates actin cytoskeletal rearrangement via ILK and potentiates survival of synovial cells via MAPK and PI3-K/Akt pathways. Importantly, ILK, ERK and Akt, playing all together in concert, are equally important for the function of synovial cells. The contribution of HSP90 to synovial overgrowth in RA is discussed in relation to spe- cific inhibition of HSP90.

Materials and methods

Antibodies and agents

Anti-HSP90 antibodies (H-114), anti-ILK antibodies (65.1), anti-Raf-1 antibodies (E-10), anti-ERK1 antibodies (K-23), anti-phospho-ERK antibodies (E-4), anti-Akt1/2 antibodies (H-136), anti-phospho-Akt1/2 antibodies (Ser473), anti-ILKβ antibodies (C-21), anti-nuclear factor-κB (NF-κB)p65 antibodies (H-286) and anti-Bcl-2 antibodies (C2) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β5α1 integrin antibodies were from Chemicon International Inc. (Temecula, CA, USA). Anti-α-parvin antibodies, anti-MEK1/2 antibodies, anti-phospho-MEK1/2 antibodies (Ser217/221), anti-PDK1 antibodies, anti-caspase 3 antibodies, anti-cleaved caspase 3 antibodies (Asp175), anti-caspase 8 antibodies (1C12), anti-caspase 9 antibodies, anti-poly(ADP-ribose) polymerase (PARP) antibodies, anti-cleaved PARP antibodies (Asp214), anti-Bid antibodies, anti-histone H2B antibodies and wortmannin were from Cell Signaling Technology (Beverly, MA, USA). Anti-Bcl-XL antibodies (2H12) were from eBioscience (San Diego, CA, USA). Anti-cytochrome c antibodies (7H8.2C12) were from NeoMarker (Fremont, CA, USA). Anti-FAK antibodies, Anti-Ras (RAS10) antibodies, anti-Rac antibodies, anti-cdc42 antibodies, PAK-1 (p21-activated kinase)- binding domain-tagged agarose, Bad agarose, anti-phosho-Bad antibodies (Ser112/136), GST-Ras binding domain-conjugated agarose beads and anti-Ras antibo- dies were from Upstate Biotechnology (Lake Placid, NY, USA).

Materials and methods

Synovial cell culture

Synovial tissues were obtained from patients with RA and OA during joint surgery. The study was approved by the ethics committee of Kobe University and written informed consent was obtained from each patient before study enrol- ment. RA synovium represented marked infiltration of macrophages and T lymphocytes and invasive growth into adjacent cartilage and bone.

Tissues were minced and stirred with 1 mg/ml collage- nase (Sigma Chemical Co.) in serum-free DMEM (Nissui, Tokyo, Japan) for 3 h, filtered through nylon mesh and washed extensively. Primary cultured synovial cell lines were established and maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 1% penicilin–streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified incubator at 37°C in the presence of 5% CO2. After 2–4 weeks of sub-culturing, cells were used in third to fifth passages at 70–80% confluence state in the dishes. Every experiment in this study was repeated by using at least three different cell lines.

Western blot

Synovial cells were lysed with buffer A [25 mM Tris pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 1.5 mM EGTA, 0.5% sodium deoxycholate, 1 mM PMSF, 10 mM sodium ortho- vanadate and 3 mg/ml aprotinin] to obtain the cytoplasmic protein. To separate the membrane protein fraction, Proteo Extract Subcellular Proteome Extraction Kit (Calbiochem, La Jolla, CA, USA) was used. Samples were then subjected to SDS-PAGE, transferred to nitro- cellulose membrane (Millipore, Bedford, MA, USA), probed with antibodies and developed by an enhanced chemiluminescence detection system (ECL; Amersham, Arlington Heights, IL, USA).

For affinity purification with PAK-1, cytosolic protein (350 μg) was incubated with GST-p21 PAK-binding domain-tagged agarose and probed with anti-Rac or
anti-cdc42 antibodies to detect PAK-1-mediated Rac or cdc42 activation, or incubated with GST-Ras binding domain-conjugated agarose beads followed by western blot with anti-Ras antibodies.

RNA interference
ILK siRNA (cat# 00288176), purchased from Qiagen (Hilden, Germany), was introduced into cultured synovial cells using Gene Eraser siRNA Transfection Reagent (Stratagene, College Station, TX, USA).

Immunohistochemistry and F-actin staining
After 24-h culture with reduced serum levels (0.5% FBS) in the presence or absence of GA (0.5 μM), synovial cells were stimulated with EGF (300 ng/ml) for 5 min. Cells were then fixed in 3.7% formaldehyde, stained with phalloidin488 (for F-actin) or Alexa Fluor594 (Molecular Probes, Carlsbad, CA, USA) conjugated anti-ILK Ab and examined under fluorescence microscopy.

Cell viability assay
Synovial cells were cultured for 24 h in a medium containing 10% FBS with or without GA (0.1, 1, 10 and 20 μM) or anti-Fas antibodies (50 and 300 ng/ml). Effects of GA on cells were also tested using a Fn-coated well (Iwaki, Tokyo, Japan). Cell viability was measured as the absorbance (450 nm) of reduced WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitriphenyl)-5-(2,4-disulphonyl)-2H-tetrazolium, monosodium salt] using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Each of the three different strains of synovial cell was tested and results were expressed as percentage relative to control cultures.

Fluorochrome nuclear staining
Cells were cultured on control or Fn-coated plates with or without GA (10 and 20 μM) for 24 h. Cells were then labelled with 1 mM Hoechst 33342 for 10 min at 37°C, and examined under fluorescence microscopy.

Fractionation of mitochondria
Synovial cells were cultured for 24 h in medium containing 10% FBS with or without GA (10 μM), wortmannin (10 nM) or PD98059 (30 μM). Cells were washed twice with cold PBS and lysed for 10 min on ice with lysis buffer B (20 mM 4-2-hydroxyethyl-1-piperazine-ethanesulfonic acid (HEPES)-KOH pH 7.5, 10 mM KCl, 1.5 mM EGTA, 1.5 mM MgCl2, 1 mM dithiothreitol, 250 mM sucrose, 1 mM PMSF, 10 mM sodium orthovanadate and 3 mg/ml aprotinin]. Cells were then homogenized by passing through a 26-gauged needles 10 times and centrifuged at 1000 g for 10 min at 4°C. The resulting supernatant was further centrifuged at 10000 g for 20 min at 4°C. The pellet (mitochondrial fraction) was resuspended in buffer C [10 mM Tris pH 8.0, 1% Nonident P-40, 5 mM CaCl2, 1 mM PMSF, 10 mM sodium orthovanadate and 3 mg/ml aprotinin] and subjected to SDS-PAGE.

Activation of NF-κB
After incubated with GA (5, 10 and 20 μM) for 24 h, cells were stimulated with 10 ng/ml of rhTNF-α for 4 h. Cells were then fixed twice with PBS, resuspended in 2 volumes of ice-cold buffer D [10 mM HEPES pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1 mM PMSF. After incubation with GA (5, 10 and 20 μM) for 24 h, cells were stimulated with 10 ng/ml rhTNF-α for 4 h. Cells were then fixed twice with PBS, resuspended in 2 volumes of ice-cold buffer D (10 mM HEPES pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1 mM PMSF and 10 mM sodium orthovanadate), and homogenized on ice. The nuclear fraction was separated from the cell lysate by centrifugation at 1000 g for 10 min at 4°C. The binding activity of NF-κB/p65 to NF-κB consensus binding site was detected using TransAM NFκB/p65 Transcription Factor Assay Kit (Carlsbad, CA, USA). Data shown are the means (±S.D.) of at least three independent experiments.

**FIG. 1** (A) Western blot of HSP90 in primary cultured synovial cells. HSP90 was expressed abundantly in synovial cells of patients with RA as compared with those with OA. (B) Western blot of integrin-α5β1 and ILK in primary cultured rheumatoid synovial cells. Expression of integrin-α5β1 in the membrane fraction was enhanced when cultured on the Fn-coated dish (left) and GA treatment (10 μM, 24 h) reduced the expression of both integrin-α5β1 and ILK (right). (C) Western blot of FAK and ILK in rheumatoid synovial cells. GA treatment (10 μM, 24 h) reduced the expression of FAK and ILK. Note that 20 μM GA was required to degrade FAK, compared with 10 μM GA for degrading ILK.
Statistical analyses were performed using one-way analysis of variance (ANOVA) and Fisher’s protected least significant difference (PLSD). P ≤ 0.05 was considered significant.

Results

HSP90 and ILK in rheumatoid synovial cells

HSP90 was abundantly expressed in the synovial cells of patients with RA as compared with those of OA (Fig. 1A). Integrin-α5β1 was expressed in increased amounts on the surface of rheumatoid synovial cells when cultured on fibronectin (Fn)-coated dishes (Fig. 1B). The expression of surface integrin-α5β1, ILK and focal adhesion kinase (FAK), another molecule downstream of integrin, was decreased by GA treatment (10 μM, 24 h), an inhibitor of HSP90 (Fig. 1B and C). It was noted that increased amounts of GA were required for the degradation of FAK as compared with those for ILK, suggesting that FAK might not be the major target of HSP90.

HSP90 modulates ILK-mediated cytoskeleton assembly in synovial cells

EGF increased cell motility with distinct rearrangement of actin fibre formation, enhanced expression and activation of ERK and ILK. Consecutively, increased ILK expression correlated with the activation of Akt [22].

Upon stimulation with EGF (300 ng/ml, 5 min), ILK was located to the periphery of cultured rheumatoid synovial cells and actin cytoskeleton was stained strongly, which were inhibited by GA (10 μM, 24 h) (Fig. 2A). While PINCH binds to the integrin-β subunit and N-terminus of ILK to enhance cell-matrix adhesion and Akt phosphorylation, and α-parvin binds to ILK kinase and actin to activate Rac and cdc42 [23], we showed that EGF increased complex formation of ILK with PINCH and α-parvin (Fig. 2B).
This was inhibited by GA, where the expression of ILK was found also be decreased. The amount of activated GTP-bound forms of Rac or cdc42, bound to PAK-1, was increased by treatment with EGF, and this was inhibited by GA (Fig. 2C), the findings suggesting that the EGF signalling conveyed from PAK-1 to Rac and cdc42 via the ILK/C150/PINCH/C150-alpha-parvin complex is inhibited by GA. Thus, HSP90 appeared to be involved in ILK-mediated cytoskeletal assembly.

HSP90 modulates MAPK and PI-3K/Akt pathways
ILK-specific siRNA strongly inhibited the expression of ILK as compared with GA, while inhibition of Akt by ILK siRNA was less significant as compared with that by GA. The extent of inhibition of the phosphorylation of ERK and Akt was similar though the extent of inhibition of ILK by GA or ILK siRNA was different, suggesting that phosphorylation of Akt, but not ERK, was affected not only by ILK but also by other upstream molecules (Fig. 3A). Western blot and GPT-Ras pull-down assays showed that cRaf, MEK, PDK and Akt, all target proteins of HSP90, were degraded dose-dependently by GA, where Ras remained unaffected (Fig. 3B).

ERK and Akt protect synovial cells from GA-induced apoptosis
Since MAPK promotes cell proliferation and Akt protects cells from apoptosis [11], we tested the effect of HSP90 and its inhibition on the viability of synovial cells. Cell survival was decreased dose-dependently with GA: the effect of GA was less significant on Fn-coated dishes, where the effect of 20 μM GA on poly-L-lysine-coated dishes was comparable to 300 ng/ml anti-Fas antibodies (Fig. 4A). The synovial cell on Fn-coated dishes appeared to be resistant to GA-induced apoptosis morphologically (Figs 4B, 5 and 6). Western blot showed that phosphorylation of ERK and expression of Akt were increased on Fn-coated dishes. Inhibition by GA of phosphorylation of ERK, expression of Akt, caspase 8, cleavage of caspase 3 and PARP was cancelled upon culture on Fn-coated dishes, indicating that activation of ERK and Akt via matrix Fn protects synovial cells from GA-induced apoptosis (Fig. 4C).

Mitochondria-related apoptosis pathway
With regard to Bcl-2 family proteins and the mitochondria-related apoptosis pathway, we found that the phosphorylation of ERK was inhibited by GA to levels comparable to PD98059, a MAPK-specific inhibitor (Fig. 5A). Expression of Akt was also inhibited by GA, where the extent of the inhibition was relatively minute compared with wortmannin, a PI3-K-specific inhibitor. GA cleaved Bid to the levels comparable with PD98059, where Bid is under regulation of MAPK. GA also inhibited phosphorylation of Bad, an agonistic molecule inducing mitochondrial apoptosis.

Upon treatment with GA, PD98059 and wortmannin, Bcl-2 shifted from the cytosol to the mitochondrial fraction (Fig. 5A). Expression of cytosolic Bcl-XL, another mitochondrial membrane-bound protein, was inhibited most strongly by wortmannin, and Bcl-XL was transferred to the mitochondrial fraction. Such transfer of cytochrome c was inhibited most strongly by GA, since GA inhibits both ERK and Akt pathways.

HSP90 inhibition interferes with the action of NF-κB
While Akt acts on IκB kinase to induce proteosomal degradation of IκBα after stimulation with TNF-α [24], we found that GA inhibited TNF-α-induced degradation of
IκBα (Fig. 5B, left). Nuclear translocation of NF-κB/p65, induced by TNF-α, was also inhibited (Fig. 5B, right). Further, ELISA showed that the binding of NF-κB/p65 at the NF-κB consensus binding site (5'-GGGACTTTCC-3') was significantly inhibited by GA in a dose-dependent manner (Fig. 5C).

Discussion

The results show that HSP90 is massively expressed in rheumatoid synovial cells, where GA, a specific inhibitor of HSP90, interferes with the rearrangement of actin filaments and enhances the apoptosis of rheumatoid synovial cells via the ILK, MAPK and PI3-K pathways. ILK has been identified to be an ubiquitously expressed kinase that directly binds integrin-α subunit [25]. Assembly of ILK with PINCH and α-parvin is essential for its localization to the focal adhesion sites on the cell membrane, through which various cellular functions including migration, proliferation and apoptosis are exerted [26-28]. We previously showed that Rac1 is activated at the periphery of the rheumatoid synovial cell [29]. Since ILK regulates cytoskeletal organization via Rho-family GTPases including cdc42 and Rac1 [30], we now show that ILK forms a

![Fig. 4](image_url)
Fig. 5 (A) Western blot of phosphorylated (p) ERK, ERK, Akt and Bcl-2 family proteins involved in mitochondria-related apoptosis induction. Synovial cells were cultured in the presence of GA (10 μM), wortmannin (Wort; 10 nM) or PD98059 (PD; 50 μM) for 24-h treatment. While Wort and PD specifically inhibited Akt and pERK, respectively, GA inhibited both pERK and Akt. GA and PD inhibited the expression of Bid, and GA and Wort inhibited the phosphorylation of Bad. Bcl-2 localized to the mitochondria from the cytosol by treatment with GA, Wort and PD in almost the same manner. The cytosolic expression of Bcl-XL was strongly inhibited by Wort, as well as GA and PD. The localization of Bcl-XL into mitochondria was strongly induced by Wort, as well as GA and PD. The cytoplasmic release of cytochrome c was most enhanced by GA. (B) Effects of GA on TNF-α-mediated NF-κB activation in synovial cells. In the cytoplasmic fraction, 10-α induced degradation of IκBα, whereas this was inhibited by co-incubating with 10 and 20 μM GA. In the nuclear fraction, maximum nuclear translocation of NF-κB/p65 was induced by 10 ng/ml TNF-α, and this was inhibited by 10 and 20 μM GA. (C) ELISA for the binding activity of NF-κB/p65 to the NF-κB consensus binding site. GA treatment inhibited the NF-κB binding in a dose-dependent manner (*1: P < 0.001; *2: P < 0.05).
complex with PINCH and α-parvin to rearrange actin fibres and potentiate PAK-1-mediated activation of cdc42/Rac1. This is inhibited by GA via degradation of ILK (Fig. 2). This observation is in line with the finding that HSP90 stabilizes ILK expression, thereby controlling the expression of intercellular adhesion molecule-1 (ICAM-1) shown in endothelial progenitor cells [31].

Akt is an endogenous inhibitor of Fas-induced apoptosis in synovial cells [32, 33], and heavily phosphorylated Akt in synovial cells enhances the stimulatory effects of TNF-α by inhibiting apoptosis [34]. While MAPK pathways are activated in rheumatoid synovial tissue [35], stimulation of ERK induces production of TNF-α in synovial cells [11]. Dominant-negative Ras as introduced into synovial cells specifically inactivates ERK without affecting JNK or p38 [36], indicating that sole suppression of ERK is sufficient for controlling the apoptosis of synovial cells among various molecules in MAPK pathways. In the present study, we show that GA inhibits either ERK by targeting cRaf/HSP90 and MEK-HSP90 complexes or Akt by targeting PDK/HSP90 and Akt/HSP90 complexes (Fig. 3B).

GA also inhibits TNF-α-induced activation of NF-κB (Fig. 5B and C). Akt has been shown to phosphorylate IκB kinase (IKK)-α [24], and NF-κB is activated when its inhibitor IκBα is phosphorylated by the IKK complex containing IKKβ and/or IKKα [37]. Further, both IKKα and IKKβ have been shown to be capable of phosphorylating p65NF-κB, which subsequently locates to the nuclei and binds NF-κB motifs [38]. Thus, our findings and previous reports, therefore, indicate that stable functioning of ERK and Akt is indeed the key for the survival of synovial cells, which is substantially supported by HSP90. We show here that the apoptosis of synovial cells as induced by GA is inhibited by the up-regulation of ERK and Akt upon culturing synovial cells on Fn-coated dishes (Fig. 4).

We next studied the relation between HSP90 and the mitochondrial apoptosis pathway by focusing on the interaction among ERK, Akt and Bcl-2 family proteins (Fig. 5A). The mitochondrial apoptosis pathway is initiated by pro-apoptotic molecules including Bid and Bad [39]. Bid links caspase 8 with Bcl-2 family proteins, followed by cleavage of Bid by caspase 8, and this process is inhibited by MAPK [40]. We now show that GA induces Bid cleavage as efficiently as PD98059. This is consistent with the previous finding that GA induced Bid cleavage in NIH3T3 fibroblasts [41]. Dephosphorylated Bad, another initiator of mitochondrial apoptosis, conveys apoptotic signal to Bcl-XL, and this process is under the control of both ERK and Akt [42, 43]. We show that GA most strongly inhibits Bad as compared with wortmannin or PD98059 (Fig. 5A). While Bcl-2 and Bcl-XL, mitochondria membrane-bound anti-apoptotic molecules, interfere with mitochondrial apoptosis signalling, we also show
that GA decreases the expression of cytoplasmic Bcl-2/Bcl-XL and increases the translocation of Bcl-2/Bcl-XL to mitochondria. Finally, in consistency with the above findings, GA did release highest amounts of cytochrome c into the cytoplasm (Fig. 5A).

In conclusion, as shown in Fig. 6, HSP90 supports not only ILK, which is responsible for rearrangement of actin fibres leading to synovial extension such as pannus, but also ERK and Akt, which are important for the survival of synovial cells. Therefore, we now show that the contribution of HSP90 in stabilizing its target molecules is far more important than previously considered in the pathogenesis of RA that potentiates a tumour-like synovial overgrowth.

**Rheumatology key messages**

- HSP90 potentiates both actin cytoskeletal rearrangement via ILK and survival of synovial cells via MAPK and PI3-K/Akt pathways.
- HSP90 is one of the important molecules in the pathogenesis of RA that induces a tumour-like synovial overgrowth.

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