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Multiple Pathways of Fas-Induced Apoptosis in Primary Culture of Hepatocytes

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Fas (Apo1/CD95) is a member of the tumour necrosis factor/nerve growth factor receptor superfamily and mediates apoptosis in various cell types (for review see [1]). Although this apoptotic activity has been clearly related to homeostasis in the immune system and pathological situations in non-lymphoid organs, the Fas signaling pathway remains mostly elusive. We and others previously showed that Fas-induced apoptosis of primary culture hepatocytes requires either an inhibitor of translation or a protein kinase inhibitor, suggesting that two distinct pathways of Fas signaling exist in hepatocytes. We report here that activation of ICE-like and CPP32-like cysteine proteases are required for Fas-mediated apoptosis, but that these pathways involve different subclasses of serine proteases and are selectively modulated by inhibitors of protein tyrosine kinases. These results confirm that distinct pathways can lead to Fas-induced apoptosis in hepatocytes. Further understanding of these pathways could facilitate the rational design of anti-apoptotic drugs in liver diseases associated with massive Fas-mediated hepatocyte apoptosis, including fulminant hepatitis.

Binding of Fas ligand (FasL) or an agonistic anti-Fas antibody induces apoptosis in Fas-bearing cells. Besides the lymphoid system in which Fas-mediated apoptosis plays a role in the maintenance of T-cells homeostasis, the apoptotic activity of Fas seems to be mainly related to pathological situations in non-lymphoid organs. Recently many studies have been devoted to the Fas-mediated apoptotic machinery and, although the signaling pathway of Fas is still not precisely defined, some steps of this pathway have been elucidated. It has been shown for example that TNFRI and Fas both contain a related intracellular ''death domain'' that is crucial to trigger apoptosis [2, 3], since it acts as an interface mediating ligand-dependent recruitment of receptors-associated proteins (for review see [4]). Among all the recently identified Fas-associated proteins, only MACH/FLICE possesses a known catalytic region, with homology to cysteine protease catalytic domain [5, 6]. Many recent works have highlighted the crucial role of these enzymes, including interleukin 1β-converting enzyme (ICE) and CPP32, in the execution of apoptosis induced by a wide range of stimuli (for review see [7]). Thus MACH/FLICE establishes a striking connection between Fas complexes and the well described cell death executors cysteine proteases. If cysteine proteases activation during Fas-mediated apoptosis is now well accepted, identification and requirement of other classes of transducing molecules in Fas signaling are still controversial. For instance Fas has been reported to couple ligand binding to a rapid induction of tyrosine and serine/threonine phosphorylations [8, 9], but in other studies, inhibition of Fas-induced apoptosis by protein kinase inhibitors could not be detected [10, 11]. However more data arguing for the role of phosphorylations in Fas-mediated apoptosis came from the Fas-insensitivity of lymphoid cells deficient in hematopoïetic cell phosphatase [12, 13], or the evidences that Fas can physically associate with phosphorylated proteins [14-17]. Another developing field of research deals with the possibility

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that Fas-induced cell death is mediated by the sphingomyelin cycle, since it has been shown that Fas activation generates ceramide in lymphoid cells [18-21]. One point which seems to emerge from all the data accumulated so far is that the signaling pathway of Fas vary from one cell type to another. In this way it can be hypothetized that all these results obtained from lymphoid cells may not account for Fas-mediated apoptosis in other non-lymphoid cells. Elucidation of Fas signaling pathway in hepatocytes could be of great interest since several dramatic liver diseases, such as fulminant hepatitis or cirrhosis, have been recently related to dysregulation of Fas and FasL expression in the liver or FasL-expressing CTL infiltrations [22]. Hepatocytes constitutively express high levels of Fas [23], and the strong susceptibility of liver cells to agonistic anti-Fas antibody has been highlighted by experiments of intraperitoneal injection of this antibody in mice, which causes their rapid death by massive liver cytolysis [24]. We recently showed that the viral large T antigen of SV40 and the anti-apoptotic Bcl-2 protein prevent this *in vivo* Fas-induced liver apoptosis [25-27], therefore suggesting original pathways of Fas-mediated apoptosis in the liver. Moreover we and others showed that Fas-induced apoptosis of primary cultures of hepatocytes requiers the presence of either an inhibitor of translation (cycloheximide (CHX)), or a protein kinase inhibitor (H7) [26, 28]. These findings suggested the possible involvement of a single pathway of Fas-induced apoptosis in hepatocytes, constitutively inhibited by a labile repressor, whose activity could be modulated by serine/threonine kinases. Alternatively, one could hypothesize that, according to the co-stimuli required for Fas apoptosis-anti-Fas antibody plus CHX (which we will call Fas plus CHX)-or-anti-Fas antibody plus H7 (which we will call Fas plus H7)-distinct pathways of dying after Fas activation exist in hepatocytes. To address the question whether the signaling pathway of Fas-induced apoptosis in liver share similarities with other cell types or is really specific, we have examined the Fas pathway in primary culture of hepatocytes.

MATERIALS AND METHODS

Reagents. CHX, TPCK, TLCK, TAME, Genistein, Herbimycin A, sodium orthovanadate, 6-DAP, okadaic acid, C_2-ceramide and Hoechst 33258 were purchased from Sigma Chemical Co. (St Louis, MO). The mouse anti-Fas monoclonal antibody was supplied by Pharmingen (San Diego, CA). YVAD-cmk and DEVD-cmk were purchased from Bale biochimie (Bale, Switzerland). The protein kinase inhibitor H-7 was from Seikagaku Kogyo Co. (Tokyo, Japan). ^{3}H-palmitate was from Amersham (UK). Collagenase A was purchased from Boehringer Mannheim (Germany).

Isolation of mouse hepatocytes. Hepatocytes were isolated by in situ collagenase perfusion as previously described [39] and had a viability exceeding 90% according to trypan blue exclusion. Following perfusion, the dissociated liver was minced, suspended in M199 medium (Gibco-BRL) and filtered through a filter of 70-mm mesh. Cells were counted and plated at a density of 5×10^5 cells per 35-mm dishes in M199 medium containing 10% FCS. After 3 hours, the medium was removed and replaced by M199 containing 10^{-8} M insulin, 10^{-6} M thyroid hormone and 10^{-6} M dexamethasone sulfate.

Assay for in vitro cytolytic activity and detection of apoptosis. Primary cultured hepatocytes were cultured in appropriate medium with 1μg/ml anti-Fas mAb, supplemented with 10 μg/ml cycloheximide (CHX) or 200 mM protein kinase inhibitor H7. All other reagents used for inhibition assays were dissolved following manufacturer recommandations. The protective effects of YVAD-cmk (Ac-Tyr-Val-Asp-chloromethylketone) and DEVD-cmk (Asp-Glu-Val-Asp-chloromethylketone) were explored by culturing primary hepatocytes with indicated concentrations respectively in dimethyl sulfoxide (DMSO) or H2O. At various time points up until 60 h, the cells were recovered and percentage of viable cells assessed by trypan blue exclusion. Approximately 200 cells in each sample were counted. To monitor apoptosis, Hoescht 33258 staining was performed as described [26] on each sample at comparable time points.

Sphingomyelin and ceramide measurement. After various incubation times with anti-Fas antibody plus CHX or plus H7, the reaction was stopped by adding cold medium and centrifuging the cells. Lipids were extracted as previously described [40]. Briefly, the cell pellets were resuspended successively in 1 ml of methanol: water: 6N HCl (100:5:1), 2 ml of chloroform and 0.6 ml of water. The mixture was then vortexed and centrifuged at 1000 $\times$ g for 5 min. The upper phase was discarded, and the lower phase was dried under N_2. The lipid residue was reconstituted with 1 ml of chloroform: methanol (2:1), vortexed, and spun at 5000 $\times$ g for 5 min to pellet insoluble material. The supernatant was evaporated, and the samples were reconstituted with 20 μl of chloroform: methanol (2:1). Lipids were chromatographed on HPTLC plates (Merck, U.S.A.) using chloroform: methanol: ammonium hydroxide solvent system (90:10:1) or chloroform: methanol: acetic acid: water (100:60:20:5). Plates were then air dried, sprayed with

En3Hance (NEN, France), exposed overnight and developed with MP-hyperfilm (Amersham, France). ^{3}H-palmitate labelled lipids were identified by co-migration with standards, scrapped off the plates, and quantitated by liquid scintillation counting.

RESULTS AND DISCUSSION

In a recent report, we showed that whatever the kind of co-stimuli-CHX or H7-, Fas-induced apoptosis of hepatocytes can be blocked by a synthetic inhibitor of ICE-like cysteine proteases, YVAD-cmk ([29] and data not shown). To determine whether other cysteine proteases could be involved in Fas-induced apoptosis, we have tested the effect of a CPP32-like cysteine protease inhibitor, DEVD-cmk, on the apoptosis of primary cultures of hepatocytes. Cells were incubated with DEVD-cmk and then treated either with Fas plus CHX or with Fas plus H7. Apoptosis was monitored by Hoechst staining which reveals the perinuclear chromatine condensation-a hallmark of apoptosis. As shown in Fig 1A, DEVD-cmk inhibited very efficiently both Fas plus CHX and Fas plus H7 induced cytotoxicity (Fig 1A). Furthermore DEVD-cmk inhibited apoptosis in a dose-dependant manner (Fig 1B): DEVD-cmk confered significant protection of hepatocytes at only 50 nM, and total protection at 5 μM. By comparison, respectively 150 μM and 300 μM of YVAD-cmk were necessary for similar rates of protection. These results indicate that, whatever the kind of co-stimuli, one or several CPP32-like proteases are involved in Fas apoptosis of hepatocytes. The discrepancies observed between DEVD-cmk and YVAD-cmk concentrations required for complete protection do not *per se* demonstrate the preponderance of CPP32-like proteases, since no data are available on permeability of these inhibitors in hepatocyte nor on CPP32-like and ICE-like proteases concentrations in these cells. Although we could not detect the activating clivage of ICE itself (data not shown), it therefore remains possible that additionnal ICE-like proteases are activated in Fas-mediated apoptosis in these cells. These findings are in agreement with results on lymphocytes or neurons which describe the involvement of CPP32 and/or ICE in Fas-induced apoptosis [30-35]. Thus an ubiquitous pathway of Fas-mediated apoptosis seems to be activation of cysteine proteases.

Participation of other classes of proteases, including serine proteases, has also been described during Fas-mediated apoptosis in Jurkat cells [10, 36]. To investigate whether such serine proteases are implicated in hepatocyte apoptosis, we examined the effects of three serine protease inhibitors, tosyl-phenylalanine chloromethylketone (TPCK), tosyl-lysine chloromethylketone (TLCK) and tosyl-argenine methylester (TAME) on Fas-induced apoptosis. Interestingly we could observe a discrepancy between the two apoptotic signaling pathways Fas plus CHX and Fas plus H7 (Table 1). TPCK and TAME inhibited the hepatocyte apoptosis induced by Fas plus CHX, while TLCK had no effect. By contrast the opposite pattern of inhibition was obtained when hepatocytes underwent apoptosis by treatment with Fas plus H7. These findings demonstrate that serine proteases also participate in Fas-induced apoptosis in hepatocytes. Furthermore, since TPCK and TLCK inhibit different subclasses of serine proteases [37], one can conclude that distinct pathways can be used in hepatocyte, involving either chymotrypsin-like proteases-Fas plus CHX pathway-or trypsin-like proteases-Fas plus H7-.

Synthetic cell-permeable analogs of ceramide can induce typical apoptotic cell death [7, 38]. Since ceramide production by activation of sphingomyelinases may account in some cell types for Fas-induced apoptosis, it was tempting to speculate in our model that the modulation of Fas-induced apoptosis could occur through a turnover sphingomyelin-ceramide. However whatever the kind of apoptotic stimuli, we could never detect any significant hydrolysis of sphingomyelin nor production of ceramide (Fig 2A). Moreover treatment of primary culture of hepatocytes with the synthetic cell-permeable analog C_2-ceramide, which was cytotoxic in control Burkitt's lymphoma cells, could never affect the viability of hepatocytes (Fig 2B). Our results are in agreement with previous data which support the idea that ceramide production in certain cells is not necessary for Fas-induced apoptosis: specific drugs that impede sphingo-

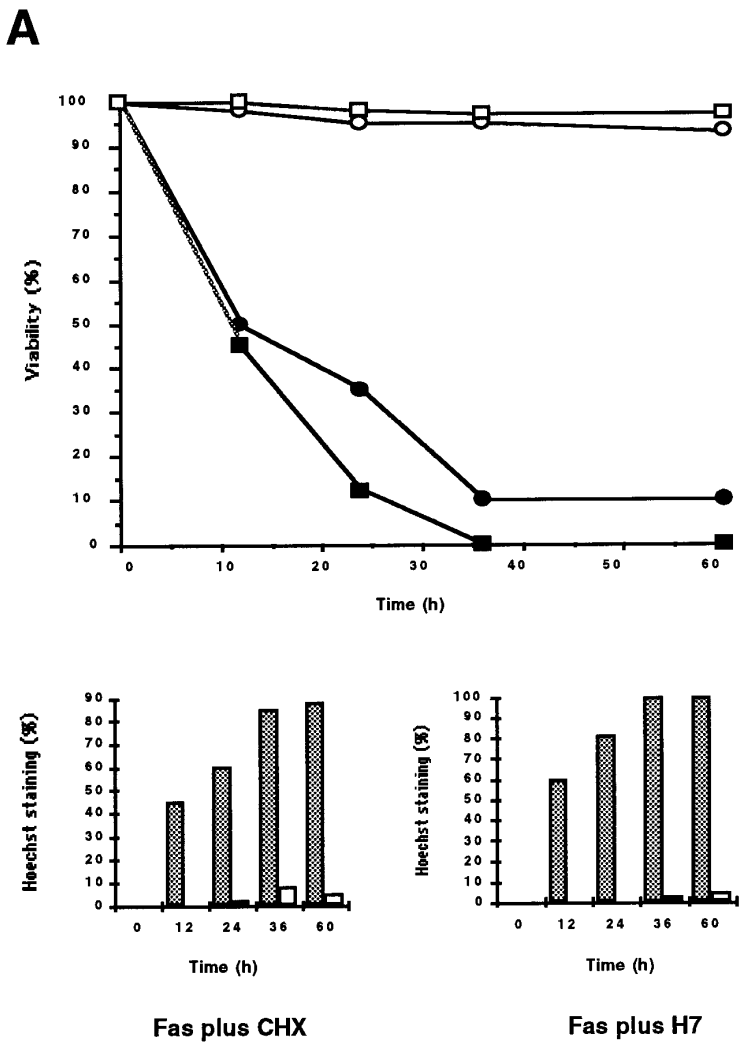

A
Viability (%)
Time (h)
Hoechst staining (%)
Time (h)
Fas plus CHX
Hoechst staining (%)
Time (h)
Fas plus H7
B
Viability (%) after 24h incubation
Inhibitors concentrations (µM)

TABLE 1
Effects of Serine Proteases Inhibitors on Fas Apoptosis of Hepatocytes

Treatment	Apoptosis (% Hoechst staining)
Fas + CHX	55 ± 5
+ TPCK 25 μM	32 ± 6
+ TPCK 100 μM	2 ± 2
+ TLCK 25 μM	53 ± 7
+ TLCK 100 μM	55 ± 4
+ TAME 2 mM	19 ± 3
+ TAME 10 mM	3 ± 3
Fas + H7	47 ± 5
+ TPCK 25 μM	49 ± 5
+ TPCK 100 μM	45 ± 4
+ TLCK 25 μM	29 ± 6
+ TLCK 100 μM	4 ± 4
+ TAME 2 mM	48 ± 5
+ TAME 10 mM	44 ± 4

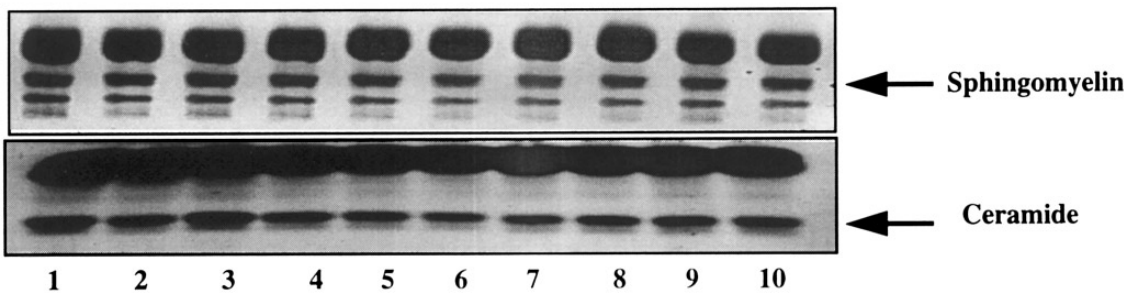
Primary cultures of hepatocytes were incubated either with anti-Fas antibody plus CHX or with anti-Fas antibody plus H7, supplemented with indicated concentrations of serine proteases inhibitors. The data represent the mean (± S.E.M.) of percent apoptotic hepatocytes as measured by Hoechst staining and obtained from at least 3 experiments.

myelinase activation cannot block Fas-induced apoptosis in a fibrosarcoma cell line [11], and ceramide production was not found in Fas apoptosis of Jurkat cells [10].

Since H7 is a potent serine/threonine kinase inhibitor, we finally wanted to assess whether phosphorylations could modulate the Fas-signaling pathway in hepatocytes. A number of protein kinase and protein phosphatase inhibitors were tested (Table 2). Among all inhibitors tested, only inhibitors of protein tyrosine kinases, namely genistein and herbimycin A, significantly affected Fas-induced apoptosis. These products rendered hepatocytes more susceptible to Fas plus CHX, but such a modulation was not observed when hepatocytes were treated with Fas plus H7. Interestingly, treatment of hepatocytes with anti-Fas antibody and both CHX and H7 resulted in additionnal cytotoxicity (data not shown), which support our hypothesis that Fas pathways with these co-stimuli are not overlapping. Our data support the idea that protein kinase inhibitors, without being necessary, can modulate the sensitivity of hepatocytes to Fas-induced apoptosis. Such a modulation could account for the resistance to Fas-mediated apoptosis induced by the ectopic expression of the large T antigen of SV40 [27]: T antigen

FIG. 1. Role of cysteine proteases in Fas apoptosis of hepatocytes. (A) Primary cultures of hepatocytes were incubated either with anti-Fas antibody plus CHX, in the absence (filled circles) or presence (open circles) of DEVD-cmk, or with anti-Fas antibody plus H7, in the absence (filled squares) or presence (open squares) of DEVD-cmk. In each case apoptosis was monitored by Hoechst staining and correlated with viability data as shown on histograms. Percent apoptotic hepatocytes (Hoechst staining) and viability (blue trypan exclusion) were obtained from triplicate experiments. (B) Dose-dependent inhibition of Fas apoptosis by YVAD-cmk and DEVD-cmk. Primary hepatocytes were incubated for 24 h with anti-Fas antibody plus CHX alone (open bars) or with various concentrations of YVAD-cmk (hatched bars) or DEVD-cmk (spotted bars).

A



	1	2	3	4	5	6	7	8	9	10
ceramide (cpm)	19224	16584	18574	15984	15436	16521	17025	18547	17598	18574
sphingomyeline (cpm)	10251	9874	11520	10847	11023	10123	9784	10471	12354	11789

B

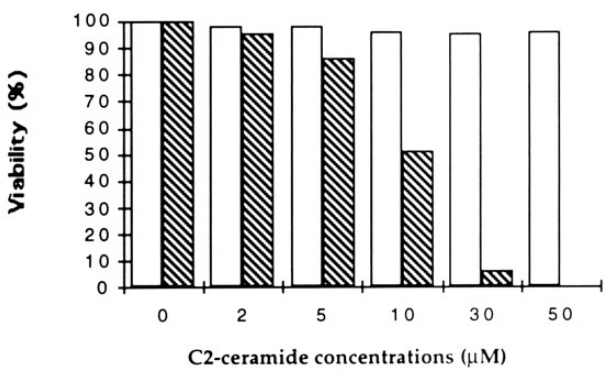


FIG. 2. Role of ceramide in Fas apoptosis of hepatocytes. (A) Autoradiograms of ceramide and sphingomyelin from an HPTLC of total lipids extracted from hepatocytes labelled with ^{3}H-palmitate. Hepatocytes were incubated either with anti-Fas antibody plus CHX (lanes 3 to 6) or with anti-Fas antibody plus H7 (lanes 7 to 10). Cells were recovered at different times of incubation: 2 min (lanes 3 and 7), 10 min (lanes 4 and 8), 20 min (lanes 5 and 9), and 1 hour (lanes 6 and 10). As a control untreated hepatocytes were recovered 2 min and 1 hour after the beginning of the experiments (lanes 1 and 2). Each spot of ceramide or sphingomyelin was scraped off the plates and quantitated by liquid scintillation counting. Quantification of changes in ceramide and sphingomyelin levels are summarized in the table. Results are given as means of at least 3 independent experiments. (B) Cytotoxicity of the synthetic analog C2-ceramide. Primary cultures of hepatocytes (open bars) were incubated with various concentrations of the synthetic cell-permeable analog C2-ceramide and viability was assessed by trypan blue exclusion. As a control C2-ceramide sensitive Burkitt's lymphoma cells (hatched bars) were incubated under the same conditions. Results are means of 3 independent experiments.

TABLE 2
Effects of Kinase and Phosphatase Inhibitors on Fas Apoptosis

Treatment	Apoptosis (% Hoechst staining)
Fas + genistein	0
Fas + Herbymicine A	0
Fas + sodium orthovanadate	0
Fas + 6-DAP	0
Fas + Acide okadaïque	0
Fas + CHX	55 ± 5
+ Genistein 1 μM	80 ± 4
+ Genistein 10 μM	99 ± 1
+ Herbymicine A 100 nM	85 ± 6
+ Herbymicine A 4000 nM	98 ± 2
+ sodium orthovanadate 300 μM	54 ± 3
+ 6-DAP 3 μM	56 ± 3
+ Acide okadaïque	55 ± 7
Fas + H7	47 ± 7
+ Genistein 1 μM	49 ± 5
+ Genistein 10 μM	48 ± 2
+ Herbymicine A 100 nM	50 ± 6
+ Herbymicine A 400 nM	52 ± 4
+ sodium orthovanadate 300 μM	44 ± 3
+ 6-DAP 3 μM	49 ± 7
+ Acide okadaïque	46 ± 3

Primary cultures of hepatocytes were incubated either with anti-Fas antibody plus CHX or with anti-Fas antibody plus H7, supplemented with indicated concentrations of inhibitors. Genistein and herbimycin A are tyrosine kinase inhibitors. Sodium orthovanadate is a tyrosine phosphatase inhibitor. 6-DMAP is a serine/threonine kinase inhibitor and okadaic acid a serine/threonine phosphatase inhibitor. The data represent the mean (±S.E.M.) of percent apoptotic hepatocytes as measured by Hoechst staining and obtained from at least 3 experiments.

is a serine/threonine phosphorylated protein, which inhibits selectively the Fas plus CHX cytotoxicity but not Fas plus H7 induced apoptosis. In view with our present findings, one can thus assume that T antigen could act by quenching phosphorylated substrates required for Fas activation.

In conclusion our findings provide evidence that the CPP32-like and ICE-like cysteine proteases play a crucial role in Fas-induced apoptosis in hepatocytes, like in all other cell types tested so far. In contrast ceramide production does not seem to be involved in this process. Our results also demonstrate that Fas-induced apoptosis in hepatocytes can use at least two distinct pathways. These pathways are selectively modulated by protein tyrosine kinase inhibitors, and imply different serine proteases. Although Fas seems to be expressed quite ubiquitely, high levels of Fas are detected mostly in the lymphoid system, heart, lung and liver [23]. The constitutive expression of such an apoptotic transducing receptor in non-lymphoid organs raises many questions by regards to its physiological significance. In liver, Fas-induced apoptosis is likely to be mainly related to pathological situations. For instance in patients with fulminant liver failure or secondary cirrhosis, infiltration of FasL-expressing CTL have been related to massive death of hepatocytes [22]. It can be assumed that the extreme variability of individual responses to liver agressions may result from modulation of hepato-

cytes sensitivity to Fas-mediated apoptosis. Thus it would be interesting to correlate the implication of the modulative agents we have described and the diversity of stimuli that can account *in vivo* for these liver damages. More complete elucidation of these pathways could also be of great interest for the design of new therapeutic agents against liver failure involving hepatocyte apoptosis, including viral and inflammatory liver diseases.

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