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Original Paper

Elevated Plasma Levels of Urokinase Plasminogen Activator Receptor in Non-small Cell Lung Cancer Patients

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The urokinase plasminogen activator (uPA) is involved in extracellular matrix degradation during cancer invasion. Binding of uPA to a specific cell surface receptor (uPAR) is a key step in this process. We have previously reported that high levels of uPAR in squamous cell lung cancer tissue extracts are associated with poor prognosis (Pedersen *et al.*, *Cancer Res* 1994, 54, 4671–4675). Recently we found that uPAR is present in blood plasma from healthy donors as determined by enzyme-linked immunosorbent assay (ELISA) and chemical cross-linking. We now report that uPAR in plasma from 17 patients with non-small cell lung cancer (NSCLC) was significantly higher than in 30 healthy controls ($P = 0.0004$), while no significant increase was found in plasma from 14 patients with small cell lung cancer (SCLC). The increased levels of uPAR in the plasma from NSCLC patients is likely to be due to release of uPAR from the tumour tissue, and may, therefore, be related to prognosis. © **1997 Elsevier Science Ltd.**

Key words: lung cancer, plasma, uPAR

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INTRODUCTION

DEGRADATION OF the extracellular matrix caused by a concerted action of proteolytic enzymes plays a critical role in cancer invasion [1–3]. One of these enzymes is the serine protease, plasmin, which can degrade most components of the extracellular matrix directly [1], and it is, in addition, involved in activation of several matrix metalloproteinases [4]. Plasmin is formed by activation of plasminogen, in a process regulated by several molecules, including urokinase-type plasminogen activator (uPA), type 1 and 2 plasminogen activator inhibitors (PAI-1 and PAI-2), and a specific cell surface uPA receptor (uPAR), which binds both uPA and its virtually inactive proenzyme, pro-uPA [2, 5].

Concomitant binding of pro-uPA to uPAR and plasminogen to, as yet, poorly identified cell surface binding sites strongly enhances plasmin generation [6]. Human uPAR is a single polypeptide chain, highly glycosylated protein with a molecular weight of 55–60 kDa [7, 8]. It consists of three homologous domains, the amino-terminal domain 1 being important for binding to an EGF (epidermal growth factor)-like domain in the uPA molecule [9]. At the carboxy terminal, uPAR is anchored to the cell surface by a glycosylphosphatidylinositol moiety [10]. PAI-1 and PAI-2 inhibit both free and receptor-bound uPA, and binding of the inhibitors to receptor bound uPA results in internalisation of the uPA–inhibitor complexes mediated by the α-2-macroglobulin receptor [11–13].

uPA, PAI-1 and uPAR are all present in lung cancer tissue [14–18]. We have developed a sensitive uPAR enzyme-linked immunosorbent assay (ELISA) [19], and found that high levels of uPAR in tumour extracts from patients with squamous cell lung cancer are associated with poor prognosis [18]. Recently, we found that uPAR is present in blood plasma from healthy donors [20]. In this study, we investigated uPAR plasma levels in patients with non-small cell lung cancer (NSCLC).

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PATIENTS AND METHODS

Patients and healthy controls

The study included plasma from 30 healthy volunteers and 31 patients with lung cancer. All patients had biopsy-verified malignant tumours and radiographically verified tumours with diameters larger than 3 cm at the time of blood sampling. The patients were divided into two groups according to the histological type of their lung cancer: 17 patients with NSCLC and 14 patients with small cell lung cancer (SCLC) (WHO classification). Blood plasma was consecutively collected at the outpatient section of the Department of Oncology, Rigshospitalet, Denmark, from patients enrolled for chemotherapy (SCLC: combination chemotherapy, NSCLC: phase I-II chemotherapy studies). The study was approved by the local ethical committee (ref. 01-411/94), and informed consent was obtained from all subjects included.

Reagents

The amino-terminal fragment (ATF) of uPA was a kind gift from Dr. A. Mazar, Abbott Laboratories. CNBr-activated Sepharose 4B was purchased from Pharmacia Biotech. *N, N'*-disuccinimidylsuberate was from Pierce. All other reagents were as described previously [8, 19, 21].

Human plasma

For preparation of plasma, blood was collected by venipuncture using a vacuum technique, into siliconised tubes containing 1 vol of 0.13 M sodium citrate to 9 volumes of blood. The tubes were immediately placed on ice and within 30 min centrifuged at 1800*g* for 30 min. The plasma was stored at −80 °C until analysis. A pool of equal volumes of plasma from 10 different, randomly selected subjects was prepared within each of the three groups: controls, NSCLC and SCLC. For phase separation, a pool of 485 ml of plasma obtained from two healthy individuals was used.

uPAR ELISA

Levels of uPAR were determined using a sandwich ELISA [19] with a polyclonal catching antibody and a mixture of three different biotinylated monoclonal detecting antibodies (R2, R3 and R5). Anti-uPAR R3 and anti-uPAR R5 are directed against different epitopes on domain 1 of uPAR, while anti-uPAR R2 is directed against an epitope on either domain 2 or 3 of uPAR [22]. This assay detects free uPAR and uPAR in complex with uPA [19]. uPAR was measured in fmol by calibration with a standard preparation of recombinant soluble uPAR (s-uPAR) in which the uPAR concentration had been determined by amino acid analysis [19]. The intra- and interassay variations were 7 and 13%, respectively. Except when otherwise indicated, plasma samples were diluted 10-fold before performing the uPAR ELISA. The protein content of the plasma samples was determined by Bio-Rad Protein Assay (Bio-Rad, Richmond, California, U.S.A.) using bovine serum albumin as standard. Results were expressed as fmol uPAR/mg total protein. The investigators were blinded to diagnosis when performing the ELISA assay.

Immunoabsorbtion and recovery of uPAR

Samples of plasma were diluted 5-fold and incubated with 25% (vol/vol) protein A-Sepharose for 2 h at 4°C to absorb human IgG. The supernatant was divided into two parts and incubated overnight with either a mixture of two monoclonal anti-uPAR antibodies (R4 and R9) different from those used for detection in the ELISA, or an irrelevant monoclonal antibody, anti 2,4,6-trinitrophenol (TNP). After incubation, antigen–antibody complexes were precipitated by incubation with protein A-Sepharose for 1 h at 4°C and the supernatants were assayed in the uPAR ELISA.

Recovery of uPAR in plasma was tested by addition of known amounts of s-uPAR (0–32.8 pM) to the different pools diluted 20-fold.

Immunoaffinity chromatography of plasma uPAR

uPAR was immunopurified from a 485 ml pool of plasma from two healthy donors, using a 2 ml column of 1.3 mg polyclonal anti-uPAR coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech). The purification was monitored by cross-linking to ^{125}I-ATF followed by SDS–PAGE (polyacrylamide gel electrophoresis) and fluorography [8]. The three eluted fractions (each 1 ml) which gave the strongest signal were pooled and dialysed against 15 mM sodium phosphate, pH 7.4, containing 14 mM NaCl, freeze-dried and dissolved in 300 µl PBS (150 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl). Buffer (900 µl), containing 0.1 M Tris-HCl pH 8.1, 1% Triton X-114, 10 mM EDTA, 10 µg/ml aprotinin and 1 mM PMSF were added to 100 µl of this uPAR-containing eluate, and were then subjected to temperature-induced phase separation [8, 23]. The temperature-induced phase separation was repeated on the resulting detergent and water phases. The final detergent and water phases were cross-linked with ^{125}I-ATF using *N, N'*-disuccinimidylsuberate, and analysed by SDS–PAGE and fluorography [8].

Statistical methods

For descriptive statistics, the GraphPad Instat Software was used. The non-parametric Mann–Whitney and Kruskal–Wallis (ANOVA) tests were used to test differences in content of uPAR between two or more groups.

RESULTS

Plasma from lung cancer patients contain authentic uPAR

The uPAR ELISA is based on catching with polyclonal antibodies raised against a highly purified preparation of a soluble recombinant uPAR variant [19], and detection with a mixture of the three monoclonal uPAR antibodies R2, R3 and R5. We have previously demonstrated that plasma from healthy donors contains uPAR, and that the ELISA can be used to quantitate this uPAR [20]. When pools of plasma from 10 NSCLC patients and 10 SCLC patients were assayed with the uPAR ELISA, virtually linear dose–response curves were obtained with concentrations of plasma up to at least 10% (results not shown). The ELISA recovered virtually all of a standard preparation of purified recombinant s-uPAR [19] added to 20-fold diluted pools of plasma from NSCLC patients (Figure 1) and SCLC patients (results not shown).

In order to test whether the signals obtained with the ELISA in the plasma from lung cancer patients are due to authentic uPAR or are caused either by non-immunological interactions or by immunological reaction with molecules different from uPAR, but cross-reacting with the antibodies used in the ELISA, the plasma pools were absorbed with a mixture of two monoclonal uPAR-antibodies, R4 and R9,

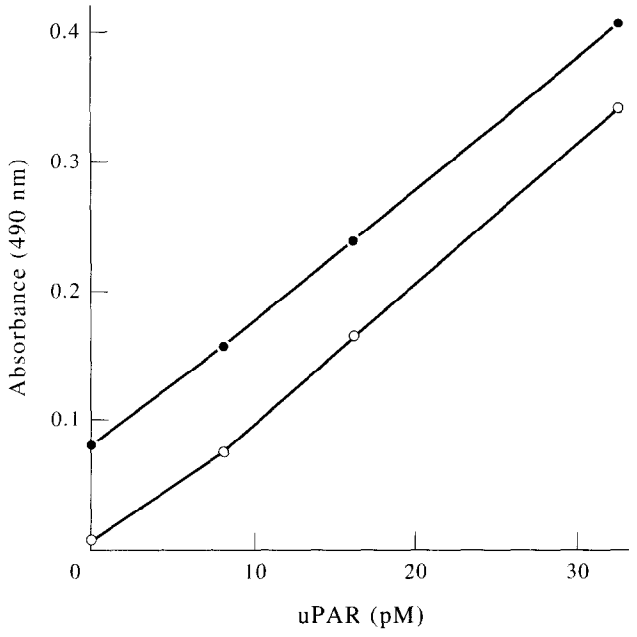

Figure 1. Recovery in an ELISA of uPAR added to plasma from cancer patients. Recombinant soluble uPAR in concentrations ranging from 0–32.8 pM was assayed either alone (○-○) or in the presence of plasma pooled from NSCLC patients and diluted 1:20 (●-●). The recovery of uPAR was calculated to be 106% as based on the slopes of the dose-response curves. The absorbance of the buffer control has been subtracted.

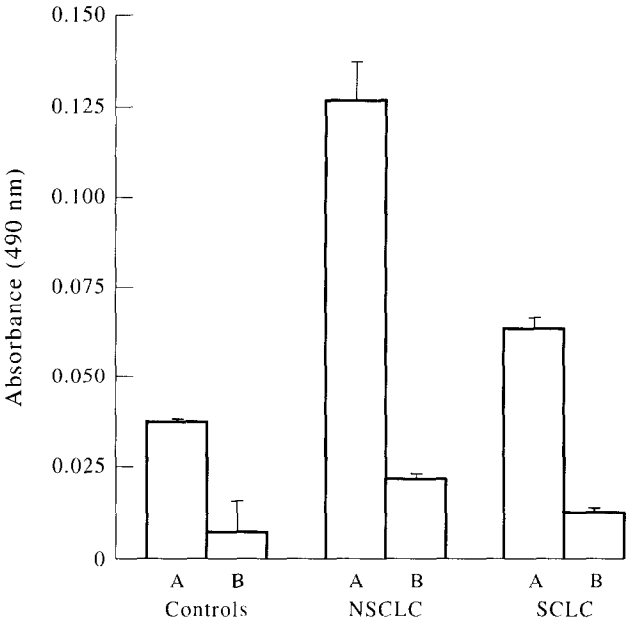

Figure 2. Absorbtion of uPAR immunoreactivity from plasma with monoclonal antibodies different from those used for ELISA. Pools of control, NSCLC and SCLC plasma were diluted 5-fold and each divided into two equal parts. One part was absorbed with a mixture of R4 and R9 monoclonal anti-uPAR IgGs immobilised on Sepharose (B), while the other part was absorbed with monclonal IgG of irrelevant specificity (anti-TNP) (A). The supernatants of the samples were assayed in duplicate with the uPAR ELISA. The mean absorbance and S.D. are indicated for each sample. The absorbance of the buffer control has been subtracted.

which recognise epitopes on the uPAR molecule that are different from those recognised by any of the three monoclonal antibodies used in the ELISA [19]. As shown in Figure 2, this absorption almost completely abolished the signal obtained in the uPAR ELISA, indicating that the signal is due to a molecule which not only reacts with the polyclonal uPAR antibody and at least one of the monoclonal antibodies R2, R3 and R5, but also reacts with at least one of the monoclonal antibodies R4 and R9. On this basis, we concluded that the ELISA measures authentic uPAR in plasma from lung cancer patients.

Plasma uPAR in NSCLC patients

The ELISA was then used to measure uPAR levels in plasma samples from 30 healthy donors and 31 patients with advanced lung cancer, 17 of whom were classified as NSCLC and 14 as SCLC. For calibration, a standard of purified recombinant uPAR was used in which the concentration has been previously determined by amino acid analysis [21], and the values were normalised according to the total protein content in the individual plasma samples. As shown in Figure 3, plasma uPAR levels in the 30 healthy donors varied within a rather narrow range of 0.62–2.1 fmol/mg protein with a median of 1.0. These values are in good agreement with our previous results on plasma from another group of healthy donors [20]. In the cancer patients, a larger variation in plasma uPAR levels was observed. The range in NSCLC patients was 0.66–7.3 fmol/mg protein with a median of 1.6, and in SCLC patients the range was 0.52–3.3 fmol/mg protein, with a median of 0.99. The Kruskal–Wallis rank sum test showed that there were statistically significant differences in plasma uPAR levels among the three groups ($P = 0.002$).When we analysed for statistically significant differences between the individual groups with the Mann–Whitney test, a highly significant difference was found between controls and NSCLC patients ($P = 0.0004$), and even if the one NSCLC patient with very high plasma uPAR was excluded (median value reduced from 1.6 to 1.5), the difference remained statistically highly significant ($P = 0.0007$). No statistically significant difference was found between controls and SCLC patients ($P = 0.98$), in spite of 2 SCLC patients having rather high uPAR values. The difference between SCLC patients and NSCLC patients was statistically significant ($P = 0.03$).

Ligand binding uPAR in normal plasma is water soluble

Intact native uPAR is an integral membrane protein, which has a glycolipid anchor and, therefore, is strongly lipophilic [10]. In ascites fluid from ovarian cancer patients, uPAR is however, present in a water-soluble ligand-binding form, which probably has been formed by cleavage with proteases or lipases of intact receptor [24]. To gain further information on the uPAR found in plasma, uPAR from a 485 ml pool of plasma from healthy donors was partly purified by immunoaffinity chromatography and reconstituted in a buffer containing the anionic detergent Triton X-114. This uPAR preparation was subjected to temperature-induced phase separation under conditions at which we have previously found that water-soluble forms of uPAR will be recovered in the water phase and lipid soluble forms in the detergent phase [8]. The two phases were analysed for the presence of uPAR by chemical cross-linking to a radiolabelled preparation of the uPAR binding amino terminal fragment (ATF) of uPA, followed by SDS–PAGE and

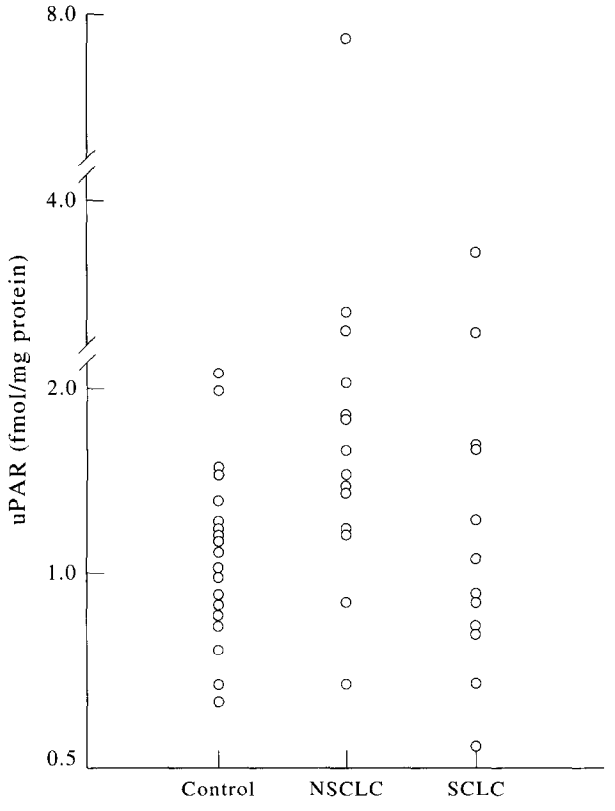

Figure 3. uPAR levels determined by ELISA in plasma from 30 normal donors, 17 NSCLC patients and 14 SCLC patients. Patients with the same value are represented by one data point only. uPAR concentrations in 10-fold diluted plasma samples were assayed in duplicate by ELISA, calibrated with a standard recombinant uPAR preparation and normalised with respect to the protein content of the individual samples.

fluorography [8]. A cross-linking uPAR–ATF complex was readily detected in the water phase, but not in the detergent phase (Figure 4), indicating that the main part of ligand binding uPAR present in normal plasma is water soluble. For ethical reasons, a similar experiment could not be performed on plasma from cancer patients, since too much plasma was required from the patients.

DISCUSSION

This study shows that there are significantly increased levels of uPAR immunoreactivity in plasma from NSCLC patients, while these levels in plasma from SCLC patients are not significantly different from those of healthy controls. Absorption experiments with monoclonal uPAR antibodies reacting with epitopes different from the monoclonal antibodies used in the ELISA indicated that the uPAR immunoreactivity measured represents authentic uPAR, and thus that the NSCLC patients do have increased plasma levels of uPAR.

We have previously found that normal plasma contains full-length three-domain uPAR capable of ligand binding [20]. The present study confirms this finding. For ethical reasons, it was not possible to study whether ligand binding three-domain uPAR is also present in plasma from lung cancer patients, but this appears likely. We do not know whether all the uPAR immunoreactivity measured in either normal plasma or plasma from lung cancer patients is due to the three-domain receptor or whether part of it is due to cleavage products [25, 26].

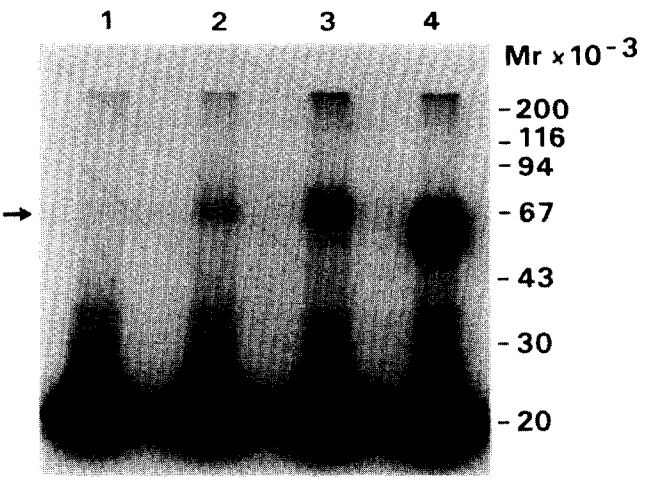

Figure 4. Phase separation of uPAR from normal plasma. uPAR was immunopurified from 485 ml plasma pooled from two healthy donors, and subjected to temperature-induced phase separation with Triton X-114 as described in Materials and Methods. Fifteen microliters of detergent phase (lane 1) and water phase (lane 2) were cross-linked to ^{125}I-ATF and analysed by SDS–PAGE and fluorography. As controls, detergent phase extracts from 7.5×10^5 U937 cells (lane 3) and 1 ng purified recombinant soluble uPAR (lane 4) were cross-linked to ^{125}I-ATF and analysed in parallel. Electrophoretic mobility of standard proteins are indicated to the right. Arrow to the left indicates the uPAR–ATF complexes.

The phase separation showed that the ligand-binding three-domain uPAR in normal plasma is water soluble. We assume that this is also the case in plasma from the lung cancer patients, although we could not test this assumption directly. Cell membrane uPAR is furnished with a GPI moiety which makes it very hydrophobic and anchors it to the membrane. uPAR lacking the GPI moiety is produced by leucocytes in patients with the rare disease, paroxysmal nocturnal haemoglobinuria [27] and has been prepared by recombinant techniques [21]. Both these forms of uPAR are water soluble [27], and water soluble three-domain uPAR can also be produced by treatment of cell membrane uPAR with the phospholipase PI-PLC of the endoproteinase asp-N [10, 27]. It, therefore, appears likely that the soluble uPAR found in normal plasma is formed from cells by the action of extracellular phospholipases or proteases, similar to that reported for other GPI-anchored proteins [28]. The identity of such putative enzymes involved in solubilisation of uPAR remains to be clarified. Another, but less likely, possibility is that the water soluble uPAR represents an alternatively spliced variant of uPAR, lacking the GPI moiety but retaining its ligand binding ability. Alternatively spliced mRNAs encoding human and murine uPAR variants with these characteristics have previously been identified [29, 30], but although these mRNA variants are expressed in both cell cultures and intact animals, corresponding native proteins have not been identified. Amino acid analysis of soluble plasma uPAR is needed to solve this question.

A water-soluble form of uPAR has previously been identified in ascites fluid and plasma from patients with ovarian cancer [24]. In that study, uPAR was not detected in plasma from healthy donors [24]. This apparent discrepancy with the results presented here is probably due to differences in sensitivity of the detection methods used in the two studies.

uPAR is consistently present in NSCLC tissue [26], and it is likely that the increased plasma levels in the NSCLC patients are due to release of uPAR from the cancer tissue. All the patients were treated with antineoplastic drugs at the time of the study, and it cannot be determined from the present data whether this treatment may have caused the release of uPAR, e.g. from dead cancer cells, or whether the plasma uPAR levels were independent of the antineoplastic treatment. It may be argued that antineoplastic drugs do not cause increased plasma uPAR, since normal uPAR levels were found in plasma from SCLC patients who also received chemotherapy. Differences between the regimes used in the two types of lung cancer and differences in their biology weaken this argument.

The difference in plasma uPAR levels between NSCLC and SCLC patients in itself points to a difference in the role of the uPA system in these two types of cancer. The cellular expression of uPA and uPAR in lung cancer tissue is not yet clear and further investigations concerning the localisation of the components are needed [31]. In many other types of cancer, such as colon adenocarcinoma, ductal mammary carcinoma and squamous cell skin cancer uPA and uPAR are consistently expressed at invasive foci [5]. Non-malignant stromal cells are, similarly to the production of matrix matalloproteases, strongly involved in the production of uPA and uPAR, and there are pronounced differences in the cellular expression pattern of the two molecules between various types of cancer [5]. uPA and uPAR are both expressed by cancer cells in squamous cell skin cancer while uPA is expressed by fibroblasts and uPAR by tumour infiltrating macrophages in most cases of ductal breast cancer. The lower plasma uPAR levels in SCLC compared with NSCLC patients, may be a reflection of the fact that there is no or only a relatively low uPAR expression in SCLC tissue, or that uPAR in this type of cancer is expressed by cells from which it is not released into the blood stream.

uPA and PAI-1 levels in tumour extracts are strong prognostic factors in different types of cancers, including breast, colon and lung cancer [17, 18, 32–38]. With respect to uPAR, we have recently found that high levels in tumour extracts, as measured by the uPAR ELISA, predicts poor prognosis in patients with squamous cell lung cancer [18] and breast cancer [39], and similar findings have been reported for colon cancer [40]. As discussed above, the increased plasma uPAR levels in NSCLC patients may be due to release of uPAR from the tumour tissue and may, therefore, reflect the uPAR level in the tumour tissue and hence be related to patients prognosis. If such an association exists, the requirement of only a plasma sample will substantially increase the clinical usefulness of uPAR measurements in these patients. The relationship of plasma uPAR to clinical parameters such as age, gender, smoking history, stage of disease and the effect of chemotherapy remains to be investigated. We are currently exploring the possibility of uPAR in plasma as a prognostic marker and also the potential value of plasma uPAR in monitoring progression in NSCLC patients.

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