Clinica Chimica Acta 402 (2009) 160–163

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Inter-α-trypsin inhibitor heavy chain 4 is a novel marker of acute ischemic stroke

Rajpal S. Kashyap [a], Amit R. Nayak [a], Poonam S. Deshpande [a], Dinesh Kabra [a], Hemant J. Purohit [b], Girdhar M. Taori [a], Hatim F. Daginawala [a,*]

[a] Biochemistry Research Laboratory, Central India Institute of Medical Sciences, 88/2 Bajaj Nagar Nagpur-10, India
[b] Environmental Genomics Unit, National Environmental Engineering Research Institute, Nehru Marg, Nagpur-440020, India

ARTICLE INFO

Article history:
Received 8 April 2008
Received in revised form 7 January 2009
Accepted 7 January 2009
Available online 22 January 2009

Keywords:
ITIH4
Acute ischemic stroke

ABSTRACT

Background: Inflammation and inflammatory markers play an important role in acute ischemic stroke (AIS). In an earlier study, we discovered a 120-kDa protein that was highly expressed in healthy participants, but either was not expressed or was barely expressed in AIS patients. The purpose of the present work was to isolate, characterize, and evaluate this 120-kDa protein in blood samples of AIS patients. In addition, we investigated how the identified protein compared with protein S-100ßß, neuron-specific enolase (NSE), and cytokine interleukins IL-2 and IL-10.
Methods: The 120-kDa protein band was analyzed via LC-MS/MS analysis. Then, the 120-kDa protein was evaluated using an enzyme-linked immunosorbant assay in serum samples from AIS patients, which were collected 0, 24, 48, 72 and 144 h after admission.
Results: The amino acid sequence of an internal fragment identified the 120-kDa protein as inter-α-trypsin inhibitor heavy chain 4 (ITIH4). The ITIH4 protein was completely absent in AIS patients as compared to those in the control group, and serum levels returned to normal in AIS patients as their condition improved. Expression of S-100 ßß, NSE, IL-2, and IL-10 were highly correlated with ITIH4 expression.
Conclusions: ITIH4 is an anti-inflammatory protein, and suggests that further investigation into its potential use in the diagnosis and prognosis of AIS is warranted.

1. Introduction

Acute ischemic stroke (AIS) is a clinical condition accompanied by oxidative stress and inflammation. It occurs when there is a sudden interruption in the blood supply to the brain. The cells die due to lack of oxygen and other nutrients [1,2]. With modern imaging techniques, like CT and MRI, it has become possible to detect ischemic damage, thus opening avenues for revascularization of the ischemic area of the brain [3]. Revascularization is possible if the stroke patient is brought to a well-equipped stroke unit in a particular window of time. However, it is difficult for patients to reach such a centre within this window, even in developed countries. In addition, a small infarct or infarcts that are difficult to detect via CT could still lead to a neurological deficit, suggesting that alternative diagnostic methods are needed [4–6].

Over the past few decades, analyses of brain damage biomarkers have attracted researchers' attention in a variety of central nervous system (CNS) disorders [7–10]. With increased interest in clinical proteomics, the use of comparative investigations of differential protein expressions is more common than ever before for diagnostic and prognostic assessments of disease states [11]. In previous work from our laboratory, by analyzing the total sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein profile of this serum, we have identified a 120-kDa serum protein band that is unique to serum samples collected from healthy subjects, but that is absent in AIS patients [12]. These earlier investigations led us to hypothesize that molecular identification of the 120-kDa serum protein would allow for the development of specific reagents and protocols for the diagnosis of AIS and the determination of patients' prognosis. The purpose of the present work was to isolate, characterize, and evaluate the identified 120-kDa protein in samples collected from AIS patients at pre-determined time points to assess trends in 120-kDa values over time. In addition, we report that this novel protein correlated with protein S-100ßß (an injured glial cell protein marker), neuron-specific enolase (NSE; a neuronal cell damage marker), and cytokine interleukins IL-2 (pro-inflammatory marker) and IL-10 (anti-inflammatory marker) expression.

2. Materials and methods

2.1. Subjects

Thirty-two patients (18 males, 14 females) aged 22–76 y (58±17 y), who were admitted to the Central India Institute of Medical Sciences (CIIMS), Nagpur, India within 24 h of the onset of ischemic stroke symptoms were included in the present study.

Abbreviations: ITIH4, inter-α-trypsin inhibitor heavy chain 4; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LC-MS/MS, liquid chromatography tandem mass spectrometry; AIS, acute ischemic stroke; NSE, neuron specific enolase.

* Corresponding author. Tel.: +91 712 2236441; fax: +91 712 2236416.
E-mail address: hfd_ciims@rediffmail.com (H.F. Daginawala).

0009-8981/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.cca.2009.01.009

Diagnosis was based on the WHO definition of Stroke: rapidly developing signs of focal (or global) disturbance of cerebral function lasting >24 h (unless interrupted by surgery or death), with no apparent non vascular cause, history, neurological examination and computerized tomography (CT). Patients with transient ischemic attack, haemorrhage, malignancies, severe renal or hepatic diseases and other types of brain injury were excluded from the present study. Neurological deficit was assessed using the National Institute of Health Stroke Scale (NIHSS) score during the hospitalization period, and functional recovery was assessed using the Modified Rankin scale (mRS) at the time of discharge. Twenty age- and sex-matched healthy subjects (control group) who did not report recent infection (i.e. within one month), other serious illness, or head trauma, were included as controls. All patients were admitted to the Intensive Care Unit (ICU), where the ambient temperature was between 20 and 25 °C. The patients received antiplatelet agents (aspirin 150 mg and clopidregel 75 mg once a day). Two patients were excluded because not all tests could be completed. Six patients were thrombolysed using intravenous recombinant tissue plasminogen activator, and 2 patients were treated with decompressive hemicraniectomy and duroplasty for malignant middle cerebral artery syndrome. Antiodema measures, such as mannitol 20%, 0.25–0.5 g/kg over 20 min (not exceeding a total of 2 g/1 kg of body weight in 24 h), were used for 15 patients (mean NIHSS 17.3), with symptoms of raised intracranial pressure. Two patients were treated with oral glycerol in addition to mannitol, IV fluids, and other supportive measures, including treatment of concurrent illnesses like hypertension and diabetes mellitus. Out of the thirty patients, twenty five survived and were discharged (Improved group), while five patients expired after 144 h (Expired group). The protocol for this study was reviewed and approved by the Institutional Ethics Committee of Central India Institute of Medical Sciences.

2.2. Sample

Venous blood was collected from healthy subjects and AIS patients at 0 h (i.e., at the time of admission), 24, 48, 72, and 144 h after admission. Blood was allowed to clot, and after centrifugation (100 ×*g*, 10 min), the serum was separated and stored at −20 °C until it was used.

2.3. One dimensional polyacrylamide gel electrophoresis

All obtained blood samples were subjected to SDS-PAGE, which was performed with a vertical slab gel electrophoresis system (Broviga, India) using the standard Laemmali method [16]. A 4% stacking gel and 10% running gel were used. Electrophoresis was carried out at 250 V and 50 mA. The resultant one-dimensional PAGE gel was stained with Coomassie blue, and the 120-kDa band was excised from the gel for analysis.

2.4. Liquid chromatography tandem mass spectrometry

The excised 120-kDa protein was sent to Pro-Tech, Australia for LC-MS/MS analysis. Pro-Tech, Australia characterized this protein as per the following protocol: Each gel piece was destained and washed before in-gel digestion. The 120-kDa band was excised, digested, and treated with trypsin after reduction; alkylation agents were added prior to analysis with LC-MS/MS.

In-gel digestion was carried out in 50 mmol/l NH_4HCO_4 buffer, pH 8.5, at 37 °C for about 4 h. The volume of the digestion buffer was approximately 20–50 µl, depending on the volume of the gel piece. The amount of proteolytic enzyme (Promega trypsin, modified, sequencing grade) used depended on both the size of the gel piece and the estimated amount of protein within the gel band. Typically, 200 ng to 1 µg trypsin was used per gel band. Acetonitrile (ACN), in a volume equal to 3–5 times the volume of digestion buffer, was then added to the digestion mix to extract the peptides, which were then centrifuged at high speed for 5 min. The supernatant was transferred to a clean microfuge tube with a gel-loading pipette tip and dried in a SpeedVac on medium heat. Alkylation agents were added prior to analysis with LC-MS/MS. The dried sample was dissolved in 0.5% acetic acid (HOA_C) for LC-MS/MS analysis. A Finnigan (ThermoFinnigan, San Jose, CA) LCQ ion trap MS coupled in-line with a high pressure liquid chromatography (HPLC) system was used for LC-MS/MS. A 75 µm (ID)× 10 cm length, 3 µm packing C18 capillary column, packed in house, was connected to a specially designed nanospray device that is capable of delivering a stable electrospray at flow rates of 100 nl/min to 1500 nl/min. Mobile phases were as follows: Solvent A (2% ACN, 97.5% H_2O, 0.1% formic acid) and Solvent B (90% ACN, 9.5% H_2O, 0.1% formic acid). For this analysis, the ion trap MS was set to operate in a data-dependent mode with Automatic Gain Control (AGC) turned on. The MS/MS data were first evaluated against several internal quality control (QC) standards. After passing the QC standards, the MS/MS data was loaded into the proprietary ProtQuest search engine to search the most recent non-redundant protein database. Then, the results from the ProtQuest search were manually analyzed.

The endoproteinase trypsin (sequencing grade) was obtained from Promega or Roche (Indianapolis, IN). Ammonia bicarbonate (analytical grade) and HOAC (>99.8% purity) were obtained from Sigma. ACN, methanol (MeOH), and water were of HPLC grade and were obtained from Sigma (St. Louis, MO).

Table 1
Baseline characteristics of acute ischemic stroke patients

Age (y)	58 ± 17
Male (%)	18 (56%)
Female (%)	14 (44%)
Hypertension (%)	19 (59%)
Tobacco addiction (%)	4 (12%)
Diabetes mellitus (%)	13 (41%)
Current smoking (%)	8 (25%)
History of coronary artery disease (%)	10 (31%)
Previous stroke history (%)	8 (25%)
Rheumatic disease (%)	3 (9%)

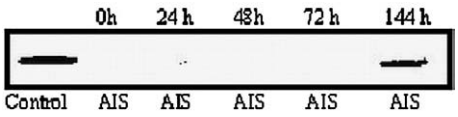

Fig. 1. Protein electrophoretogram of serum samples from acute ischemic stroke (AIS) patients that were collected at 0, 24, 48, 72, and 144 h.

2.5. Production of antibody to ITIH4

Female rabbits were produced sites along the back with eluted ITIH4 protein antigen (200 g/ml in PBS), which was emulsified in an equal volume of complete Freund's adjuvant. Equivalent booster doses were given at 4-week intervals (Banglore Genei, Banglore, India). Blood was collected from the marginal ear vein 2 weeks after each booster dose. Serum was obtained from this blood by allowing it to clot at room temperature for 3 to 5 h, followed by overnight storage at 4 °C.

2.6. ITIH4 estimation

Indirect enzyme-linked immunosorbent assays (ELISA) were performed as described by Kashyap et al. In brief, 100 µl (1:100) serum samples taken from stroke patients at each time point were added to individual microtiter wells and blocked with 2.5% BSA in PBS. After washing with PBS, the polyclonal antibody against ITIH4 protein was added, and plates were incubated at 37 °C for 60 min. The wells were washed, followed by addition of the secondary antibody (goat anti rabbit immunoglobulin G-Horseradish peroxidase; IgG-HRP) and incubation for 60 min at 37 °C. After another wash, antibody reactivity was detected via the addition of 100 µl tetramethylbenzidine-hydrogen peroxide (TMB/H_2O_2) substrate solution to the wells, which were then incubated at room temperature for about 20 min. The reaction was stopped with 100 µl of 2.5 N H_2SO_4, and the absorbance of each well was read at 450 nm.

2.7. NSE and S-100 ßß estimation

An *in vitro* assay to quantitatively determine the protein NSE/S-100 ββ concentration in human serum was performed as per the manual instructions (Can Ag NSE/ S-100 EIA; Sweden).

2.8. ELISA assessment of cytokine production

ELISAs were used to measure the cytokines produced (used according to manufacturer's instructions; BenderMed System, Austria). In brief, anti-cytokine interleukin (IL)-2 and anti-IL-10 monoclonal coating antibodies were adsorbed onto microwells. After 2 h of incubation at room temperature, wells were washed and blocked with 0.5% BSA/PBS. After blocking for 1 h at room temperature, the serum sample and a biotin-conjugated, anti-cytokine antibody were added to the coated wells. Following another 2 h of incubation, streptavidin-HRP was added to bind to biotin-conjugated anti-cytokine. After 1 h of incubation, streptavidin-HRP was removed during washes, and substrate solution reactive with HRP was added to the wells. A coloured product was formed in proportion to the amount of cytokine present in the sample. The reaction was terminated by the addition of 4 N H_2SO_4, and the absorbance was measured at 450 nm.

2.9. Statistical analysis

Quantitative data have been analyzed using the χ^2 test to compare the AIS and control groups. Data are expressed as means ± SD for assessing the differences at different time points. Differences between groups at a given time were assessed with the Mann–Whitney-*U*-test. S-100, NSE, ITIH4, IL-2, and IL-10 values at different time intervals were compared with controls using the Med Calc Software. *P*-values <0.05 were considered to be statistically significant.

3. Results

Table 1 shows the Baseline characteristics of the AIS patients at the time of admission.