β2-Microglobulin is potentially neurotoxic, but the blood brain barrier is likely to protect the brain from its toxicity

Sofia Giorgetti1, Sara Raimondi1,2, Silvia Cassinelli1,2, Monica Bucciantini3,4, Massimo Stefani3,4, Gina Gregorini5, Giulia Albonico6, Remigio Moratti6, Giovanni Montagna7, Monica Stoppini1,8 and Vittorio Bellotti1,2,8

1Department of Biochemistry, University of Pavia, 2Laboratori di Biotecnologie, IRCCS Policlinico San Matteo, Pavia, 3Department of Biochemistry and 4Research Centre on the molecular basis of neurodegeneration (CIMN), University of Florence, Florence, 5Reparto di Nefrologia e Dialisi, Ospedale Civile di Brescia, Brescia, 6Servizio di Analisi Chimico Cliniche, IRCCS Policlinico San Matteo, 7Salvatore Maugeri Foundation, IRCCS, Rehabilitation Institute of Pavia, Division of Nephrology and Haemodialysis, Pavia and 8Consorzio Interuniversitario Istituto Nazionale Biostrutture e Biosistemi (INBB), Roma, Italy

Abstract

Background. In dialysis-related amyloidosis, β2-microglobulin accumulates as amyloid fibrils preferentially around bones and tendons provoking osteoarthritis. In addition to the pathologic role played by the amyloid fibrils, it can be speculated that a pathogenic role is also played by the high concentrations of soluble β2-microglobulin because it is toxic for certain cell lines like HL60 and mitogen for other cells such as the osteoclasts. The discovery that β2-microglobulin can influence the biology of certain cells may lead to the assumption that it might affect neuronal cells that are quite sensitive to amyloidogenic proteins in the oligomeric state. Such a concern might be supported by clinical evidence that haemodialysis is associated with the risk of a cognitive impairment.

Methods. The cytotoxicity of β2-microglobulin on the SH-SY5Y neuroblastoma cells was assayed by the MTT test. The β2-microglobulin concentration was determined in the cerebrospinal fluid of four different patients by means of immunonephelometry and western blot.

Results. Oligomeric β2-microglobulin is cytotoxic for the SH-SY5Y cells at a concentration that can be easily reached in the plasma of patients on haemodialysis. However, the β2-microglobulin concentration, measured in the cerebrospinal fluid of a haemodialysis patient, appears to be independent of its plasma concentration and is maintained under the lower limit of cytotoxicity we have determined in the cell culture.

Conclusions. Although β2-microglobulin is potentially neurotoxic, it is unlikely that this protein plays a role in the pathophysiology of cognitive impairment observed in haemodialysis patients due to the protective effect of the blood brain barrier that maintains a low concentration of β2-microglobulin in the cerebrospinal fluid.

Keywords: blood brain barrier; cerebrospinal fluid; cytotoxicity; dialysis-related amyloidosis; oligomers

Introduction

The plasmatic level of β2-microglobulin (β2-m) dramatically increases during haemodialysis and remains chronically high throughout the duration of the treatment [1]. Other conditions, such as lymphoproliferative diseases, are associated with increased plasma levels of β2-m, though without reaching the persistently high levels typically found in haemodialysis patients. The main concern with the high concentration of β2-m relates to its capacity to generate amyloid deposits that are mainly located in the musculoskeletal system, although other organs (but not the brain) can be affected. The preferential deposition of fibrils over tendons and bones is most likely mediated by the interaction of β2-m with collagen and heparin [2,3].

β2-m isolated from the natural fibrils corresponds to the full-length unmodified polypeptide chain associated with partially oxidized and deamidated minor species [4]. A minor, but highly pathogenic, component of the fibrils is an N-terminal truncated form that is not detectable in the plasma [5]; conversely a form cleaved at Lys58 can be detected in the plasma of dialyzed patients [6] but has not been found in amyloid fibrils [5].

Most systemic amyloidoses generally spare the brain with the exception of some variants of TTR, characterized by a selective meningeal involvement [7,8]. Although in β2-m amyloidosis the brain is not affected by amyloid deposition, the central nervous system (CNS) is most likely damaged during the haemodialytic procedure; as a matter of fact a statistically significant increase of cognitive impairment is reported in patients requiring chronic haemodialysis [9]. Up until now, the pathological basis of the cognitive impairment has been ascribed to the high susceptibility of
these patients to atherosclerosis and haemodynamic abnormalities, but other factors, including a direct role of β2-m, have been proposed [9]. The effects of β2-m on different cells are pleomorphic; β2-m can induce apoptosis in some cell lines such as HL60 [10], whereas it can stimulate osteoclastogenesis when added to the colony-forming unit of granulocytes and macrophages [11].

In this paper, we report the effect of β2-m and its oligomers arising early in the path of fibrillization on a cultured SH-SY5Y neuroblastoma cell line. We found that β2-m oligomers (but not the monomers) are cytotoxic, as revealed by the reduction of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, a general indicator of cell physiological stress. We also determined a dose–response curve of β2-m cytotoxicity. The latter triggered the apoptotic, rather than the necrotic, cell death, as assessed by the caspase-3 and LDH assays, respectively. Finally, we determined the β2-m cytotoxicity. The latter triggered the apoptotic, rather than the necrotic, cell death, as assessed by the caspase-3 and LDH assays, respectively. Finally, we determined the β2-m concentration in the cerebrospinal fluid (CSF) of patients displaying haemodialysis patient, and found very low values when compared with the corresponding plasma values. These data, never reported in the literature so far, are of relevance on the issue of the toxic activity of β2-m in the CNS. Our findings support the idea that β2-m, in its oligomeric form, is potentially toxic to neuronal and maybe other brain cells, but that, possibly due to the blood brain barrier, β2-m never reaches in the CSF concentrations as high as needed to nucleate aggregation into early toxic oligomers.

Methods

Production of recombinant β2-m

Expression and purification of recombinant β2-m was carried out as previously reported [12]. The experimentally determined average molecular mass of the purified protein was consistent with the calculated mass for β2-m with an N-terminal methionine residue. Natural β2-m purified from urine was supplied by CALBIOCHEM (LaJolla, CA, USA) (cat. no. 475823).

Inhibition of MTT reduction

Human SH-SY5Y neuroblastoma cells were obtained from A.T.C.C. (Manassas, VA, USA) and cultured in 1:1 Ham’s F-10:DMEM medium. Aggregate cytotoxicity was assessed by the MTT reduction inhibition assay. Mitochondrial dehydrogenase cleavage of MTT was used to determine cell survival in quantitative colorimetric assays. Cells were plated on 96-well plates at a density of 6000 cells/well in 200 µL of fresh medium. After 72 h, the cells were exposed for 24 h to different concentrations of β2-m (in the 0.1–20.0 µM range) or with vehicle for control. At the end of the incubation, the cell culture medium was removed and the cells were incubated for 2 h with 100 µL of a 0.5 mg/mL MTT solution in DMEM without phenol red. Then, the cell lysis solution (100 µL/well; 20% SDS, 50% N,N-dimethylformamide) was added and the samples were kept overnight at 37°C in a humidified incubator. The blue-coloured formazan produced from MTT cleavage by active mitochondria dehydrogenases and dissolved in the lysis buffer was quantified spectrophotometrically at 570 nm using an automatic plate reader (Bio-Rad, Hercules, CA, USA).

LDH release

Membrane integrity in cells exposed to β2-m was evaluated by measuring LDH release to the culture medium. LDH activity was measured by the CytoTox-ONE(tm) Homogeneous Membrane Integrity Assay, according to the instructions provided by the manufacturer. The assay couples the diaphorase-catalyzed reduction of resazurin to resorufin to the LDH-catalyzed oxidation of lactate to pyruvate. The intensity of the resulting fluorescence (560 Ex/590 Em) is a measure of the LDH activity in the sample. Briefly, the cells, plated and cultured as described for the MTT reduction assay, were treated for 24 h with 10 µM β2-m or with vehicle for control. The reagent was added to the culture medium, and the increase in fluorescence emission was measured after 2 h using a Fluoroskan Ascent FL fluorescence plate reader (Thermo Scientific, Waltham, MA, USA). For each sample, the fluorescence value was normalized with respect to the ‘total LDH activity’ determined after cell lysis.

Caspase-3 activity

Cells were exposed for 24 h to 10 µM β2-m, or with vehicle for control, lysed with a 20 mM Tris–HCl buffer, pH 7.4, containing 250 mM NaCl, 2.0 mM EDTA, 0.1% Triton X-100, 50 µg/mL leupeptin, 5.0 µg/mL aprotinin, 0.5 mM PMSF, 4.0 mM vanadate, 1.0 mM DTT for 20 min in ice. Lysis was completed by sonication, and total protein content was determined in the clarified lysate using the Bradford reagent. Then, 50 µg of total protein was diluted in 0.5 mL of 50 mM HEPES–KOH buffer, pH 7.0, containing 10% glycerol, 0.1% 3-[3-cholamidopropyl]-dimethylammonium]-1-propane sulfonate, 2.0 mM EDTA, 10 mM DTT, in the presence of 50 µM of the caspase-3 fluorometric substrate Ac-DEVD-AMC. The incubation was carried out for 2 h at 37°C, and fluorescence values (380/460 nm) were recorded at the end of the incubation. To determine non-specific substrate degradation, the same assay was carried out by pre-incubating samples for 15 min at 37°C in the presence of 100 nM of the caspase-3 inhibitor Ac-DEVD-CHO before substrate addition.

Statistical analysis

The Dunnett multiple comparisons test was performed on data coming from MTT, LDH and caspase-3 assays.

β2-m assay

Quantitative determination of β2-m in samples was performed on the BNII System (Dade Behring, Marburg, GmbH, D) by means of particle-enhanced immunonephelometry. Reference values in blood for this method are up to 1800 mcg/L (<97.5th percentile).
CSF and plasma proteins analysis

Fresh plasma and CSF samples were obtained from a patient on haemodialysis, two patients affected by chronic lymphatic leukaemia (CLL) and a subject without systemic diseases in which the CSF specimen was obtained in the course of spinal anaesthesia for orthopedic surgery. The two patients affected by CLL were in stages I and II according to the Rai classification and were submitted to lumbar puncture following episodes of vomiting and headache in order to exclude the CNS involvement. The CNS involvement was then excluded because the cell number was normal, no clonal population was detected and the protein content as well as the IgG–albumin index was normal.

An aliquot of 6.25 µL of plasma was mixed with 10 µL of a solution containing SDS (10% w/v) and DTE (2.3% w/v). The sample was heated to 95°C for 5 min and then diluted to 500 µL with a solution containing 8 M urea, 4% w/v CHAPS, 40 mM Tris, 65 mM DTE and a trace of bromophenol blue. Sixty microlitres (45 µg) of the final diluted plasma sample was loaded on the immobilize strip for the first dimension separation in 2D-PAGE. The protein separation in 2D-PAGE was performed as previously described [4].

Two hundred fifty microlitres of CSF was mixed with 500 µL of ice-cold acetone and centrifuged at 10 000 g at 4°C for 10 min. Subsequently, the pellet corresponding to 60 µg of total proteins was diluted with 350 µL of the solution used for plasma and then processed using the same procedure described for plasma samples.

SDS-PAGE and immunoblotting

SDS–PAGE was performed according to Laemmli [13] using 15 µL of plasma and CSF samples were diluted 1:10. After SDS–PAGE or 2D-PAGE, the proteins in the gel were transferred onto a PVDF membrane and analysed as previously described [14,4].

Results

β2-m aggregates are cytotoxic

The cytotoxicity of β2-m was studied using human neurotypic SH-SY5Y neuroblastoma cells previously shown to be sensitive to the toxicity of amyloid protein aggregates [15,16]. The biological effect of β2-m was assayed by the MTT reduction assay. In viable cells, MTT undergoes reduction by mitochondrial dehydrogenases (sucinate–tetrazolium reductase system) to insoluble formazan, which serves as an indicator of the amount of metabolically active cells; we found that MTT reduction in the exposed neurotypic SH-SY5Y cells decreased with increasing protein concentrations reaching a maximum toxic effect of 75 ± 5% (n = 21) in cells treated for 24 h with 20 µM β2-m with respect to the untreated control cells. Figure 1a shows the decrease of SH-SY5Y cell viability after this treatment. Our data indicate a dose dependence of the cytotoxic effect in the assayed β2-m concentration range (1–20 µM) with an IC50 value ~4.6 µM under our experimental conditions. All the experiments have been carried out with recombinant β2-m. However, in order to exclude any possible difference with natural β2-m, the evaluation of toxicity at a β2-m concentration of 10 µM was also carried out with β2-m purified from urine and the results were perfectly reproduced.

It is worth noting that sample cytotoxicity was abrogated by protein filtration through 0.02 µm Anopore™ nanofilters (Whatman Inc., UK), which efficiently remove β2-m oligomers from the solution, as it was previously assessed by dynamic laser light scattering [17]. Moreover, aggregates formed again progressively in the filtered β2-m sample stored at 25°C, and after 4 days DLS measurements of the aged sample were the same as those obtained before filtering [17]. The filtered and aged protein sample was endowed with the same cytotoxicity to the SH-SY5Y cells as that of the fresh, non-filtered β2-m sample (Figure 1b). These evidences suggest that these β2-m oligomers, present under physiological conditions in the β2-m solution, are in dynamic equilibrium with the monomer and play a key role as triggers of cell damage and death.

Next, we sought to ascertain whether cell damage by β2-m oligomers triggered a necrotic or an apoptotic response. The possible necrotic cell death was investigated by measuring LDH release from the cells treated with 10 µM β2-m for 24 h (Figure 1c). We found a modest (15%) increase of LDH release as compared to the controls in cells treated with β2-m. The apoptotic activation was measured by assaying the caspase-3 activity in the SH-SY5Y cells exposed to the same amount of β2-m for 24 h. We found a 40% increase in caspase-3 activity in the cells treated with 10 µM β2-m with respect to the vehicle-treated controls (Figure 1d).

Determination of the β2-m concentration in the CSF

We determined the β2-m concentration in the CSF of patients with different plasma concentrations of β2-m (Figure 2). Patient A displayed the highest level of β2-m in plasma; he was under chronic haemodialysis for 2 years because of end-stage renal failure due to AA amyloidosis associated with Familial Mediterranean fever. From the onset of haemodialysis, the plasma concentration of β2-m has been monitored several times and it was always in the range of 20–40 µg/mL (1.7–3.4 µM). The value reported here corresponds to the day in which the patient was submitted to a lumbar puncture justified by acute onset of the comatose state in the context of multi-resistant staphylococcus aureus sepsis due to pneumonia. The CSF examinations (standard tests and cultures) were negative, and the comatose state resolved spontaneously in a few days. Patients B and C are affected by lymphatic leukaemia in different stages of the disease, which is characterized by a significant increase of the plasma β2-m concentration, such that the protein is even considered a biomarker of the disease severity [18]. Finally, patient D can be considered a non-diseased subject, with normal β2-m levels in plasma and CSF. In order to exclude that any artefact related to the assay performed on different biological fluids might affect the consistency of the assay, we have analysed the plasma and CSF of the haemodialysis patient by western blot after SDS–PAGE. Direct visualization of the immunostained bands (Figure 2) confirmed the discrepancy in the β2-m concentration between plasma and CSF. These data suggest that the blood brain barrier might
Cytotoxicity of β2-microglobulin

Fig. 1. Cytotoxicity of β2-m on neuroblastoma cell line. (a) Concentration-dependent cytotoxic effect of β2-m (filtrated or not filtrated) after 24 h of cell treatment was measured by the MTT assay as described in the Methods section. The percentage of SH-SY5Y cell viability compared to untreated controls cells is shown on the y-axis. (b) Cytotoxic effect of β2-m filtrated and aged 1 week. (c) Cell death was determined by using the LDH assay. LDH release from SH-SY5Y cells 24 h treated with 10 µM of β2-m. The percentage of LDH release is shown on the y-axis. Untreated cells (cn) were used as a negative control, while cells treated with TritonX-100 were used as a 100% positive control. (d) Evaluation of SH-SY5Y apoptosis after 24-h cell exposure to β2-m. The cells' extracts were used to measure the caspase-3 activity by using the fluorescent substrate Ac-DEVD-AMC. Data obtained from three different experiments carried out under identical conditions were normalized with respect to the absorbance of controls (cells not treated) that were taken as 100%.

protect against the increase of β2-m in the CSF following marked increase in the plasma; however, if we take into account that β2-m is also produced in the CNS [19], the lack of β2-m accumulation in the CSF during haemodialysis shows up the existence of an efficient system of removal of β2-m from liquor even against a high concentration gradient. To verify whether brain β2-m might be post-translationally modified in CSF or proteolytically degraded in a peculiar way, we carried out a 2D gel electrophoresis followed by an immunoblot (Figure 3). This technique was previously used to identify proteolysed species of β2-m, such as the N-truncated amyloidogenic form (ΔN6β2-m) or that cleaved at position 58 [5]. Apparently β2-m in the CSF and plasma has the same characteristics in term of molecular weight, isoelectric point and charge, and we are unable to detect any fragment or polypeptide cleavage form. The three main spots of Figure 3 have mobility consistent with the previously characterized forms oxidized at Met 99 (intermediate band) and deamidated species (most acidic) in which Asn17 is converted into Asp [4].

Discussion

The data presented in this study confirmed the cytotoxic activity of β2-m in vitro in a dose-dependent fashion [10–20] and in particular its pro-apoptotic activity on neuronal cell lines. The minimal concentration at which β2-m is still cytotoxic is above 1 µM. These data are consistent with previous observations derived from the investigation of β2-m cytotoxicity on the HL-60 human leukaemia cell line in which the IC50 was ∼1 µM [10]. Moreover, when the SH-SY5Y cell line was tested, the IC50 of β2-m was ∼4.6 µM. The removal of β2-m oligomers through filtration of the protein samples with a 20 nm pore size filter abrogated the cytotoxic effect of β2-m. These data suggest that the monomeric form is not toxic and emphasize the importance of the oligomeric species of the protein in determining the pathogenetic effect of β2-m.

The discovery that β2-m is cytotoxic for the neuronal cell line would cause concern regarding the possible role of β2-m in causing the cognitive impairment associated with haemodialysis. However, because the cell toxicity of β2-m is dose dependent, we considered worthy of interest to obtain information about the concentration of β2-m in CSF of patients presenting a persistently high concentration of β2-m in plasma. In fact, although the β2-m concentration has been previously measured as a marker of inflammatory diseases affecting the brain [21], it was never measured from the perspective of evaluating the effect of the blood brain barrier in determining the plasma/CSF ratio. In particular, we have extensively searched in the literature the
information regarding the concentration of β2-m in CSF of patients under haemodialysis, but, to our knowledge, these data were never reported. We have been able so far to measure β2-m in the CSF only in one dialysis patient; however, the results obtained with this single patient are comparable with those obtained in other two patients with normal renal function but a high level of β2-m in plasma. These data suggest that an efficient system that permits the elimination of β2-m from CSF also when its concentration in the plasma is very high is present in the brain. We have demonstrated that β2-m in CSF has the same physical and chemical characteristics as the protein present in the plasma; furthermore, no fragment was detected. It is likely that β2-m produced in the brain is eliminated through the generic system of arachnoid villi, in which the latter acts as ‘one way valve’ and the pressure difference between the CSF and dural venous sinuses provides the dominant driving force for CSF protein absorption. This system apparently works well against a gradient of concentration, in fact it is able to transfer into the plasma also proteins with a plasma/CSF concentration

<table>
<thead>
<tr>
<th>patient</th>
<th>plasma mg/L</th>
<th>μM</th>
<th>CSF mg/L</th>
<th>μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>22</td>
<td>1.88</td>
<td>2.06</td>
<td>0.17</td>
</tr>
<tr>
<td>B</td>
<td>9.06</td>
<td>0.76</td>
<td>1.58</td>
<td>0.13</td>
</tr>
<tr>
<td>C</td>
<td>6.25</td>
<td>0.54</td>
<td>2.21</td>
<td>0.18</td>
</tr>
<tr>
<td>D</td>
<td>2.05</td>
<td>0.17</td>
<td>1.05</td>
<td>0.089</td>
</tr>
</tbody>
</table>

**Fig. 2.** Comparative assay of β2-m in plasma and CSF in a patient on haemodialysis (A), two patients affected by chronic lymphatic leukaemia (B, C) and a subject with a normal level of β2-m in plasma (D). Western blot immunostained with a polyclonal anti-human β2-m antibody after SDS–PAGE of plasma and CSF of subjects A (lanes 2 and 4) and D (lanes 1 and 3); lane 5: molecular weight standards. Detection of β2-m was performed using a chemiluminescent procedure (ECL plus western blotting detection reagent, Amersham Bioscience).

**Fig. 3.** Western blot developed with a polyclonal anti-human β2-m antibody after 2D-PAGE of CSF (panel A) and plasma (panel B) of patient A. Detection of β2-m was performed using a chemiluminescent procedure.
ratio > 1. Apparently, a plasma concentration of β2-m, even 10-fold higher than in CSF, does not affect the β2-m re-absorption.

In conclusion, in this report we have shown for the first time that the concentration of β2-m in CSF of haemodialysis patients is similar to that found in subjects with normal renal function, demonstrating that the blood brain barrier and the system of CSF protein absorption are capable of maintaining the β2-m concentration under the minimal level required for the cytotoxicity.

Acknowledgements. This study was supported by Fondazione Cariplo (Progetto Nobel and project number 2007-5151); Italian MIUR (PRIN 2006058958, FIRB RBNE03PX83), Ministero della Salute progetto stra-gico Malattie Rare; EU grant EURAMY, Regione Lombardia and Ente Cassa di Risparmio di Firenze. We are indebted with Giampaolo Merlini and Laura Obici for their precious suggestions.

Conflict of interest statement. None declared.

References

Received for publication: 1.7.08
Accepted in revised form: 15.10.08