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Correlation between circulating proteasome activity, total protein and c-reactive protein levels following burn in children

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ABSTRACT

Aim of the study: To characterize burn-induced changes following burn in children by analyzing circulating proteasome (c-proteasome) activity in the plasma in correlation with total protein and c-reactive protein levels in the plasma, and the severity of the burn.

Methods: Fifty consecutive children scalded by hot water who were managed at the Department of Pediatric Surgery after primarily presenting with burns in 4–20% TBSA were included into the study. The children were aged 9 months up to 14 years (mean age 2.5 ± 1 years). Patients were divided into groups according to the pediatric injury severity score used by American Burns Association. Plasma proteasome activity was assessed using Suc-Leu-Leu-Val-Tyr-AMC peptide substrate, 2–6 h, 12–16 h, 3 days, 5 days, and 7 days after injury. 20 healthy children consecutively admitted for planned inguinal hernia repair served as controls.

Results: Statistically significant elevation of plasma c-proteasome activity was noted in all groups of burned children 12–16 h after the injury. We found a strong negative correlation of c-proteasome activity with total protein levels, and positive correlation with CRP levels 12–16 h after burn. We also found stronger correlation between c-proteasome activity and severity of burn, than CRP level and severity of burn 12–16 h, and 3 days after the burn. Correlations were statistically significant.

Conclusions: This study characterized circulating 20S proteasome activity levels after burn. C-proteasome activity elevate after burn and correlate negatively with plasma total protein level, thus plasma 20S proteasome activity could be additional biomarker of tissue damage in burn in pediatric population.

1. Introduction

Burn is associated with negative nitrogen balance and whole-body protein loss, mainly reflecting a catabolic response in skeletal muscle to severe burns [1]. The stimulated protein degradation, in particular myofibrillar protein degradation, is the most important component of muscle catabolism following burn [1]. Intracellular protein breakdown is regulated by multiple proteolytic pathways, including lysosomal, calcium-dependent and ubiquitin–proteasome-dependent pathways; the latter is the most important energy dependent proteolytic

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mechanism [2]. In this proteolytic pathway, proteins are marked with polyubiquitin chain, and then directed to the 26S proteasome complex for the degradation [2,3]. The catalytic core of this complex, the 20S proteasome is a barrel-shaped particle composed of four stacked rings [2,3]. Each ring consists of seven subunits, the outermost of which are called α-subunits and the innermost of which are called β-subunits. The hydrolytic sites are located on three β-subunits [3]. Continued muscle protein breakdown by proteasomes results in muscle wasting and fatigue, which may impair recovery and lead to an increased risk of thromboembolic and pulmonary complications if ambulation is delayed and respiratory muscles are affected [4]. Recently, proteasomes have been detected in normal serum or plasma, and elevated circulating proteasome (c-proteasome) concentrations or activity have been described in patients with various pathological conditions, e.g. hematologic malignancies, autoimmune disorders and critical illness [5,6].

The purpose of this novel human study was to characterize burn-induced changes following burn in children by analyzing circulating proteasome activity in correlation with total protein and c-reactive protein levels in the serum, and the severity of the burn.

1.1. *Patients*

The study was approved by the local Ethics Committee. The study population comprised 50 patients with burns who were consecutively admitted to the Department of Pediatric Surgery of Medical University of Bialystok between 2010 and 2012, and whose parents gave informed consent for both clinical and biochemical follow-up. Patients were aged 9 months to 14 years (median = 2.07 ± 1.91 years). There were 16 girls and 34 boys. Patients were divided into three groups depending on the severity of the injury according to American Burns Association: first group – children with minor burns $n = 16$ (<5% TBSA [TBSA – total body surface area] burn, <2% full thickness burn), second group – patients with moderate burns $n = 21$ (5–10% TBSA burn, 2–5% full-thickness burn), third group – patients with severe burns $n = 13$ (>10% TBSA burn, >5% full-thickness burn).

Burn patients were managed according to the conventional lines of burn treatment in our department. All patients requiring surgical intervention received standard surgical care and postoperative treatment according to the standard treatment protocols of our clinic. Injury severity was assessed by the percentage of at least second-degree burned TBSA.

Exclusion criteria were hospital admission later than 6 h after injury, severe preexisting infections, other diseases that required long-term medication.

20 healthy children aged 1–14 years (median = 4.36 ± 2.03 years), consecutively admitted to the Department of Pediatric Surgery for planned inguinal hernia repairs between 2010 and 2012 (6 girls, 14 boys), and whose parents gave informed consent for both clinical and biochemical follow-up served as controls.

2. Methods

Venous blood samples (1–2 mL) were drawn after admission, 2–6 h, and 12–16 h after the injury, and in the morning of the subsequent days 3, 5 and 7 along with the routine laboratory work-up. Blood samples were collected in plasma test tubes (EDTA tubes), were prepared according to the standard hospital procedures, and stored at −80 °C until further analysis. After all blood samples were collected and patient data recorded, proteasome plasma concentrations were determined with the investigators blinded to the patient-related data.

The thawed plasma samples were centrifuged to remove fibrinogen, and then each sample was diluted to the protein concentration of 5.0 mg/mL with 100 mmol/L Tris–HCl (pH 7.5) supplemented with inhibitors of other proteases: ethylene diamine tetraacetic acid (EDTA, 1 mmol/L), trans-epoxy-succinyl-leucylamide-(4-guanidino)-butane (E-64, 10 μmol/L) and pepstatin (1 μmol/L) (Sigma, USA). Total protein concentration in plasma samples was determined using the Bio-Rad assay reagent with bovine serum albumin as the standard (the Bio-Rad protein assay is a simple colorimetric assay for measuring total protein concentration using a dye-binding method based on the Bradford assay).

The proteasome activity in the plasma was measured using the assay designed to measure chymotrypsin-like protease activity (ChT-L) of proteasomes. The sodium dodecyl sulfate (SDS) was an artificial proteasome activator for the fluorogenic peptide substrate the Suc-Leu-Leu-Val-Tyr-AMC. The reaction mixture (total volume, 30 μL) contained a 100 mmol/L Tris–HCl buffer (1 mmol/L EDTA, EGTA, 0.05% SDS, pH 7.5), and a 10 μL 0.5 mmol/L Suc-Leu-Leu-Val-Tyr-AMC substrate. 10 μL of non-activated plasma sample or 10 μL of plasma sample activated for 15 min in room temperature with 10% SDS was added to the reaction mixture. To confirm the specificity of the assay, the plasma was pre-incubated with the selective proteasome inhibitor epoxomicin (1.0 μmol/L) for 15 min before the addition of a substrate. The samples were incubated at 37 °C for 30–60 min, since during this time a linear relation between time and product generation was obtained. After incubation, the reaction was stopped by the addition of 1 mL of 100 mmol/L monochloroacetate – 30 mmol/L sodium acetate, and the samples were centrifuged to remove any insoluble material. Fluorescence of the released AMC was determined at 380 nm excitation and 460 nm emission wavelength in a Hitachi F-2000 fluorimeter. The 20S proteasome ChT-L activity was calculated from the differences between the fluorescence in the sample incubated with the substrate and the sample incubated with 0.1% dimethyl sulfoxide (DMSO). One unit of the 20S proteasome ChT-L activity was expressed as the amount of AMC released from the substrate per minute (pmol/min). All assays were performed in triplicates.

For identification of 20S proteasome antigen, a plasma sample (20 μg of total protein per line) was electrophoresed by SDS-polyacrylamide gel electrophoresis (PAGE) in a 10% separation gel under reducing conditions, and then proteins were electroblotted onto nitrocellulose membranes. The nitrocelluloses were incubated for 1 h with the monoclonal antibody reacting with the human β5 subunit (C2) of the 20S proteasome (Affiniti Research Products Ltd., UK). The immunoreactive proteins were visualized with an alkaline phosphate-conjugated anti-rabbit IgG as the secondary antibody, and p-nitrophenyl phosphate as the phosphatase substrate (Sigma, USA). Optical density for 20S beta 5 subunit (A) and