



Original article

Serum and subcellular proteasome in Moroccan patients reached hematological malignancies

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ABSTRACT

Background: The regulated degradation of intracellular proteins plays an essential role in most biological processes, especially in the control of cell proliferation and differentiation. In eukaryotic cells, intracellular proteolysis is ensured largely by a multi-enzyme system, called the ubiquitin / proteasome system. This system involves hundreds of components that allow the recognition and labeling of proteins destined to degradation by the 26S proteasome. The aim of this work was to analyze and establish a link between the concentration of the proteasome and its catalytic activity in both serum and intracellular level in a wide range of patients with different hematological neoplastic in a Moroccan population newly diagnosed. **Methods:** Proteasome levels were measured using a sandwich ELISA assay in normal donors and in patients with Acute and Chronic Leukemia, multiple myeloma, lymphoma. **Results:** The increase of serum proteasome levels was more pronounced in patients with myeloma (7112 ± 733 ng / ml). The catalytic activity of circulating proteasome was very low as well in controls than in patients. The intracellular proteasome has revealed an optimal value in individuals suffering from chronic leukemia (6303 ± 726 ng/ml), followed by patients with multiple myeloma (5910 ± 336 ng/ml). The activity of intracellular chymotrypsin-like of proteasome was more important in the patients. **Conclusions:** Although the serum proteasome level is a potential new tool for the monitoring of patients with liquid cancer, the intracellular assay proves to be beneficial since it allows a dosage at the source and allows estimating the predictive toxicity risk score in patient treatment.

KEYWORDS: Proteasome, moroccan patients, hematological malignancies, subcellular, serum, ELISA technique.

INTRODUCTION

The cellular metabolism is tightly regulated by changes in the concentration of specific proteins. Mammalian proteins, which are responsible for most cell functions, are constantly degraded and replaced by new proteins synthesized in order to renew the stock of active molecules in the cell [1]. This degradation allows the elimination of misfolded from mutations or metabolic damage proteins [2].

The ubiquitin-proteasome system, responsible for the degradation of the majority of cellular proteins involved in the regulation of many biological processes: cell cycle regulation (p21et p27), apoptosis, transcription, antigen presentation, signal transduction, control of protein integrity and angiogenesis [3].

The proteasome exists in different forms in the cell. The 26S proteasome, including the leading role in the biology of eukaryotes, has been clearly established through the development of specific inhibitors [4], is formed by the association of the 20S proteasome (the proteolytic core) with one or two regulatory complexes called 19S complex (or PA700), which joins the ends of the catalytic body [5], [6].

Localized mainly in the nucleus and cytoplasm of eukaryotic cells, the proteasome can be found in peripheral blood (circulating proteasome) from a cell lysis or migration process of prosomal units, first from the nucleus and

cytoplasm passing through the membrane and after they are in the extracellular medium [7].

In oncology, numerous studies have shown that dysregulation of proteasomal degradation of certain proteins can cause disruption of cell proliferation and lead to a process of carcinogenesis [8], [9]. Thus, the proteasome has been identified as being responsible for the ubiquitin-dependent degradation of the tumor suppressor protein p53. Excessive degradation of p53 prevents its activation, which promotes the development of a tumor phenotype [10]. The proteasome has recently acquired a major interest as a drug target for the treatment of certain cancers [11]. Indeed, inhibition of its proteolytic activity has the direct consequence, to induce cell cycle arrest and apoptosis, and, more significantly in cancer cells at the level of normal cells [12].

Moreover in Morocco no study has been performed on the proteasome, even more on the relationship between the proteasome and the onset or progression of a disease given in the Moroccan population.

In this work, we proposed to study the variations of proteasome subcellular and in circulating, in Moroccan patients with liquid cancers disease. The study and analysis of the proteolytic potential of the 20s proteasome (chymotrypsin-like activity) was conducted in parallel with the measurement of concentration of proteasome.

The objective of our work can be summarized in these points:

- 1) To determine whether there were differences in serum proteasome levels between normal samples and samples from patients with different types of neoplastic diseases

- 2) To compare the evolution of the intracellular and serum proteasome in patients with hematological malignancies.
- 3) Research the correlation of the proteasome serum levels and the markers of cell lysis or malignant disease activity (Lacticdehydro-genase [LDH], beta 2-microglobulin, CRP)
- 4) 4)To assess the intracellular and serum activity of chymotrypsin-like both in control subjects and patients with hematological malignancies.

MATERIALS AND METHODS

Patients:

The study included 204 Moroccan patients, including 59 controls, 29 men and 33 women, with a mean age 42 ± 17 years. As well as 145 patients with different forms of hematological malignancies (all newly diagnosis and untreated), whose 69 women and 76 men, with an average age of 47.9 ± 15.3 years, including patients suffering from: Acute lymphoblastic leukemia (ALL, n = 14); Acute Myeloid Leukemia (AML, n = 45); Chronic lymphocytic leukemia (CLL, n = 9); Chronic myeloid leukemia (CML, n = 10) suffering from Multiple Myeloma (MM, n = 18); Non-Hodgkin's Lymphoma (NHL, n = 28); Hodgkin's disease (HD, n = 21) (see Table 1).

The blood sample is recovered, after obtaining consent of all recruited patients between April 2012 and June 2014, to the service of Hematology and Oncology Pediatric - Hospital 20 August Casablanca –Hospital Centre- University IBN ROCHD Casablanca.

Table 1: Clinical characteristics of the study population

Diagnostic	No. of persons	Average age	Male	Female	Untreated	Treatment
Healthy donors	59	42±17	29	33	0	0
Patients						
ALL	14	31.4 ± 14.8	8	6	6	8
AML	45	40.6 ± 16.5	24	21	32	13
CML	10	45.4 ± 13.3	5	5	6	4
CLL	9	58.4 ± 16.2	6	3	4	5
MM	18	60.3 ± 10.3	9	9	10	8
NHL	28	51.8 ± 18.3	12	16	16	12
HD	21	48 ± 18.2	12	9	11	10
Total	145	47.9 ± 15.3	76	69	85	60

ALL : acute lymphoidleukemia; AML : acute myeloidleukemia ; CLL : chroniclymphocyticleukemia ; CML : chronicmyeloidleukemia ; MM : multiple myeloma ; NHL : non-Hodgkin'slymphoma ;HD : Hodgkin'sdisease

Preparation of samples:

The Serum is recovered from a fresh sample of blood human and cooled gradually to 4 ° c and -20 ° c. Lymphocyte layer are recovered by lysis of red cells or by gradient Ficoll.

Lysis of the cells:

Briefly we broke out the red blood cells from the blood sample, by adding a solution of Tris/ EDTA (20/5) after centrifugation, we eliminate the supernatant and repeat this

maneuver until obtaining a clear cell layer. Added quickly 200 µl of Lysis buffer (10 mM NaCl, 10 mM KCl, HEPES 10 mM, EDTA 1 mM PH 7.1, DTT 0.1 mM, 1% Triton, fortified with Anti proteases (PMSF 2 nM) on each sample. Cells are exploded with periods of 20 s break alternated with 30 s rest to avoid the excessive heating of the samples that may cause a denaturation of enzymes. The Protocol is repeated 3 times. The extracts can be stored at -20 ° C until their use [13].

Quantification of the proteasome 20s human by ELISA:

Determination of the serum and intracellular, 20s Proteasome by the technique of Indirect ELISA [14] based on recognition in "sandwich" of complexes of 20s proteasome by specific antibodies of the subunit α of the catalytic core: MCP20 (Enzolifesciences).

First, there is the 'Coating' of the monoclonal Ab MCP20 (1/3000) to the microplate 1h at 37 °c. Non-specific sites are blocked by addition of 200 µl of PBS - BSA 2%. Are deposited the standard range and the samples to be determined (100 µl / well). Subunits of the proteasome captured are detected by second polyclonal Ab I571 (1: 5000). The revelation is made after adding conjugate labeled with peroxidase. After the addition of the substrate (OPD), is the follow-up to the OD at 492 nm on an ELISA reader (ELx800 UV). The results are expressed in concentration of Proteasomes in: ng/ml, after comparison with the standard curve obtained with the standard of the proteasome 20s purified human range.

Catalytic activity of the Proteasome (Chymotrypsin-like):

Catalytic activity (chymotrypsin-like) of the 20s proteasome, is determined by measuring substrate

RESULTS

Quantification of the level of the serum Proteasome 20S in patients with hematological malignancies:

Serum Proteasome measured by ELISA, in 145 patients (with an average age 47.9 ± 15.3 years) suffering from malignant Hemopathy is represented in fig. 1.

It is observed that the serum level of the 20s proteasome is high in all forms of malignant hematologic diseases included in the study, compared to the control value (1624 ± 601 ng/ml); a growing evolution of proteasome circulating is observed in patients with acute leukemia (LAM and LAL): 4416 ± 980 ng/ml; $p < 0.0001$, followed by patients suffering from chronic leukemia (CML and LLC): with a serum of 4491 ± 174 ng/ml; $p < 0.0001$, patients with Lymphoma (Hodgkin's and non-Hodgkin) reported a serum from: 5337.5 ± 663 ng/ml; $p < 0.0001$. Also note that patients of Multiple Myeloma (MM) have the average concentration of the highest serum Proteasome noted during the study (7112 ± 733 ng/ml, $P < 0.0001$).

Correlation between the serum concentration of Proteasome and some biological parameters in patients with hematologic malignancy:

Lactate dehydrogenase (LDH):

Analysis of the correlation between the rate of Lactate Dehydrogenase (LDH), and the concentration of the

fluorogenic (AMC) released after degradation of the peptide Succinyl-leu-leu-val-tyr-amido-4-methyl-Coumarin "Suc-LLVY-AMC" (Sigma Aldrich), after 3 h of incubation of the substrate (40 µM) in Proteasome assay Buffer (25 mM HEPES (pH 7.5), 0.5 mM EDTA), with the same volume of the sample containing the proteasome (serum or cell lysate) at 37 ° C [15].

The fluorescence emitted after cleavage of the AMC is measured on a Fluorometer (Heofer Scientific Instruments). The excitement of peptides, coupled at the AMC, is done through a 360/40 nm filter that the wavelength is between 340nm and 380nm with a maximum at 360nm. The fluorescence emitted after digestion of peptides coupled to the AMC reads on the 460/40nm filter. To convert the unit of fluorescence (UF) issued µmol of used fluorophores, a standard AMC range established from a stock solution of AMC at 10 - 3 m. The excitement of the AMC is done at a wavelength of 360/40 nm and the signal is then recovered on the 460/40 nm filters.

Other biological parameters analyzed:

In Patients with hematological malignancies, three biological parameters were tested and analyzed, in order to establish possible statistical correlation with the level of circulating and intracellular 20s proteasome: Lactate Dehydrogenase (LDH) as global marker of cytolysis, C - reactive protein (CRP), which reflects the level of movement of Interleukin-6, and $\beta 2$ - micro globulin.

Statistics: Comparison of proteasome 20s serum and intracellular concentrations, appealed to the STUDENT'S test.

Proteasome in patients with hematologic malignancies, shows a significant correlation ($R^2 = 0.9265$; $P < 0.005$) (fig. 2).

Precisely a very strong correlation is observed in patients with multiple myeloma ($R^2 = 0.9433$; $p < 0.005$).

CRP and β -2 microglobulin:

Analysis of the correlation between CRP (mg/l) and β -2-microglobulin (mg/l), and the rate of circulating proteasome (ng/ml) in patients with hematologic Cancers, reports no significant correlation ($R^2 = 0.0063$), ($R^2 = 0.0141$).

Quantification of the level of intracellular Proteasome 20S in patients with hematological malignancies:

The rate of intracellular Proteasome measured of by ELISA assay, in 145 patients (47.9 ± 15.3 years), reached to hematological malignancies is represented in fig. 3.

We note that all patients with hematological malignancies have rates of intracellular proteasome significantly ($P < 0.0001$) higher than the control subjects (2949 ± 223 ng/ml). However, patients of chronic leukemia (LLC and CML) exhibit the highest intracellular level (6303 ± 726 ng/ml), followed by patients with Multiple Myeloma: (5910 ± 336 ng/ml). Patients with acute leukemia (ALL and AML) reports rate of intracellular proteasome: 5363 ± 369 ng/ml. Patients with Lymphoma (NHL and MDH) displayed a rate of intracellular proteasome from: 5026 ± 827 ng/ml.

Figure 1: Evolution of serum Proteasome (ng/ml) by type of Pathology

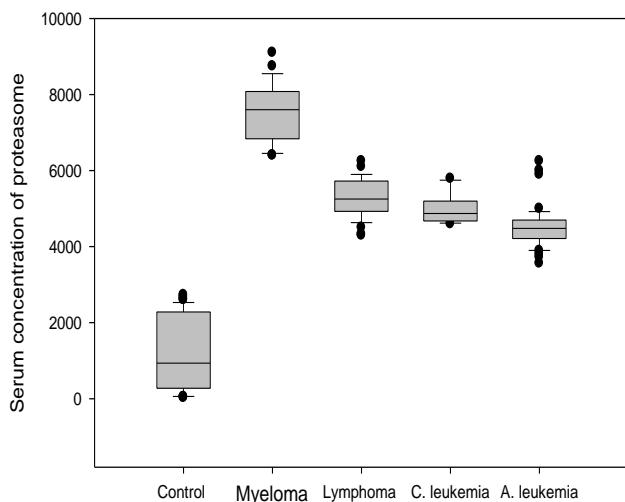
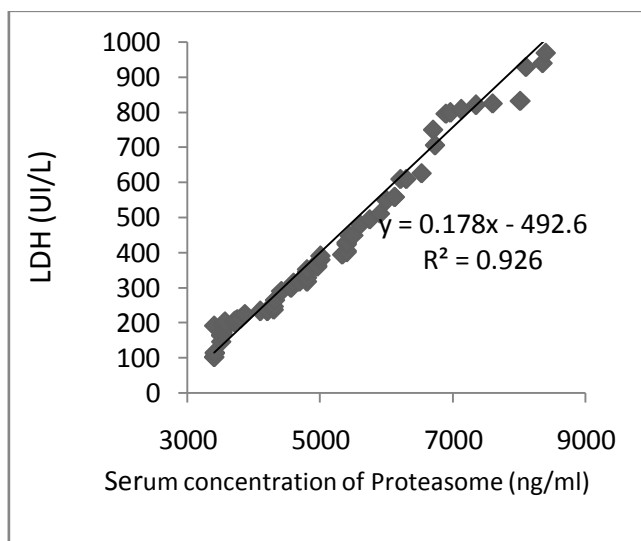


Figure 2: Correlation between serum proteasome rate and level of LDH in cancer patients (liquid tumors)



Evaluation of catalytic activity of the Proteasome:

The results of the activity of level of chymotrypsin-like in serum and intracellular in control subjects and in patients with hematological malignancies, is represented in fig.4. In all subjects, the chymotrypsin-like activity is significantly higher at the intracellular level (control 1168 ± 96 UF, P < 0.001; patients: 1803 ± 120 UF, P < 0.0001) in comparison to the activity recorded at serum level (control: 491 ± 96 UF; patients: 630 ± 69 UF).

The diseased subjects expressed a chymotrypsin like significantly greater compared to controls both activities at

Figure 3: Evolution of the intracellular levels of Proteasome (ng/ml) by pathology

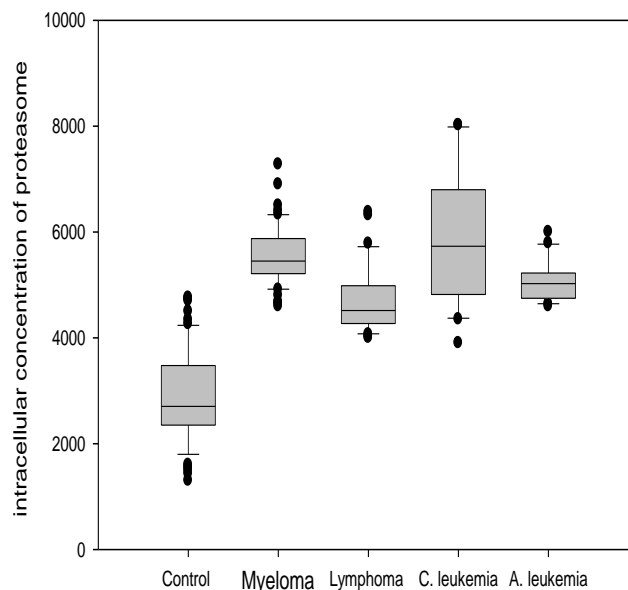
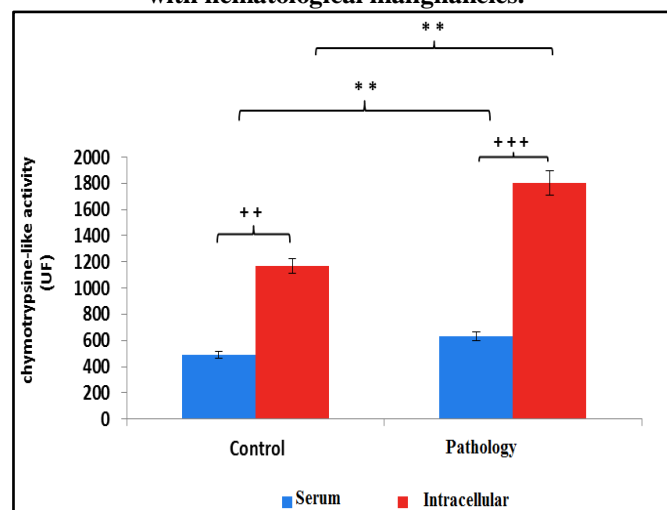


Figure 4: Quantification of chymotrypsin-like activity in intracellular and serum in control subjects and patients with hematological malignancies.



** P < 0.001; **+: P < 0.0001 (comparison between serum and intracellular proteasome in healthy subjects), *: P < 0.001: comparison between control and sick

serum level (control: 491 ± 96 UF / patients: 630 ± 69 UF; P = 0.0003) and intracellular (control: 1168 ± 96 UF; Patient: 1803 ± 120 UF; p < 0.001).

▪ Evaluation of catalytic activity of serum and intracellular Proteasome in various pathologies:

Table 2 represents the catalytic activity of the proteasome in patients with various forms of hematological malignancies, compared with controls. Proteasome chymotrypsin-like activity (serum) was significantly increased in all patients, with a rate of proteolysis more pronounced in patients with LA (activity of 785 ± 99 UF, p <

0.001). No significance was observed in patients with LC. At the intracellular level, proteolytic activity is identical between different pathologies but significantly ($p < 0.0001$) higher in comparison to the controls.

Correlation between the activity rate and the concentration of intracellular proteasome: In order to follow the evolution of the chymotrypsin-like activity of

intracellular proteasome, we investigated the correlation between these two parameters in the various forms of hematological malignancies. We observe a strong correlation between the rate of intracellular proteasome (ng/ml) and its catalytic activity (chymotrypsin-like) in UF, for patients with Lymphoma ($R^2 = 0.9721$) and acute leukemia ($R^2 = 0.9157$) and myeloma ($R^2 = 0.9002$).

Table 2: Determination of chymotrypsin-like activity in serum and intracellular proteasome by type of Pathology.

Diagnosis	Serum chymotrypsin-like activity (UF)	P-value	Intracellular chymotrypsin-like activity (UF)	P-value
LA	785 ± 99	P < 0.001	1648 ± 186	P < 0.0001
LC	552 ± 103	P = 0.061	1676 ± 173	P < 0.0001
Lymphoma	674 ± 141	P < 0.05	1784 ± 156	P < 0.0001
Myeloma	659 ± 110	P < 0.05	1702 ± 127	P < 0.0001
Control	491 ± 90	-	1168 ± 96	-

Data are means ± SEM. The values of the patients were compared to healthy subjects (Controls). LA (Acute leukemia); LC (Chronic leukemia).

DISCUSSION

The rapid proliferation of malignant cells is associated with dysfunction of one or more mechanisms of "checkpoint". These alterations lead to the rapid accumulation of malignant cells in a large number of defective proteins [16]. The relationship between clinical state and concentration of circulating proteasomes was found in patients with systemic lupus erythematosus, rheumatoid arthritis [17] and with different solid tumours (carcinoma of breast, stomach, kidney, colon, lung, testes and liver, respectively [18].

We projected through this study to analyze and establish a link between the concentration of the proteasome and its catalytic activity in both serum and intracellular level in a wide range of patients with different hematological neoplastic in a Moroccan population newly diagnosed. In the first time, we demonstrated that the serum proteasome levels are significantly elevated in patients compared to healthy donors. The increase was more pronounced in patients with myeloma (7112 ± 733 ng / ml), and lymphoma (5337.5 ± 663 ng / ml), the LA and LC is last with an average of (LA: 4416 ± 980 ng / ml; LC: 4491 ± 174). In parallel, a positive correlation between the level of serum LDH and proteasome in various neoplasie analyzed.

To measure the concentration of circulating proteasome by the ELISA technique, we detected the 20S proteasome [19]. The 20S complex is the central core of multiple proteasome subtypes [10], it could be expected that the detection of 20S proteasome components would reflect the level of the total proteasome expression [20], [21]. The technique of immune histochemical [9] demonstrated that proteasome components are strongly expressed in most of the corresponding malignant cells in certain hematologic malignancies. In addition Wada and al. reported that, proteasome concentrations are elevated in the culture media of human leukemia cell lines [9]. Thus, the investigators concluded that proteasome serum levels could mainly

originate from the malignant cell population and reflect the tumor burden [22].

Our data from patients with hematologic malignancies are also in accordance with previously published data showing that serum proteasome levels are elevated in patients with solid tumors and with different types of blood disease [18], [21]. Several lines of evidence have shown that proteasome subunits in both leukemia and MM cells are abnormally higher than those normal [22], [23]. The increase of circulating proteasome observed in hematology neoplastic may reflect tumor cell lysis [19]. This is consistent with the positive correlation that we found with serum LDH patients. It is known that myeloproliferative disorders imply a high rate of intramedullary apoptosis [20], which also may explain the increased levels of plasma proteasome seen in patients. Adams J. [16] reported that the rapid and extensive storage of mutant proteins, and / or misfolded is certainly the source of the dependence of malignant cells vis-à-vis an active proteasome.

In the present study, all patients were newly diagnosed and previously untreated. Increased circulating proteasome was high between 70 to 82% in comparison to control. In the pretreated patients, the increase was low only between 33-50 % [14]. Thus the reported circulating Proteasome levels may be changed during therapy [24]. Our results on MM patients approach those of Jakob and al. [21] whose patients were untreated. Although the majority of work was in agreement on increased serum proteasome in patients with hematologic malignancies [25], the use the concentration of circulating proteasomes as a character prognosis remains problematic because the absolute values of the proteasome are very different from one laboratory to another. Nevertheless, our result (mean serum 1264 ± 601 ng / ml) is similar to the mean serum (1500 ± 367 ng / ml) reported by the recent work of M de Martino and al. in healthy persons [26].

The fluctuations observed in the absolute values of the proteasome serum can be technical or originate from the nature molecular of circulating proteasome. To fully understand the source of these variations we have proceeded to analyze both the proteasome intracellular and the chymotrypsin-like activity. The intracellular assay proves to be beneficial since it allows a dosage at the source and certainly functional and intact proteasome at the molecular level.

The work of Bureau and al.[14] describes the process externalization outside the cell of the subprosome units, first in the cell membrane and then in the extracellular medium. Thus, explaining the significant presence of proteasome 20 circulating in the peripheral blood of the control subjects.

A possible relationship between the intra and extracellular of Proteasome is thus confirmed. Nevertheless sub units prosomal externalized outside the cell could not be functional since the catalytic activity of the 26S proteasome requires entity (Complexes regulators 19S + 20S proteasome) [27].

The most likely explanation for this difference in the level of serum values in clinically healthy people can be attributed to the fact that the extracellular proteasome is inactivated and defective [28]. The study performed on a Moroccan population revealed an optimal value in individuals with chronic leukemia (6303 ± 726 ng / ml), followed by patients with multiple myeloma (5910 ± 336 ng / ml). Patients with acute leukemia and lymphoma express the respective values of 5363 ± 369 ng / ml and 5026 ± 827 ng / ml. Patients with MM in contrast to other pathologies, have the particularity of a higher than the intracellular proteasome serum.

In this sense, the work of Amesterdam and al. [29], suggests a modification of the subcellular distribution of certain components of the proteasome during differentiation of malignant cells. Proteasome inhibition is now considered a unique and effective way to kill cancer cells that can tolerate conventional chemotherapy. The proteasome inhibitor approved for clinical application and is now widely used for the treatment of multiple myeloma (MM) [16]. Another study indicated that B-CLL cells are about 10 times more sensitive to Lactacystin than normal peripheral B lymphocytes. These results strongly suggest that proteasomes could be used as a drug target for myeloma and lymphoma therapy [30].

The development fast and continuous new-targeted oncology Therapeutics is a hope for patients, but also a source of new side effects. Intracellular measurement of proteasome allows estimating the predictive toxicity risk score. Indeed, observed after treatment by proteasome inhibitors (PS341) gastrointestinal toxicity is dose-dependent. Treatment is well tolerated if the proteasome inhibition does not exceed 80% of the basal state; beyond, appear the changes of pressure blood pressure and heart rate [31], [32].

In order to determine the optimal dose of a proteasome inhibitor in studies of phases II, and I myeloma proteasome activity is measured in white blood cells or tumor samples [31]. Catalytic activity is the main function of the proteasome, its analysis allows both to provide information

on the status of the proteasome intra and extracellular and respond to the effectiveness of a treatment.

In the literature, it has been clearly demonstrated that inhibition of proteasome activity has to result to induce the arrest of the cell cycle and apoptosis, and more significantly in cancer cells than level of normal cells [4]. Many works describe 3 catalytic activities of Proteasome: trypsin-like, chymotrypsin-like and PGPH-like [33]. The orientation assay of chymotrypsin-like activity of the proteasome takes account of fundamental role of this activity in the cycle of proteolytic degradation. Indeed, the data of literature confirms that only inhibition of chymotrypsin-like activity is sufficient to allow a significant reduction in the rate of protein degradation. Whereas inactivation of other sites trypsin and PGPH-like has little effect on the total proteolysis [6].

Our results showed a decrease in the catalytic activity after release of the proteasome in the extracellular medium as well in controls than in patients. Some studies allow an understanding the effect of the aging process on the proteasome activity in muscle cells of the rat and the Human fibroblasts [34]. These studies suggest that this failure of proteasome activity is due to a modification of some proteins, especially of the subunits of the proteasome and the reduction of the expression regulatory complex (PA28, PA700). The proteasome released into circulation, thus loses its catalytic potential by the disintegration of its sub units effectors and/or regulatory [7], [35].

We report that in intracellular the chymotrypsin like activity of proteasome is more important in patients. It has recently been demonstrated that protein degradation by the proteasome is a biological process that is essential for the survival of cancer cells. Increased proteasome activity in tumor cells provides a certain resistance to apoptosis [36], [37]. Apoptosis is a feature common to most malignancies tumors. It is currently established that treatment with proteasome inhibitors provides favorable results in the treatment of certain liquid tumors particularly myeloma [23].

The proteasome appears as a key element of neoplastic differentiation. Quantitative and especially qualitative variations seem to play an important pathophysiological role. It seems to be in blood original and sensitive tumor marker. To reduce side effects and improve patients' conditions, we propose to adjust the dose of treatment with proteasomes inhibitors, which will be established following an estimation of concentration and the catalytic activity of the proteasome cell level.

Conflict of interest

We have not a financial relationship with the organization that sponsored the research. We declare that we have no conflict of interest.

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