Molecular alterations in proteasomes of rat liver during aging result in altered proteolytic activities

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Abstract Aging induces alterations of tissue protein homeostasis. To investigate one of the major systems catalysing intracellular protein degradation we have purified 20S proteasomes from rat liver of young (2 months) and aged (23 months) animals and separated them into three subpopulations containing different types of intermediate proteasomes with standard- and immuno-subunits. The smallest subpopulation III and the major subpopulation I comprised proteasomes containing

immuno-subunits $\beta 1i$ and $\beta 5i$ beside small amounts of standard-subunits, whereas proteasomes of subpopulation II contained only $\beta 5i$ beside standard-subunits. In favour of a relative increase of the major subpopulation I, subpopulation II and III were reduced for about 55 % and 80 %, respectively, in aged rats. Furthermore, in all three 20S proteasome subpopulations from aged animals standard-active site subunits were replaced by immuno-subunits. Overall, this transformation resulted in a relative increase of immuno-subunit-containing proteasomes, paralleled by reduced activity towards short fluorogenic peptide substrates. However, depending on the substrate their hydrolysing activity of long polypeptide substrates was significantly higher or unchanged. Furthermore, our data revealed an altered MHC class I antigen-processing efficiency of 20S proteasomes from liver of aged rats. We therefore suggest that the age-related intramolecular alteration of hepatic proteasomes modifies its cleavage preferences without a general decrease of its activity. Such modifications could have implications on protein homeostasis as well as on MHC class I antigen presentation as part of the immunosenescence process.

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Introduction

As the liver is responsible for numerous vital functions of the organism, a myriad of enzyme proteins catalysing these reactions has to be maintained and in case of varying functional demands to be adapted by finely tuned protein turnover rates (Goldberg and St John 1976). With increasing age of rodents, the rate of protein synthesis in liver was found to be decreasing, increasing or changing biphasically (increase after initial decrease or decrease after initial increase) depending on the experimental set-up (Ward and Richardson 1991). Data published on protein breakdown rates in liver of rodents at different ages are also heterogeneous although decreasing rates parallel to protein synthesis were generally thought to be most likely (Ward and Richardson 1991; Short and Nair 2000) and to explain best that aging-induced changes in liver functions seem to be limited (Kitani 1994, 2007; Zeeh and Platt 2002). In fact, microinjected as well as endogenous proteins were degraded significantly slower in mice liver of old animals as compared to young (Ishigami and Goto 1988, 1990; Ikeda et al. 1992).

With regard to basal and induced protein degradation in liver, lysosomal autophagy is known to be a major pathway (Ueno et al. 2012), and in rat liver autophagic proteolysis was reported to be maximal at the age of 6 months before declining with increasing age (Donati et al. 2001). This observation parallels the biphasic run of the rate of overall protein degradation in aging rat (Ward 1988). Mechanistically, autophagy is divided into three different types — macroautophagy, microautophagy and chaperone-mediated autophagy (Cuervo 2004) — and at least two of them, macroautophagy and chaperone-mediated autophagy, are known to be compromised in liver tissue during aging (for review, see Rajawat et al. 2009; Hubbard et al. 2012).

The second major pathway responsible for intracellular protein degradation is catalysed by the ubiquitin–proteasome system (UPS), whose involvement in the aging process has been intensively investigated (for review, see Carrard et al. 2002; Chondrogianni and Gonos 2005; Gaczynska et al. 2001; Merker et al. 2001; Stolzing and Grune 2001; Vernace et al. 2007). The proteolytically active core of the UPS is the 20S proteasome, a cylinder-shaped complex composed of four seven-membered rings of α - and β -subunits. Their stoichiometry is $\alpha_7\beta_7\beta_7\alpha_7$, and three of the β -subunits, β_1 , β_2 and β_5 , contain the proteolytically active sites

catalysing the caspase-, trypsin-, and chymotrypsin-like activities, respectively. In liver tissue of aging mice and rats, all or some of these activities when measured with fluorogenic peptide substrates have been found to decrease (Sahakian et al. 1995; Shibatani et al. 1996; Conconi et al. 1996; Breusing et al. 2009; Dasuri et al. 2009; Hayashi and Goto 1998; Rodriguez et al. 2010) or decrease temporarily before increasing again (Petersen et al. 2010; Keller et al. 2000). However, no change of activity was measured in liver of aged as compared to young mice in one investigation (Huber et al. 2009), an observation corroborating the data published by Scrofano and colleagues, who determined liver proteasome activity in young and old mice by use of β -lactoglobulin and oxidized ribonuclease as substrates (Scrofano et al. 1998).

Activity measurements of proteasomes in all of these investigations were performed in tissue extracts or with extracts partially fractionated by chromatographic or centrifugation steps (Hayashi and Goto 1998; Rodriguez et al. 2010). Only Conconi et al. (1996) measured the activity of 20S proteasomes after their purification to apparent homogeneity.

In vivo 20S proteasomes associate various regulatory complexes like 19S regulator, PA28, PA200, and thus exist in different forms, the most abundant of which are 26S/30S proteasomes (20S proteasomes in complex with one or two 19S regulators, respectively), hybrid proteasomes (19S regulator/20S proteasome/PA28) and 20S proteasomes attached to one or two PA28 activator complexes (Rechsteiner and Hill 2005). Their activity towards short fluorogenic peptide substrates is different, an aspect taken into consideration in some of the investigations. In some investigations, a parallel decrease of 20S and 26S proteasomes was found in liver of aging rat (Hayashi and Goto 1998; Dasuri et al. 2009), whereas a decline of PA28 and 19S regulator (Rodriguez et al. 2010) or just of the 19S regulator subunit Rpn10 was suggested to be responsible for a decreasing activity during aging (Huber et al. 2009).

Another reason for alteration of proteasome activity is the replacement of proteasomal subunits, β_1 , β_2 , and β_5 , by the immuno-subunits, β_{1i} , β_{2i} and β_{5i} , respectively. Thus, irrespective of whether they are present as complexes of 26S/30S or any other form of proteasome in the cell, 20S proteasomes exist in two major forms designated standard- and immuno-proteasomes, which exhibit the three proteolytic activities to different extents (Gaczynska et al. 1994; Ustrell et al. 1995; Boes et al. 1994). An incomplete replacement of standard-subunits by

immuno-subunits results in the formation of a third population designated intermediate-type proteasomes, which can be further subdivided into subpopulations according to the extent of standard by immuno-subunit replacement (Klare et al. 2007; Dahlmann et al. 2000; Guillaume et al. 2010). We have shown previously that proteasomes of rat liver all comprised intermediate-type subunit compositions (Dahlmann et al. 2000; Schmidt et al. 2006). Taking advantage of their different surface hydrophobicity, we separated 20S proteasomes from rat heart into three subpopulations two of them comprising intermediate-type and one containing standard proteasomes (Kloss et al. 2009). In the present investigation, we found that 20S proteasomes from liver of young and aged rats could also be separated into three subpopulations. In liver of aged rat, these subpopulations differed with regard to their relative amounts, their content of immuno-subunits and their proteolytic activities measured with several oligo- and polypeptide substrates, indicating that 20S proteasomes are subject of a process of molecular and functional conversion in liver of aged organisms.

Material and methods

Animals and antibodies

Young (2 months) and aged (23 months) male Wistar rats were purchased from Harlan Winkelmann GmbH (Borchen, Germany) and sacrificed by carbon dioxide. Their livers were excised and frozen at -80°C .

For immunohistochemistry (IHC) and western blotting the following antibodies were used: pan-proteasome antisera against 20S proteasome from rat skeletal muscle and rat liver were rabbit 37 (Dahlmann et al. 1985) and rabbit 87 (laboratory stocks), respectively. Serum K43/7 (laboratory stock) was raised against subunit $\beta 1$, antibodies towards subunits $\beta 5$, $\beta 1i$ and $\beta 5i$ were from Abcam (UK) and against $\alpha 1$ from Cappel (Belgium).

Determination of proteasome activity and of proteasome amount

Proteasome activity was determined using fluorogenic peptide substrates as described elsewhere (Dahlmann et al. 2000). Briefly, 20 μl of peptide substrates Suc-LLVY-MCA (200 μM), Bz-VGR-MCA (200 μM) and Z-LLE-MCA (400 μM) were mixed with 20 μl of enzyme solution and after incubation for 30–120 min at 37°C

fluorescence of methylcoumarylamide (MCA) released was measured to determine the chymotrypsin-, trypsin-, and caspase-like activities, respectively.

Quantitative determination of 20S proteasomes was performed by Rocket immunoelectrophoresis (Weeke 1973).

Electrophoretic techniques and western blotting

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12.5 % gels and then stained with Coomassie brilliant blue. First dimension on the two-dimensional gel electrophoresis (2D-PAGE) was performed under non-equilibrium pH gradient conditions with pH gradient from 3 to 10 (O'Farrell et al. 1977). After electrophoresis, proteasomes were blotted onto PVDF membranes, which were blocked with 5 % milk powder dissolved in 20 mM Tris, 0.5 M NaCl, 1 mM NaN_3 , pH 7.5. Subunits were detected using antibodies specific for subunits $\beta 1i$, $\beta 5i$ and $\beta 5$ as well as subunit $\beta 1$. Normalisation was performed using an antibody against subunit $\alpha 2$ and $\beta 4$. Densitometric analysis was performed using the ImageJ software.

Purification of 20S proteasomes and separation of proteasome subpopulations

By ten strokes in a glass–Teflon Potter–Elvehjem tissue grinder 50 g of rat liver was homogenized in a 4-fold volume of TEAD buffer (20 mM Tris, 1 mM EDTA, 1 mM NaN_3 , 1 mM DTT, pH 7) and centrifuged for 60 min at $20,000\times g$. The resulting supernatant (liver extract) was used for determination of protein and 20S proteasome content, for measurement of chymotrypsin-, trypsin- and caspase-like activities as already described as well as for purification of 20S proteasome as described elsewhere (Schmidt et al. 2006). Briefly, this consists of the following steps: a fractionated protein precipitation with ammonium sulphate, chromatography on DEAE-Sephacel, gel filtration on Superose 6 and anion exchange chromatography on Mono Q. In the last purification step, a hydrophobic interaction chromatography was performed on a Resource-Phe column for separation of proteasome subpopulations (Kloss et al. 2009).

Digestion of peptide substrates

20S proteasome (0.5 μg) was incubated with 40 μM solutions of synthetic peptides dissolved in TEAD buffer

Fig. 1 Protein content, proteasome concentration and proteolytic activities in liver extracts and proteolytic activities of purified 20S proteasomes from liver of young and aged rats. Data obtained with young (2 months) and aged (23 months) rats are indicated by *black* and *white columns*, respectively. **a** Protein concentrations in liver extracts. **b** 20S proteasome concentrations measured in liver extracts by means of rocket immunoelectrophoresis. **c** Proteolytic activities measured by means of Suc-LLVY-MCA (chymotryptic),

Bz-VGR-MCA (tryptic), and Z-LLE-MCA (caspase) in liver extracts. **d** Proteolytic activities measured in 20S proteasomes purified from rat liver extracts by use of the same substrates as detailed in panel **c**. Data are shown as mean value $\pm$ SD for three different rats of each age in panels **a–c** and for six young and three aged rats in panel **d**, respectively. Unpaired Student's *t*-test was performed to determine statistical significance (*p* values are given only for comparisons that were significantly different, *p* > 0.05)

at 37 °C over a period of 8 h. Digestion was stopped at different time points by adding trifluoroacetic acid at a final concentration of 0.3 % and frozen at –20 °C. The kinetics of substrate degradation was measured with ESI-MS as described elsewhere (Mishto et al. 2010). Briefly, substrate was separated from digestion products by C18 reversed phase HPLC (HP1100, Agilent Technologies, Germany) and online detected by an ESI-MS analyses performed with an LCQ ion trap mass spectrometer (Thermo Fisher Scientific, Germany). Peptides were identified by their molecular masses calculated from the *m/z* peaks of the multiple charged ions, confirmed by tandem mass spectrometry sequencing analyses and the peaks' area was quantified to estimate the relative amount. All peptides were synthesized using Fmoc solid phase chemistry as previously described. The sequence enumeration for the peptides gp100_{40–52} (sequence: RTKAWNRLPEW), gp100_{35–57} (sequence: VSRQLR TKAWNRLYPEWTEAQR) and gp100_{201–229} (sequence: AHSSSAFTITDQVPFVSVSQRLRALD GGNK) is referred to the human gp100^{PMEL17} sequence, for the peptide pp89_{16–40} (sequence: RLMYDMYPH FMTNLGPSEKRVWMS) is referred to the murine cytomegalovirus pp89 protein (Liepe et al. 2010; Boes et al. 1994).

Immunohistochemistry

The specificity of the antibodies towards immuno-proteasome subunits has been preliminary tested on livers of one wild-type mouse (1.5 years), three $\beta 1i^{-/-}$ mice (8 weeks, 22 weeks and 18 months) and two $\beta 5i^{-/-}$ C57/BL6 mice (23 weeks and 12 months) (Fig. S1). In order to identify the different cell types expressing immuno-subunits in rat liver, we performed IHC assays by staining eight rat livers (four organs for each age) with antibodies specific for $\beta 1$, $\beta 1i$, $\beta 5i$ subunits and for the entire proteasome (pan-proteasome).

All liver samples were formalin-fixed and routinely processed. From paraffin blocks 3-mm-thick sections were cut for haematoxylin–eosin stain and IHC. Antibodies against pan-proteasome, proteasome subunit $\beta 1$, as well as immuno-subunits $\beta 1i$ and $\beta 5i$ were employed. Histological samples were treated as reported previously (Vasuri et al. 2010).

Statistics

All statistical analyses were performed using SIGMASTAT 3.0. Observed differences in values between the animals of the two age groups were analysed for

statistical significance by using the impaired Student's *t*-test. All data are indicated by means $\pm$ SD. The number of experiments is detailed in the figure captions.

Results

20S proteasomes in liver extracts from rats of two different stages of age

Initially, we determined the total protein and proteasome content in liver tissue extracts of young (2 months) and aged (23 months) rats. Liver tissues from 23-month-old rats compared with those of young rats revealed a slightly lower, however, statistically not significant protein concentration (mg protein/g tissue) (Fig. 1a). Determination of 20S proteasome concentration by immunoelectrophoresis in the same tissue extracts revealed that the proteasome concentration was substantially declined by about 50 % in 23 months as compared to 2-month-old rats (Fig. 1b). We also measured proteolytic activities in the liver extracts using Suc-LLVY-MCA, Bz-VGR-MCA, and Z-LLE-MCA as substrates for proteases with chymotryptic, tryptic and caspase-like activity, respectively. None of the three activities was significantly different between both age groups (Fig. 1c). Considering that, in addition to proteasomes, liver tissue contains a large number of different proteolytic enzymes, which may also hydrolyse these short fluorogenic peptide substrates, we isolated 20S proteasomes to investigate whether their specific activities and molecular properties are subject to age-dependent alterations.

Therefore, 20S proteasomes were purified to apparent homogeneity from the liver tissue of young and aged rats by using a five-step chromatographic purification procedure. As shown in Fig. 1d, the specific proteolytic activities of the purified 20S proteasomes differed between the two age groups with respect to a lower caspase like activity in aged as compared to young rats.

Separation of 20S proteasome into subpopulations

Present knowledge about the substrate specificity of proteasomes suggests that changes in their activities result from intra-molecular alterations like subunit replacements within the proteasomal cylinder (Huber et al. 2012). As described above, 20S proteasomes can contain standard- or immuno-subunits or a mixture of both and thus exist as standard- immuno- and intermediate-

proteasomes, respectively. Each subpopulation exhibits a different pattern of chymotryptic, tryptic and caspase-like activity and they also differ in their activity towards the 25mer polypeptide pp89_{16–40} (Dahlmann et al. 2000; Klare et al. 2007). Therefore, we studied whether there exist age-dependent alterations in the subunit composition of rat liver proteasomes that might explain the partial loss of the measured caspase-like activity.

Standard-, immuno- and intermediate-proteasomes can be separated due to their differences in surface hydrophobicity (Kloss et al. 2009; Dechavanne et al. 2013). Accordingly, we have subjected rat liver 20S proteasomes to hydrophobic interaction chromatography and thereby dividing them into three subpopulations designated I, II, and III with subpopulation I being the predominant form of 20S proteasomes (Fig. 2a,b). While subpopulations II and III were apparently pure, subpopulation III still contained some high molecular mass contaminations (Fig. 2c).

Age-dependent differences in ratios and subunit composition of 20S proteasome subpopulations

After subdividing the proteasomes of 2- and 23-month-old rats into subpopulations, we compared the concentrations of the three subpopulations (μ g protein/g tissue) in rats of both age groups. While the difference in concentration of subpopulation I between both age groups reached no statistical significance, there was a significant decrease in subpopulations II and III from 2 to 23 months of age (Fig. 2d). Calculating the percentage of each subpopulation in relation to the total proteasome population, subpopulation I was determined to be increased by about 50 % in 23-month-old as compared to 2-month-old rats. On the other hand, the percentage of subpopulation II declined by about 55 % and subpopulation III was decreased even by 80 % in aged as compared to young animals (Fig. 2e). These data show that in liver of aged rats proteasome subpopulation I increased at the expense of a significant loss of proteasome subpopulations II and III.

Next, we investigated the content of active site-containing subunits in the three proteasome subpopulations and possible alterations in liver of aged rats. By means of 2D PAGE of 20S proteasomes, we had previously shown that liver contains proteasomes exclusively of an intermediate-type subunit composition, i.e., they contain standard as well as immuno-subunits (Schmidt et al. 2006). Applying the same technique, we analysed the

subunit composition of the three proteasome subpopulations of young and aged rats, respectively, and assigned the proteasome subunits according to our earlier investigations (Fig. 3a) (Schmidt et al. 2006). Relative quantification of the active site containing subunits $\beta 1$ and $\beta 1i$ as well as of $\beta 5$ and $\beta 5i$ was performed by using

Fig. 2 Separation of rat liver 20S proteasomes into their subpopulations. **a** 20S proteasomes from liver extracts were purified chromatographically as described elsewhere (Kloss et al. 2009) and in the final step of the purification were separated into three subpopulation by hydrophobic interaction chromatography using a gradient from 1 to 0 mol/l ammonium sulphate. **b** Proteasome activity was measured with Suc-LLVY-MCA as substrate. Only the fractions of the chromatogram containing proteasomes are shown in **a** and **b**. **c** Fractions comprising the peaks I, II, and III were pooled, dialysed and aliquots subjected to SDS-PAGE. The gel was Coomassie stained to check the purity of the three proteasome subpopulations. **d** The amount of each proteasome subpopulation was measured by Bradford assay and data obtained from three preparations each of 2-month-old (black bars) and 23-month-old (white bars) rats, respectively, are shown. **e** The percentage of each subpopulation of the whole proteasome content was calculated and compared for 2-month-old (black bars) and 23-month-old (white bars) rats. **d**, **e** Mean values $\pm$ SD are shown from preparations of three animals of each age group. Statistically significant differences between young and aged rats are documented by *p* values

subunits $\alpha 2$ and $\beta 4$ as reference spots. As shown in Fig. 3b and c, the proteasome subpopulation I of both age groups contained subunit $\beta 1i$ and $\beta 5i$. Most importantly, the corresponding standard subunits $\beta 1$ and $\beta 5$, which were present in 2-month-old rats, were completely absent in subpopulation I of 23-month-old rats, indicating that this intermediate-type subpopulation was replaced by immuno-proteasomes in liver of aged rat. Subpopulation II comprises proteasomes containing high amounts of standard subunits $\beta 1$ and $\beta 5$; its small content of $\beta 5i$ was increased in 23 months as compared to 2-month-old rats. The subunit pattern of proteasomes of subpopulation III of young rats was similar to that of subpopulation II; however, in aged rats no $\beta 5$ was found and a small amount of $\beta 1i$ was detectable. Due to its limited amount, alterations in proteolytic subunits were difficult to quantify in subpopulation III. Considering that in 23-month-old rats subpopulation I comprises the majority of proteasomes consisting of pure immuno-proteasomes and the small amounts of subpopulations II and III are of intermediate subunit composition, one can conclude that aging leads to a predominance of immuno-subunit containing proteasomes in rat liver tissue.

Peptide hydrolysing activity of proteasome subpopulations

The fluorogenic peptide-hydrolysing activities of 20S proteasomes are largely determined by their content of active-site containing standard and immuno-subunits. Therefore, we measured the specific activities of the three

Fig. 3 Analysis of standard and immuno-subunits content in proteasome subpopulations. **a** Thirty micrograms of purified liver 20S proteasomes from rats of 2 months of age was subjected to 2D PAGE and the gel stained with Coomassie. The proteasome subunits were indicated according to our spot analyses performed elsewhere (Schmidt et al. 2006). **b** 20S proteasome subpopulations (I, II, III) purified from rat liver of 2 (2 mo) and 23 (23 mo) months

of age were subjected to 2D PAGE and the gels then Coomassie stained. Only the sections containing the subunits $\beta 1$ and $\beta 1i$, $\beta 5$ and $\beta 5i$, as well as $\alpha 2$ and $\beta 4$ are shown. **c** The spots of subunits $\beta 1$, $\beta 1i$, $\beta 5$, and $\beta 5i$, respectively, were densitometrically quantified and their amounts relative to subunits $\alpha 2$ and $\beta 4$ plotted in dependence of animal age. Data are means $\pm$ SD relative to the spots of subunits $\alpha 2$ and $\beta 4$

proteasome subpopulations purified from rat livers of the two different age cohorts. As shown in Fig. 4, a comparison of the hydrolysing activities towards short fluorogenic peptide substrates revealed that subpopulations I and II exhibit higher specific activities than subpopulation III. At both age stages, the chymotrypsin-like activity was the predominant activity in all three proteasome subpopulations. In subpopulation II, this activity decreased significantly by more than two thirds in aged as compared to young rats. In subpopulation I, the decrease in chymotrypsin-like activity was statistically not significant. An alteration of the trypsin-like activity was observed only in subpopulation III, where an increase from 2- to 23-month-old rats was measured. The caspase-like activity

of the three subpopulations did not show any considerable age-dependent alterations. The decline of caspase-like activity in subpopulation II from young to aged rats did not reach statistical significance due to high interindividual variance.

In the total population of liver 20S proteasomes, we measured a significant lower caspase-like activity in 23-month-old rats as compared to 2-month-old rats. This phenomenon most likely results from the significant loss of subpopulations II and III as well as from the transition of intermediate-type proteasomes to immuno-proteasomes that exhibit lower caspase-like activity than standard and intermediate-type proteasomes (Dahlmann et al. 2000; Klare et al. 2007; Kloss et al. 2009).

Fig. 4 Measurement of proteasomal activity with fluorogenic peptides. Hydrolysis of fluorogenic peptide substrates Suc-LLVY-MCA (Suc-LLVY), Bz-VGR-MCA (Bz-VGR) and Z-LLE-MCA (Z-LLE) for chymotrypsin-, trypsin- and caspase-like activities was measured with the three proteasome subpopulations (I, II, and III) isolated from liver of 2-month-old (*black columns*) and 23-month-old (*white columns*) old rats. Next, 100 nM of each subpopulation was incubated with substrate for 2 h at 37 °C. Data are means $\pm$ SD of three separate preparations of each age group. Student's *t*-test was performed, and *p* values are shown only for statistically significant ($p < 0.05$) differences in activities

Degradation of polypeptide substrates by rat liver 20S proteasomes

Although fluorogenic tri- and tetrapeptide substrates are generally used to determine the three proteasome activities, they hardly resemble natural proteasome substrates that commonly are polypeptides and proteins of different sizes. Therefore, we investigated the degradation of polypeptide substrates of different length and amino acid composition, i.e., polypeptide 30mer, gp100_{201–230}, the 23mer peptide gp100_{35–57}, and the 13mer peptide,

gp100_{40–52}, which were delineated from the human melanocyte glycoprotein, gp100 (Liepe et al. 2010), as well as the 25mer polypeptide pp89_{16–40} derived from the murine cytomegalovirus IE protein pp89 (Boes et al. 1994). The different polypeptide substrates were applied to in vitro degradation by the three proteasome subpopulations from young and aged rats and the relative amount of non-degraded substrate overtime was measured by mass spectrometry (Fig. 5a). To compare the substrate degradation rates of the different proteasome subpopulations, we assessed the time required for 50 % substrate degradation (Fig. 5b). Irrespective of the subpopulation, proteasomes from old rats degraded pp89_{16–40} and gp100_{201–230} polypeptide substrates much faster than proteasomes of young rats, suggesting that the higher content of immuno-subunits in proteasomes of the aged rats favoured the degradation of these peptides. In contrast, analysis of the degradation rates of the 23mer gp100_{35–57} peptide showed a completely different pattern, since no significant difference in degradation rate could be observed neither between proteasomes of the three subpopulations nor of the two age groups. The degradation of the 13mer gp100_{40–52}, i.e., the shortest substrate, by proteasome subpopulation III was considerably slower than by subpopulations I and II and this was independent of age. This result resembles the overall activities measured with the fluorogenic peptide substrates in so far as subpopulation III showed the lowest activity also towards these substrates (Fig. 5a,b).

Production of MHC class I-restricted epitopes

Because of the relevance of hepatocytes in the MHC class I-restricted antigen presentation, we investigated the generation of MHC class I-restricted epitopes by liver 20S proteasome of young and aged rats. We focused on the peptides pp89_{22–28} and gp100_{208–217}. The first is the epitope presented on the MHC class I L^d molecule (Boes et al. 1994) whereas the latter is the precursor of the HLA-A*02-restricted gp100_{209–217} epitope (Chapiro et al. 2006). The epitope pp89_{22–28} was produced in higher amount by liver proteasome of aged compared to young rats, in agreement with the faster degradation of the parental substrate pp89_{16–40} and the previously described cleavage preference at the C-terminal leucine₁₅ epitope anchor residue (Boes et al. 1994) (Fig. 6). Conversely, the epitope precursor gp100_{208–217} was generated more effectively by liver proteasome of young as compared to aged rats (Fig. 6),

thereby diverging from the trend of substrate hydrolysis (Fig. 5a). This higher efficiency in the gp100_{208–217} production by the liver proteasome of young compared to aged rats is well in agreement with the previously reported less efficient generation of the epitope by immuno-subunit containing proteasomes (Chapiro et al. 2006).

Cellular distribution of proteasomes in liver tissue

As immunohistochemical localisation of proteasomes had revealed their presence throughout rat liver lobules in hepatocytes, Kupffer cells, sinusoidal endothelial cell and bile duct epithelial cells (Tanaka et al. 1986; Rivett et al. 1992; Tanaka et al. 1989) and proteasomes containing

Fig. 5 Degradation of polypeptide substrates by rat liver proteasomes. **a** First, 40 μ M of four different peptide substrates, pp89_{16–40}, gp100_{201–230}, gp100_{35–57}, and gp100_{40–52} were each digested with 500 ng of the three 20S proteasome subpopulations isolated from livers of 2-month-old (black circles) and 23-month-old (white circles) rats at 37 °C for 8 h. At the times indicated, the

amount of non-degraded substrate was measured by ESI mass spectrometry. Data are means $\pm$ SD of digestion experiments with proteasomes from two independent preparations. **b** Incubation times to obtain 50 % degradation of the four substrates were read in **a** and plotted in dependence of subpopulations isolated from livers of 2-month-old (black columns) and 23-month-old (white columns) rats

Fig. 6 Generation of MHC class I-restricted epitopes by rat liver proteasomes. The peptides pp89_{22–28} and gp100_{208–217} are a MHC class I-restricted epitope and an epitope precursor, respectively. They were generated by 20S proteasome subpopulations isolated from livers of 2-month-old (*black circles*) and 23-month-old (*white circles*) rats during the degradation of the polypeptides pp89_{16–40} and gp100_{201–230} in the condition described in Fig. 5. The signal intensity of the peptides was measured by mass spectrometry in an extracted ion chromatogram

immuno-subunits, $\beta 1i$ and $\beta 5i$, were found in the cytoplasm, nucleus and microsomal fraction of rat liver cells (Palmer et al. 1996; Brooks et al. 2000), we were interested whether the relative rise of immuno-subunits during aging is reflected in their tissue distribution. Therefore, sections of liver tissue of rats of both ages were histochemically investigated by staining with antibodies to subunit $\beta 1$, $\beta 1i$, and $\beta 5i$ as well as with an antibody raised against total 20S proteasomes purified from rat liver. As shown in Fig. 7, pan-proteasome and $\beta 1$ -specific antibodies bound to the cytoplasm of both hepatocytes and bile duct epithelial cells in all the animals studied. The nuclear presence of proteasomes was found in all cases as well, with a variable percentage of positive hepatocytes. In particular, antibodies against liver 20S proteasomes showed a mean of 26.8 ± 17.5 % of positive nuclei (range 10–60 %), and nine (75 %) cases had strong positivity. Similar data were obtained with a polyclonal antibody to 20S proteasome of rat skeletal muscle (data not shown). Finally, antibodies against the

standard subunit $\beta 1$ showed a mean of 28.3 ± 14 % of positive nuclei (range 10–60 %), and four (33 %) cases had a strong positivity.

In addition, $\beta 1i$ and $\beta 5i$ subunits were detected in cytoplasm of all liver cell types, although their positivity was more pronounced in Kupffer cells, lymphocytes (when present) and vascular endothelial cells. Antibody to $\beta 1i$ showed a mean percentage of positive nuclei of 20.0 ± 18.3 % (range 5–60 %), with four (33 %) cases strongly positive. Antibody to $\beta 5i$ showed a mean percentage of positive nuclei of 26.7 ± 15.4 % (range 5–60 %), with six (50 %) cases strongly positive. No remarkable difference in cell type expression emerged comparing livers from rats of different ages (data not shown).

Discussion

Preservation of the cellular proteome, i.e., protein homeostasis, is a prerequisite for functional maintenance

of a living organism. During aging, this is not necessarily the case, for example in skeletal muscle the disequilibrium between the rates of protein synthesis and degradation causes sarcopenia (Millward et al. 1997). Although there is no equivalent phenomenon known in liver tissue, it has been reported that the liver volume decreases with age (Zeeh and Platt 2002; Schmucker 2005), although the volume of individual hepatocytes in rat liver rises till the age of 6 or 16 months before declining again with increasing age to the volume measured in young rats (David 1985; Schmucker et al. 1978). Our investigation showed

that the protein concentration was diminished by about 25 % in liver tissue of aged as compared to young rats paralleled by a 40 % decrease in the content of 20S proteasome. Such a change in proteasome content did not correspond to a decrease of the chymotryptic, tryptic and caspase-like activities in total liver extracts of aged rats. To evaluate this in correlation to contrasting results of other studies (Breusing et al. 2009; Dasuri et al. 2009; Hayashi and Goto 1998; Rodriguez et al. 2010), we have to consider that hydrolysis of short fluorogenic peptides in crude tissue extracts always reflects the sum of activities of

Fig. 7 Immunohistochemical localisation of proteasome-subunits in rat liver. Immuno-proteasome subunits are expressed in different liver cell types, independent of age. Representative fields of immunohistochemistry performed with antibodies against $\beta 1i$ subunit (a, e), $\beta 5i$ subunit (b, f), $\beta 1$ subunit (c, g) and pan-proteasome (d, h) on rat livers of 2-month-old (a–d) and 23-month-old (e–h) rats are shown. Both the cytoplasm and nucleus of hepatocytes contained subunits $\beta 1$, $\beta 1i$ and $\beta 5i$, although with small differences. These cells, which represent the majority of the parenchyma, are not tagged. Other cell types, expressing standard- and immuno-proteasome subunits are indicated as follows: 1 Kupffer cells, 2 bile duct epithelial cells. Magnification, $\times 40$

different proteases (Puente and Lopez-Otin 2004), which could be differently affected by aging or protein extraction protocols and therefore mask the real age-dependent alteration of proteasome activities. Indeed, our analysis of 20S proteasomes purified to apparent homogeneity and measurement of their peptide hydrolysing activity using fluorogenic peptide substrates revealed a significantly decreased caspase-like activity in 20S proteasome population purified from livers of aged as compared to young rats, which is in agreement with results of other investigators (Shibatani et al. 1996; Conconi et al. 1996; Hayashi and Goto 1998; Petersen et al. 2010; Rodriguez et al. 2010). This phenomenon most likely not only reflects alterations in the specific activities of the 20S proteasome subpopulations but also in their concentrations, keeping in mind that each subpopulation has a specific pattern of activities.

Although short fluorogenic peptides are often used to determine proteasome activities, they have only limited similarity to physiological proteasomal substrates, which are proteins and polypeptides. Therefore, to test the impact of age on proteasome activity, we also measured the *in vitro* degradation rate of four polypeptides by 20S proteasome subpopulations of livers from young and aged rats. In agreement with the fact that the contribution of each proteasomal active site to protein degradation varies from substrate to substrate (Kisselev et al. 2006), the velocity in degrading the polypeptide substrates was differently affected by the age of the animals and varied from subpopulation to subpopulation and from polypeptide to polypeptide. This finding may also explain the non-uniform age-dependent alterations observed for the degradation rates of specific liver proteins by other investigators (Ward and Richardson 1991; Scrofano et al. 1998).

In addition, pp89_{16–40} and gp100_{201–230} polypeptides were degraded faster by all 20S proteasome subpopulations from aged rats, while no age-dependent effects were observed for the substrate gp100_{35–57} and its 13mer derivative gp100_{40–52}. Because the tested polypeptides have different sequences and lengths, it may well be that liver 20S proteasome subpopulations of aged rats have a higher affinity to the pp89_{16–40} and gp100_{201–230} polypeptide substrates than proteasomes from young rats. However, we did not detect a clear impact of polypeptide length on the degradation rate by 20S proteasomes in agreement with our previous observation (Mishto et al. 2008) and the degradation rates of polypeptides with similar length but different sequence, such as pp89_{16–40} and gp100_{35–57}, were differently affected by the age of

the animals. Previously, we have also shown that the impact of the six proteasomal catalytic sites varies between these latter substrates (Mishto et al. 2012). These two facts suggest that rather the sequence than the length of the substrate polypeptides is the reason for the age-dependent differences in proteasomal degradation rate. Such a different affinity for cleavage sites of polypeptide substrates can be explained by the alterations in the content of immuno-proteasome subunits (Huber et al. 2012; Guillaume et al. 2010) and the relative content of subpopulations in liver 20S proteasomes of aged as compared to young rats. Therefore, the age-dependent decrease in proteasomal activity measured with fluorogenic peptide substrates by us and other investigators may not reflect the actual capability of proteasomes to cope with polypeptide substrates.

20S proteasomes from rat liver are intermediate-type proteasomes (Schmidt et al. 2006), and by subjecting them to hydrophobic interaction chromatography they can be subdivided into subpopulations due to their different content of standard- and immuno-subunits and possibly due to post-translational modifications (Bardag-Gorce et al. 2004; Zong et al. 2008). In liver of 2-month-old rats, enzymes of subpopulation I contain both immuno-subunits $\beta 1i$ and $\beta 5i$ in excess to their standard homologues $\beta 1$ and $\beta 5$, whereas proteasomes of subpopulation II do not contain $\beta 1i$ but only $\beta 5i$ as well as $\beta 1$ and $\beta 5$ in the main. Subpopulations with a similar immuno-subunit content have been detected in human liver (Guillaume et al. 2010). The proteasomes of the minor subpopulation III have a similar subunit composition as that of subpopulation II and contain some non-proteasomal contaminations. Parallel to the total amount of 20S proteasomes, the concentration ($\mu\text{g/g}$ tissue) of subpopulations II and III were decreased in rats of 23 as compared to 2 months of age. Consequently, in aged rats subpopulations II and III were much less relevant, while subpopulation I became the predominant one. In addition to this quantitative change in the ratio of the three subpopulations, a qualitative alteration occurred. In aged animals immuno-subunits are incorporated in subpopulation I at the expense of standard subunits. Similarly, a rise of subunit $\beta 5i$ was observed in subpopulation II and of subunit $\beta 1i$ in subpopulation III in aged as compared to young rats. All alterations together entailed that the relative proportion of immuno-proteasomes were increased at the expense of intermediate-type proteasomes in 23-month-old as compared to 2-month-old rats.

The expression of immuno-proteasome subunits in rat liver might result from the interaction of the organism with pathogenic insults, since specific pathogen-free mouse liver does not contain proteasomal immuno-subunits unless experimentally infected with a virus and bacterium (Khan et al. 2001). The liver of human adults, usually exposed lifelong to different pathogens, also contains all three proteasome immuno-subunits in contrast to fetal human liver (Vasuri et al. 2010; French et al. 2011; Guillaume et al. 2010). These data and our observations with liver tissue of young and aged rats suggest that the progress in age of an organism results in a conversion from standard-proteasomes (in the prenatal state) to intermediate-type and finally immuno-proteasomes in aged subjects, although a study on human liver aging would be compulsory to confirm such a hypothesis.

Immuno-proteasomes are constitutively present in lymphoid tissues whereas non-lymphoid cells have to be induced by cytokines like interferons to express immuno-subunits (Kruger and Kloetzel 2012). Actually, the sinusoidal endothelial cells and Kupffer cells are responsible for removing pathogens and toxins entering the liver from the gastrointestinal tract via the portal vein and from the systemic circulation via the hepatic artery, respectively, and thus they form sort of an immunological barrier shielding the hepatocytes (Gao et al. 2008; Knolle and Gerken 2000). Determination of IFN γ mRNA by RT-PCR in liver of 2- and 23-month-old rats revealed a slightly higher mRNA concentration in aged as compared to young rats (data not shown), corroborating data by Singh and co-workers, who have measured elevated levels of several cytokines including IFN γ in liver tissue of 24-month-old as compared to 3-month-old mice (Singh et al. 2008, 2011). This went along with an enrichment of large clusters formed by natural killer cells, macrophages and lymphocytes. Our histochemical investigations showed that immuno-subunits were located in the cytoplasm and nuclei of vascular endothelial cells, Kupffer cells and lymphocytes. However, immuno-subunits were similarly detectable in hepatocytes, thereby indicating that these cells can respond to an elevated cytokine level. Therefore, the shift of liver proteasomes from intermediate- to immuno-proteasomes seems to be a general age-dependent process that is not reliant on an immune response against specific pathogens but rather is part of the complex phenomenon of inflamm-aging (Franceschi et al. 2000).

The alteration of proteasome activities in livers of aged rats could also have implications for the MHC

class I antigen presentation by hepatocytes. Indeed, we showed that the production of a representative MHC class I-restricted epitope and an epitope-precursor by liver 20S proteasome subpopulations differed between 2- and 23-month-old rats. It is worth to note that the generation of the precursor of the epitope gp100_{209–217} followed an opposite trends than the gp100_{201–230} substrate degradation, thereby indicating that proteasomes derived from old rats had a much lower efficiency in producing such peptide. This is most likely due to altered cleavage site preference of the immuno-proteasomes formed in livers of aged rats, which are known to impair gp100 epitope generation (Chapiro et al. 2006). Because of the delivery of multiple antigens through the bloodstream to liver cells and the ability of liver to retain activated T cells and to destroy them, an alteration of the MHC class I antigen presentation upon aging in hepatocytes could have a wide range of implications thereby being a relevant aspect of immunosenescence (De Martinis et al. 2005; Mishto et al. 2003; Franceschi et al. 2000).

In addition, the shift of intermediate-type toward the immuno-proteasomes and the concomitant alteration of their hydrolytic activity in liver of aged organisms could have impact also on protein homeostasis. Indeed, it is often argued that an age-related decline in proteasome activity goes along with an accumulation of oxidized proteins that are preferred proteasomal substrates (Grune et al. 2004; Goto and Nakamura 1997). It was shown that proteasomes containing immuno-subunits have a higher capacity to degrade oxidized proteins than standard-proteasomes (Seifert et al. 2010). Therefore, the age-dependent increment in intermediate-type and immuno-proteasomes might belong to those cellular mechanisms aimed to balance the consequences of the impaired antioxidative capacity in aging liver (Hussong et al. 2010; Suh et al. 2004; Stio et al. 1994). Since an age-related increase in immuno-subunits was also observed in other tissues, e.g., in skeletal muscle (Husom et al. 2004) and brain (Gavilan et al. 2009; Mishto et al. 2006), we suggest that part of the mechanism to keep up proteostasis during aging is the formation of immuno-proteasomes (Mishto et al. 2003).

In summary, by using only specific short fluorogenic substrates, as it happened in the past, one may arrive to the misleading conclusion that proteasome activity is decreased upon aging also in liver. On the contrary, by combining assays with short fluorogenic peptides, polypeptides and focusing on MHC class I-restricted epitope generation we showed that the quality of cleavage

preferences of liver 20S proteasomes changes in old rats. However, this alteration cannot be simplified in the narrow dichotomous contest of increased/decreased activity.

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