Published in final edited form as:

Int J Radiat Biol. 2009 June ; 85(6): 483–494. doi:10.1080/09553000902883794.

Imaging of radiation effects on cellular 26S proteasome function in situ

James M. Brush¹, Kwanghee Kim¹, James W. Sayre^{2,3}, William H. McBride¹, and Keisuke S. Iwamoto¹

¹Department of Radiation Oncology, David Geffen School of Medicine at UCLA

²Department of Radiological Sciences, David Geffen School of Medicine at UCLA

³Department of Biostatistics, School of Public Health, University of California Los Angeles, Los Angeles, California, USA

Abstract

Purpose—The classical radiobiological paradigm is that DNA is the target for cell damage caused by ionising radiation. However, evidence is accumulating that other constituents, such as the membrane, organelles, and proteins, are also important targets. We have shown that the isolated 26S proteasome is one such target and here we wish to substantiate it within the cell, in situ.

Materials and methods—We used confocal microscopy to quantitatively detect and subcellularly localise radiation-induced 26S proteasome inhibition in cells expressing an ornithine decarboxylase degron that targets a fused *Zoanthus* species green (ZsGreen) fluorescent protein reporter specifically to the 26S proteasome.

Results—Exposure of cells to a range of radiation doses, even as low as 0.05 Gy inhibited 26S activity within minutes. Initially, punctate nuclear ZsGreen fluorescence was observed that became cytoplasmic after seven hours – a pattern distinct from the diffuse homogeneous fluorescence of cells incubated in the conventional proteasome inhibitor MG-132.

Conclusions—Our study clearly indicates that the 26S proteasome is a radiation target with physiological consequences and introduces a new perspective in mechanistic investigations of cellular responses to stresses.

Keywords

Proteasome; fluorescence confocal microscopy; ionising radiation; ornithine decarboxylase degron-Zs-Green

Introduction

Cells respond to stresses, such as radiation, by altering their levels of expression of critical, short-lived, regulatory proteins so as to initiate processes such as DNA transcription and repair, cell cycle arrest, and apoptosis. Activation of the signaling pathways involves addition and/or

© 2009 Informa Healthcare USA, Inc.

Correspondence: Keisuke S. Iwamoto, Roy E. Coats Research Laboratories, Department of Radiation Oncology, David Geffen School of Medicine at UCLA, 10833 Le Conte Ave., Los Angeles, CA 90095-1714, USA, Tel: +1 310 206 5595. Fax: +1 310 206 1260. kiwamoto@mednet.ucla.edu.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

removal of phosphate, acetyl, or other chemical groups and this integrates with ubiquitination that, amongst other possible outcomes, targets proteins for proteolysis through the 26S proteasome. Modification of protein stability is therefore a primary event in stress signaling.

For almost all labile native proteins, post-translational modification with polyubiquitin (poly-Ub) is the sine qua non for proteasomal recognition and degradation. This involves a complex enzymatic cascade of E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes and E3 ubiquitin-protein ligases (Chau et al. 1989, Pagano et al. 1995, Thrower et al. 2000, Dong et al. 2004). The receptor for recognition of polyubiquitinated proteins includes the S6' ATPase subunit of the 19S regulatory complex that is part of an ATPase ring that functions to assist in protein unfolding and opening the pore to the 20S core chambered protease complex to which it abuts (Hershko 1997, Varshavsky 2003, 2005, Klotzel 2004). The 20S core is a symmetrical structure of four hetero-oligomeric rings that contains the enzymatic beta subunits sequestered in the middle (alpha1-7, beta1-7, beta1-7, alpha1-7) (Tanaka et al. 1988, Tanahashi et al. 1993, Unno et al. 2002), which are highly conserved, although inducible variant replacements (Imp2, 7 and 10) have evolved, most likely to assist in development of the immune system (Takami et al. 1997).

In spite of the compelling need for exquisite specificity in the degradation of regulatory proteins, which polyubiquitin provides largely through E3 ligase function, there exist a small, limited number of proteins that can be recognised by the 26S proteasome in a ubiquitin-independent manner. The best characterised of these is murine ornithine decarboxylase (mODC) (Hoyt et al. 2005). The 37 carboxyterminal amino acids of ODC (cODC) are required and sufficient for 26S proteasome recognition and ODC degradation (Ghoda et al. 1992a, 1992b, Hoyt et al. 2005). cODC directly targets the same subunit in the 19S cap that recognises polyubiquitin chains, implying molecular mimicry (Zhang et al. 2003). In addition to this tethering site, it also has an extended unfolded region, that is required for insertion of proteins into the chamber (Takeuchi et al. 2007). Fusion of cODC to proteins destabilises them (Hoyt et al. 2003) and fusion to fluorescent proteins produces a reporter gene product that does not accumulate to any extent within most cells under normal conditions (Li et al. 1998) but can be used to assess proteasome inhibition. The validity of this approach was demonstrated in a reverse genetic screen for proteasome inhibition following small hairpin RNA (shRNA) targeting of individual proteasome subunits (Paddison et al. 2004); only shRNA that affected the 26S proteasome stabilised expression.

In this study we stably transduced human prostate cancer (PC3) cells with a ZsGreen-cODC fusion gene to assess effects of exposure to ionising radiation on proteasome function. We had previously reported that the rate of degradation of artificial fluorogenic peptides by extracted 26S, but not 20S or 20S + 19S, proteasomes (Pajonk and McBride 2001, Pervan et al. 2005) was inhibited by cell irradiation. All three major enzymatic functions, chymotrypsin-like, trypsin-like, and peptidylglutamyl-hydrolyzing-like, were affected after doses as low as 10cGy and as high as 20Gy, and as early as 30 min after irradiation (Pajonk and McBride 2001). Inhibition was incomplete; the rate of peptide hydrolysis being slowed by 40–50%. The target appears to be the 19S cap of the 26S proteasome and probably an ATPase component (Pervan et al. 2005), but other targets could not be excluded (Liu et al. 2006). It is worth noting that inhibition was seen whether whole cells or partially purified proteasomes were irradiated, suggesting that the 26S proteasome is a direct target of radiation. Free radicals were required for radiation-induced inhibition, in keeping with the known redox sensitivity of the structure (Ding et al. 2006, Jung et al. 2006, Lev et al. 2006, Poppek et al. 2006). Not surprisingly, ubiquitinated proteins accumulate after irradiation (Soldatenkov and Dritschilo 1997, Pervan et al. 2005).

We sought to confirm these findings in a more physiologic and relevant in situ system using the ZsGreen-cODC reporter gene. The study has implications for the mechanism underlying the multiple radiation-induced molecular changes in gene expression that underpin cellular consequences of exposure such as cell cycle arrest, repair, and cell death.

Methods

Cells

The human prostate carcinoma line PC3 was obtained from American Type Culture Collection (Manassas, VA, USA). This cell line was maintained at 37°C and 5% CO₂ in Dulbecco's Modification of Eagle's Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Omega Scientific, Tarzana, CA, USA) and 1% antibiotic-antimycotic (Mediatech, Inc., Manassas, VA, USA) (complete medium) prior to retroviral transduction.

Retroviral Transduction

Retroviral vectors were used to increase the efficiency of transduction to avoid clonal selection of lines that might confound interpretation of results. The ZsGreen-cODC fusion sequence from pZsProSensor-1 (BD Biosciences, San Jose, CA, USA) was cloned into the BamHI and EcoRI sites of the retroviral vector pQCXIN (BD Biosciences) using NotI-EcoRI DNA oligonucleotide adaptors (EX Clone Systems, New Orleans, LA, USA). pQCXIN/ZsGreen-cODC was transfected into GP2-293 pantropic retroviral packaging cells (BD Biosciences) and the collected retroviral supernatants were filtered and used (2×) to infect the PCS cell line in the presence of 8 µg/ml polybrene. PC3-ZsGreen-cODC cells were selected and maintained in the presence of 1 mg/ml G418 (Invitrogen).

Irradiation and drug treatment

Adherent PC3-ZsGreen-cODC cells in logarithmic growth were irradiated at room temperature using a MARK-1-30 ¹³⁷Cs-irradiator (J.L. Shepherd and Associates, San Fernando, CA, USA) at a dose rate of 4.89 Gy/min. 100 mM stock solution of proteasome inhibitor MG-132 (Sigma-Aldrich, St Louis, MO, USA) was prepared in dimethyl sulfoxide (DMSO) (Fisher Scientific, Pittsburgh, PA, USA) and stored at -20°C in 50 ml aliquots. Cells were treated with 5 µM MG-132 in complete medium and were incubated at 37°C for 3 h, unless noted otherwise, prior to imaging.

Flow cytometry

Adherent PC3-ZsGreen-cODC cells were treated as described above. Immediately prior to scoring, cells were trypsinised, washed, and suspended in PBS (phosphate buffered saline). The single-cell suspension was loaded onto a FACScalibur flow cytometer (BD Biosciences). Non-transfected PC3 parent cells were used as negative controls on the FL1 window.

Detection of ubiquitinated proteins

Isolation and immunoblotting of ubiquitinated proteins were carried out as previously described (Pervan et al. 2005). Briefly, PC3 cells were lysed in ice-cold lysis buffer [20 mM Tris-HCl (pH 7.4), 1% NP40, 137 mM NaCl, 50 µM EDTA, 1 mM PMSF, 1 µM pepstatin A, 1 µM leupeptin, and 5 mM N-ethylmaleimide] (Sigma-Aldrich) for 20 min at 4°C with occasional rocking. Cells were further disrupted by passage through a 21-gauge needle. Supernatants were collected after centrifugation at 20,000 g for 10 min and protein concentrations were determined. Cell lysates (protein, 1 mg/ml) were incubated with 25 µl of p62-derived ubiquitin-binding domain immobilised on agarose beads (Affinity Research Products Ltd., Exeter, UK). Following a 20-min incubation at room temperature, the beads were washed three times with 50 mM Tris (pH 7.5) and 0.1% bovine serum albumin. Bound

proteins were eluted in 50 μ l of the sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 25% glycerol, 0.01% bromophenol blue, 14.4 mM β -mercaptoethanol] (Sigma-Aldrich) and heated to $>95^{\circ}\text{C}$ for 4–5 min. Supernatants were collected following centrifugation at 12,000 g for 30 sec and separated by SDS-PAGE (10–20%) and immunoblotted with anti-ubiquitin or anti-I κ B α antibody (Affinity Research Products Ltd). Blots were quantitated using densitometric values from ImageJ (Rasband 1997–2008).

Confocal microscopy

Approximately 7×10^5 cells were plated each into several 35 mm glass bottom culture dishes (MatTek Corporation, Ashland, MA, USA) appropriate for confocal microscopy and incubated overnight at 37°C . ZsGreen fluorescence was detected using a TCS-SP-MP Inverted Confocal Microscope (Leica, Wetzlar, Germany). Images presented are representative of an entire viewable area and contain approximately equal numbers of cells. Once an acceptable image was achieved, the same gain/ voltage settings were used for all images within a given experiment, thus allowing for normalisation and valid quantification.

Statistics

For quantitation, in each of three different experiments, images of approximately 200 cells per field were analysed. Each 512×512 pixel image was broken into nine equal size subfields and ImageJ (Rasband 1997–2008) was used to plot a histogram of the distribution of grey levels from 0–255, with value-255 corresponding to the brightest fluorescence. A list of pixel values and a mean pixel value and standard deviation was generated for each subfield. A background-noise image was taken by selecting areas with no cells. The histogram of these images was used to determine grey level values of non-signal events that could be subtracted as background from all images. This technique effectively facilitated the exclusion of background noise evident in the areas where there were no cells. Subsequently, the standard deviations for the controls were averaged over the nine subfields and a pixel value representing one of the 256 shades of green three standard deviations above the mean was used as a cut-off point for positivity for all images. Summation of the values beyond this pixel cutoff value generated a number representing fluorescent intensity for each of the nine subfields. This was then averaged across subfields, a standard deviation was derived, and plotted for each treatment condition. The statistical significance of the difference in the fluorescence histogram between the control and irradiated samples was examined using the Analysis of variance between groups (ANOVA) test. Stata 8.0 (StataCorp LP, College Station, TX, USA) statistical software was used for the analysis with a statistical significance considered at the 5% level.

Results

ZsGreen expression

The effects of MG-132 could be detected by flow cytometry, which confirmed ZsGreen stabilisation after 5 μM of MG-132 that was seen after 5 h and that increased with time up to 24 h (Figure 1a). Specific proteasome inhibitors PS-341 (Bortezomib/ Velcade) and NPI-0052 (Salinosporamide A) also gave similar results (data not shown). The important observation to note is that the fluorescent fine structure cannot be detected by flow cytometry, which integrates the fluorescent intensity over the whole cell giving very small shifts compared to the controls (Figure 1b). The vast majority of PC3-ZsGreen-cODC retrovirally transduced cells expressed no green fluorescence by confocal microscopy, although a rare cell was positive. We believe that these represent an interesting subpopulation of cells that we are subjecting to a separate investigation. In contrast, when 5 μM of the proteasome inhibitor MG-132 was added, almost all cells were positive by confocal microscopy for ZsGreen at 24 h (Figure 2a), demonstrating that the reporter gene was working as anticipated.

Radiation inhibits 26S proteasome in PC3-ZsGreen-cODC cells

Having validated the reporter gene assay for proteasome function using MG-132, the effect of radiation on ZsGreen expression was examined in PC3-ZsGreen-cODC cells. Confocal microscopy showed that ZsGreen became evident within 10 min following 2 Gy irradiation (Figure 2a). Interestingly, the distribution of ZsGreen was different after irradiation than after MG-132 treatment. Punctate foci of fluorescence were seen after irradiation while MG-132 showed a more diffuse pattern (Figure 2a). In a time-course experiment, the foci initially were associated with the nuclear region, especially over the first 90 min after irradiation, but by 7 h had become more cytoplasmic (Figure 2a). These spatial and temporal changes were independent of dose, and indicate an unexpected dynamic potential rearrangement of the proteasomes following irradiation. Using ImageJ to quantify the images, the spectral profiles did not change significantly with time after irradiation, although the location of the fluorescence did. Figure 2b shows approximately a 3-fold increase in ZsGreen expression within 10 min of 2 Gy irradiation that was maintained up to 24 h. Statistical analysis confirmed the significance of the increased fluorescence at all time points (10 min, 30 min, 1 h, 1.5 h, 7 h, and 24 h post-irradiation) with *p*-values less than 0.001 except for times 1.5 h and 7 h, which were significant with *p*-values equal to 0.017 and 0.001, respectively.

Radiation-induced inhibition is dose-dependent at low doses

Prior investigation by our lab using extracts from cells examined the rate of liberation of the fluorogenic compound 7-amido-4-methylcoumarin (AMC) from the peptide suc-LLVY-AMC following cell or extract irradiation and concluded that radiation-induced inhibition occurred after a dose as low as 0.1 Gy and varied in a non-linear fashion with dose up to 2 Gy, as assessed 3 h post-treatment of ECV304 bladder carcinoma (Pajonk and McBride 2001). We later observed radiation-induced inhibition in PC3 prostate carcinoma cells to be dose-independent 15 min after 1–20 Gy using the same fluorogenic technique (Pervan et al. 2005). In an attempt to clarify the response in the low dose region, PC3-ZsGreen-cODC cells were irradiated with 0.05–2.0 Gy and examined by confocal microscopy 1.5 h later. The images (Figure 3a) and their graphical representation (Figure 3b) show that the proteasome is inhibited by doses as low as 0.05 Gy and inhibition is as marked at low as at high doses with no obvious linear dose-dependent relationship. Statistical analysis confirmed the significance of the fluorescence compared to the control for all dose points (0.05, 0.10, 0.20, 0.50, 1.0, and 2.0 Gy) as being <0.001.

Radiation-induced accumulation of ubiquitinated proteins

To confirm that the increased Zs-Green fluorescence reflects low-dose radiation-induced inhibition of the proteasome and subsequent manifest cellular consequences, we quantified the level of ubiquitinated proteins in the cells before and after exposure to radiation. Figure 4a demonstrates a statistically significant increase of approximately 35% in total cytosolic ubiquitinated proteins 30 min following exposure to a low dose of 0.25 Gy. Further, the same dose of radiation was sufficient to double ubiquitinated IκBα levels.

Discussion

We have utilised a ubiquitin-independent ZsGreen-cODC retroviral vector reporter system for 26S proteasome function to analyse the effects of radiation on the rate of protein degradation. We focus on the 26S proteasome because we previously showed it to be a target for radiation, whereas the 20S and 20S + 11S structures were not (Pervan et al. 2005). The ZsGreen-cODC system targets the 26S proteasome without the need for post-translational modification so the effects of proteasome inhibition can be readily interpreted without regard to the potential secondary effects of irradiation on conjugation or deconjugation systems.

To eliminate the possibility that the ODC fragment itself may be regulated or altered directly by radiation and may not reflect radiation-induced proteasome inhibition, we are presently constructing a ubiquitinated red fluorescent protein reporter, which is independent of the ODC degradation pathway and which could be used simultaneously with the ZsGreen-cODC reporter. However, in the mean-time, we do have circumstantial evidence that the radiation effect that we see is not an artifact of ODC response to irradiation. We have stably transduced the ODC reporter into the human glioblastoma cell lines U87MG and U87MG-vIII, a derivative of U87MG with constitutively active epidermal growth factor receptor (EGFR) (Kim et al. 2008). Using these cells we have demonstrated that radiation increases fluorescence in the parent U87MG but not in the U87MG-vIII as a result of differences in proteasome subunit expression (Kim et al. 2008). If the ODC were the direct target of the radiation, then one would expect both cell lines to show increased fluorescence following irradiation.

The choice of substrate for these studies is critical. Our previous studies (Pajonk and McBride 2001, Pervan et al. 2005), and these studies suggest that the rate of degradation is slowed by irradiation and inhibition is not as complete as it is with MG-132, even though MG-132 is selective in the protease it affects while irradiation affects all three major activities (Pervan et al. 2005). This is not surprising, as the target of radiation appears to be in the 19S regulatory subunit, not the enzymes themselves. In addition, it seems that this is a physiological response that might be shared by other oxidative stresses (Ding et al. 2006, Jung et al. 2006, Lev et al. 2006, Poppek et al. 2006), which adds to its relevance. From a practical viewpoint, if degradation rate is slowed, molecules with a very rapid turnover and/or a slow rate of production may not accumulate, while molecules with slow turnover and/or a fast production rate may accumulate without treatment. The fact that the vast majority of PC3-ZsGreen-cODC retrovirally transduced cells expressed no green fluorescence by confocal microscopy, while almost all did after radiation exposure indicates that this system is able to detect physiologically relevant changes in degradation rate.

The choice of detection system is also of importance. Following irradiation, ZsGreen accumulated primarily in sequestered foci localised in a compartment-specific manner within the cell, while after MG-132 treatment accumulation was more diffuse. The reason may be that irradiation affects only 26S proteasomes and all enzymatic activities are inhibited (Pervan et al. 2005), while MG-132 inhibits all proteasome species and primarily only chymotrypsin-like activity. The punctate localisation may represent sites that are particularly rich in 26S proteasomes, but what was intriguing was that the localisation of the punctate foci changed with time after irradiation and this suggests ongoing physiological processes. Up until roughly 90 min post-irradiation, the foci appear nuclear at all doses tested, whereas by 7 h the foci were more cytoplasmic with very little ZsGreen within the nucleus. Our earlier studies indicated that proteasomes in both cytoplasmic and nuclear locations could be inhibited by irradiation (Pervan et al. 2005) and therefore the temporal changes in localisation of ZsGreen might represent, in part, the migration of proteasomes to intracellular sites in response to demand. Proteasomes are known to migrate to DNA double strand breaks and may be required for their repair (Krogan et al. 2004, Jacquemont and Taniguchi 2007). They are also heavily involved in the retrotranslocation pathway for misfolded proteins in the endoplasmic reticulum - an important, possibly late, physiological adaptation to stress (Ye 2005). Most remarkable is the fact that they might be involved in cytoplasmic-nuclear shuffling, which may be a mechanism for delivery of anthracyclins into the nucleus (Kiyomiya et al. 2001). In any event, clearly, our observations illustrate a dynamic temporal and spatial reorganisation of proteasome function following ionising radiation and imply the importance of being able to spatially identify specific proteasomal responses in order to fully understand the consequences of radiation-induced inhibition.

In our studies, while previously flow cytometry was sufficient to detect ZsGreen accumulation after irradiation of PC3 cells transiently transfected with the ZsGreen-cODC reporter (Pervan et al. 2005), flow cytometry was not an efficient way to measure 26S proteasome inhibition in our stable ZsGreen-cODC-producing line after irradiation, although we were able to show accumulation 5 h after MG-132 treatment (Figure 1). This discrepancy is probably a question of copy number and protein levels. The time course we observed with flow cytometry, however, is not dissimilar to that seen by Luker et al. (2003) using the Ub4-GFP reporter gene system to chart the response to the proteasome inhibitor PS-341. On the other hand, our confocal microscopy experiments showed ZsGreen stabilisation within minutes of exposure and confirm in situ what we found earlier using artificial fluorogenic peptide substrates and extracts from irradiated cells (Pajonk and McBride 2001, Pervan et al. 2005). Furthermore, the sensitivity and high spatial resolution of our present method allows us to explain the partial inhibition of proteasome activity by radiation seen in our previous studies as the result of the total inhibition of a fraction of the cellular proteasomes rather than partial inhibition of all of them.

The rapid timing of the radiation-induced response and the effectiveness of extremely low doses are remarkable. Almost all cells responded with increased ZsGreen accumulation within minutes of exposure to irradiation at doses above and equal to 0.05 Gy. The rapidity of the effect is similar to what we have shown previously (Pajonk and McBride 2001, Pervan et al. 2005) using fluorogenic substrates, and suggests that it is almost immediate, since 10 min is the fastest we can examine slides post-irradiation. What seems slightly different is the maintenance of the response. Our previous data (Pajonk and McBride 2001, Pervan et al. 2005) showed proteasome function returning closer to baseline levels after 24 h, yet our ZsGreen-cODC studies still show inhibition at 24 h, possibly reflecting ex situ versus in situ assay differences. We observed here, as before (Pajonk and McBride 2001, Pervan et al. 2005), a degree of variability in the extent of proteasome inhibition with dose that was non-linear and undulated in this very low dose range, while still being statistically elevated above control levels. We are uncertain as to the significance of this fluctuation with dose, but generally conclude that the proteasome is a highly sensitive indicator of cellular stress following radiation insult.

Clearly, our method allows examination of both the inhibition of the 26S proteasome by and the cellular localisation dynamics of this inhibition induced by radiation – at clinically relevant doses – and should permit investigation of mechanisms. The lack of a dose-response and the existence of a basal level of function following irradiation suggest that the proteasome acts as a binary switch mechanism to change the molecular phenotype of the cell in response to stress. Obviously, radiation-induced alterations in gene expression must occur against a backdrop of altered proteolysis. In concert with this, we have shown previously and in this study that at low radiation doses, proteasome inhibition minimises NF- κ B responses by preventing I κ B α degradation, while at high doses alternative NF- κ B induction pathways are activated (Pajonk and McBride 2001, McBride et al. 2002). Here we have demonstrated that low doses of radiation doubled the level of ubiquitinated I κ B α in a relatively short period of time after exposure, which can potentially quell major cellular first-responses to stimuli that depend on NF- κ B signaling. The concept that emerges is that radiation-induced 26S proteasome inhibition is most relevant for a subset of regulatory proteins that are turning over rapidly and that have evolved to make appropriate responses to stress, cytokines, free radicals, and radiation. Proteins with a slow turnover will not be greatly affected.

Remarkably, ATPase- and Ub-independent core 20S and 20S plus 11S proteasomes are not inhibited (Pervan et al. 2005), indicating that the radiation target is in the 19S complex. In keeping with this, all 3 major proteolytic activities of the 26S proteasome are inhibited and ATPase function is affected (Pervan et al. 2005). Since the 26S proteasome is affected, the degradation of short-lived regulatory proteins is slowed, while other housekeeping functions

such as removal of damaged proteins may continue unabated. Our preliminary findings suggest the unfolded protein response is not activated, in keeping with a spatially restricted response. However, we, and others, have shown that radiation mimics the effects of proteasome inhibitors like PS-341 in generating reactive oxygen species, affecting mitochondrial function, increasing levels of ubiquitinated proteins, stabilising expression of regulatory proteins such as p27, Bax, p53, and I κ B α , and causing cell cycle arrest and apoptosis of tumour cells (Pajonk et al. 1999, Pervan et al. 2005, Ding et al. 2006, McBride et al. 2003, Papa et al. 2007) and radiation-induced 26S proteasome inhibition clearly has physiologic consequences.

Radiation-induced 26S proteasome inhibition occurs whether cells or purified 26S proteasomes are irradiated and with similar kinetics and dose response (Pajonk and McBride 2001, Pervan et al. 2005), indicating that the 26S proteasome is a direct target for irradiation. However, we cannot conclude that the mechanisms by which radiation affects 26S proteasome function within a cell are identical to those that affect purified proteasomes, at least until we are able to identify the changes that take place at the proteomic level. Further, we have shown that purified 26S proteasomes are inhibited by exposure to hydrogen peroxide, N-acetyl cysteine (Pajonk et al. 2002), glutathione, and tempol (Pervan et al. 2005). The concentration of drug is important, but low levels of free radical scavengers (Pervan et al. 2005) and hypoxia (Pajonk et al. 2006) can prevent radiation-induced inhibition suggesting that the 26S proteasome senses changes in redox. Others have shown using other systems the sensitivity of the proteasome to oxidative stress (Reinheckel et al. 1998, 2000).

The relevance of these findings for radiobiology and radiotherapy are immense. For decades the proteasome has languished in the role of garbage disposal unit for the cell. It is only relatively recently that it has become appreciated that the heterogeneity in structure, function, and spatial distribution allows division of labor between proteasomes, that the proteasome is a dynamic entity that can respond to extracellular and intracellular signals, including changes in metabolism, redox, hypoxia/reperfusion, hyperthermia, oxidative stress, as well as irradiation. The mechanisms by which such responses are made have yet to be elucidated, but may be post-translational, compositional, spatial, or occur by rapid de novo production of proteasomes. We have recently shown that radiation can alter proteasome subunit expression and therefore 26S composition with clinically relevant doses of 2 Gy in a human glioblastoma cell line (U87MG) but not in a derivative cell line (U87MG-vIII) made radioresistant with a constitutively active EGFR (Kim et al. 2008). Whether the proteasome plays a direct role in cellular radiosensitivity awaits further investigation.

Cellular compartmentalisation of proteasomes appears to be important in cell function as implied by experiments showing rapid diffusion of labeled proteasomes within either the nucleus or cytosol, but slow interchange between these two compartments, usually unidirectionally from the latter to the former (Reits et al. 1997). Moreover, addition of EGF to cells induces redistribution of proteasomes leading to accumulation in the nucleus suggesting that transport of proteasomes into the nucleus requires phosphorylation (Rivett 1998, Evdonin et al. 2001). The mechanisms of proteasome associations with specific intracellular compartments are largely unknown but a recent report has demonstrated that FK506-binding protein 38 (FKBP38), an endoplasmic and mitochondrial outer membrane protein, binds the 26S proteasomes. Knockout of FKBP38 reduced activity and abundance of organelle-associated proteasome activity (Hirsch and Ploegh 2000). There is mounting evidence for a link between proteasome inhibition and disruption of mitochondrial homeostasis and increases in reactive oxygen species (ROS), especially in a wide variety of neurodegenerative conditions (Leach et al. 2001, Sullivan et al. 2004, Nakagawa et al. 2007, Papa et al. 2007, Torres and Perez 2008). Furthermore, irradiation of cells can induce mitochondria to generate reactive oxygen and nitrogen species, which underscores the significance of links among proteasome compartmentalisation, organelle-association, and inhibition, with irradiation. We have

preliminary data suggesting that proteasome inhibition colocalises with mitochondria (data not shown). In order to study how radiation acts on this system, it is critical to be able to identify in a quantitative way the proteasomes that are contained within a cell, their post-translational modifications, their location, and their function in vivo and in vitro. Our study reported here is one of the first steps in facilitating such investigations.

Conclusion

In summary, using a ubiquitin-independent bio-fluorescent reporter, ZsGreen-cODC, we have used confocal microscopy to detect 26S proteasome inhibition in situ after exposure to ionising radiation. We found that the effect is very rapid (10 min) after even very low doses (0.05 Gy). This may be one of the first 'danger' responses a cell makes to radiation challenge. The strength of this signal may be critical for subsequent decisions by the cell, such as DNA repair and associated DNA-damage responses, apoptosis or cell cycle arrest, and it may be a target for manipulation to increase efficacy of radiotherapy, as is indicated by the enhanced radiation response of tumours exposed to proteasome inhibitor drugs, like Velcade (Pervan et al. 2001). Given the proteasome's growing role in critical cellular processes such as growth, cell cycle control, DNA damage repair, and apoptosis, the proteasome is plausibly a master controller of response to irradiation and other cellular stresses. Finally, the studies suggest caution in the interpretation of measurements of protein half-lives made using incorporation of radioactive materials into growing cells, which will expose the cells to small but not insignificant doses of radiation, until the effects of these on proteasome function are evaluated.

References

- Chau V, Tobias JW, Bachmair A, Marriott D, Ecker DJ, Gonda DK, Varshavsky A. A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* 1989;243:1576–1583. [PubMed: 2538923]
- Ding Q, Dimayuga E, Keller JN. Proteasome regulation of oxidative stress in aging and age-related diseases of the CNS. *Antioxidants and Redox Signaling* 2006;8:163–172. [PubMed: 16487050]
- Dong X, Liu J, Zheng H, Glasford JW, Huang W, Chen QH, Harden NR, Li F, Gerdes AM, Wang X. In situ dynamically monitoring the proteolytic function of the ubiquitin-proteasome system in cultured cardiac myocytes. *American Journal of Physiology - Heart and Circulatory Physiology* 2004;287:H1417–H1425. [PubMed: 15105173]
- Evdonin AL, Tsupkina NV, Konstantinova IM, Medvedeva ND. Effect of EGF on nuclear-cytoplasmic distribution of proteasomes in A-431 cells. *Tsitologiya* 2001;43:142–147. [PubMed: 11347469]
- Ghoda L, Basu HS, Porter CW, Marton LJ, Coffino P. Role of ornithine decarboxylase suppression and polyamine depletion in the antiproliferative activity of polyamine analogs. *Molecular Pharmacology* 1992a;42:302–306. [PubMed: 1513327]
- Ghoda L, Sidney D, Macrae M, Coffino P. Structural elements of ornithine decarboxylase required for intracellular degradation and polyamine-dependent regulation. *Molecular Cell Biology* 1992b; 12:2178–2185.
- Hershko A. Roles of ubiquitin-mediated proteolysis in cell cycle control. *Current Opinion in Cell Biology* 1997;9:788–799. [PubMed: 9425343]
- Hirsch C, Ploegh HL. Intracellular targeting of the proteasome. *Trends in Cell Biology* 2000;10:268–272. [PubMed: 10856929]
- Hoyt MA, Zhang M, Coffino P. Ubiquitin-independent mechanisms of mouse ornithine decarboxylase degradation are conserved between mammalian and fungal cells. *Journal of Biological Chemistry* 2003;278:12135–12143. [PubMed: 12562772]
- Hoyt MA, Zhang M, Coffino P. Probing the ubiquitin/proteasome system with ornithine decarboxylase, a ubiquitin-independent substrate. *Methods in Enzymology* 2005;398:399–413. [PubMed: 16275346]
- Jacquemont C, Taniguchi T. Proteasome function is required for DNA damage response and fanconi anemia pathway activation. *Cancer Research* 2007;67:7395–7405. [PubMed: 17671210]

- Jung T, Engels M, Kaiser B, Poppek D, Grune T. Intracellular distribution of oxidized proteins and proteasome in HT22 cells during oxidative stress. *Free Radical Biology and Medicine* 2006;40:1303–1312. [PubMed: 16631520]
- Kim K, Brush JM, Watson PA, Cacalano NA, Iwamoto KS, McBride WH. Epidermal growth factor receptor vIII expression in U87 glioblastoma cells alters their proteasome composition, function, and response to irradiation. *Molecular Cancer Research* 2008;6:426–434. [PubMed: 18337449]
- Kiyomiya K, Matsuo S, Kurebe M. Mechanism of specific nuclear transport of adriamycin: The mode of nuclear translocation of adriamycin-proteasome complex. *Cancer Research* 2001;61:2467–2471. [PubMed: 11289116]
- Kloetzel PM. Generation of major histocompatibility complex class I antigens: Functional interplay between proteasomes and TPPII. *Nature Immunology* 2004;5:661–669. [PubMed: 15224091]
- Krokan NJ, Lam MH, Fillingham J, Keogh MC, Gebbia M, Li J, Datta N, Cagney G, Buratowski S, Emili A, Greenblatt JF. Proteasome involvement in the repair of DNA double-strand breaks. *Molecular Cell* 2004;16:1027–1034. [PubMed: 15610744]
- Leach JK, Van Tuyle G, Lin PS, Schmidt-Ullrich R, Mikkelsen RB. Ionizing radiation-induced, mitochondria-dependent generation of reactive oxygen/nitrogen. *Cancer Research* 2001;61:3894–3901. [PubMed: 11358802]
- Lev N, Melamed E, Offen D. Proteasomal inhibition hypersensitizes differentiated neuroblastoma cells to oxidative damage. *Neuroscience Letters* 2006;399:27–32. [PubMed: 16584840]
- Li X, Zhao X, Fang Y, Jiang X, Duong T, Fan C, Huang CC, Kain SR. Generation of destabilized green fluorescent protein as a transcription reporter. *Journal of Biological Chemistry* 1998;273:34970–34975. [PubMed: 9857028]
- Liu X, Huang W, Li C, Li P, Yuan J, Li X, Qiu XB, Ma Q, Cao C. Interaction between c-Abl and Arg tyrosine kinases and proteasome subunit PSMA7 regulates proteasome degradation. *Molecular Cell* 2006;22:317–327. [PubMed: 16678104]
- McBride WH, Iwamoto KS, Syljuasen R, Pervan M, Pajonk F. The role of the ubiquitin/proteasome system in cellular responses to radiation. *Oncogene* 2003;22:5755–5773. [PubMed: 12947384]
- McBride WH, Pajonk F, Chiang CS, Sun JR. NF-kappa B, cytokines, proteasomes, and low-dose radiation exposure. *Military Medicine* 2002;167:66–67. [PubMed: 11873521]
- Nakagawa T, Shirane M, Iemura S, Natsume T, Nakayama KI. Anchoring of the 26S proteasome to the organellar membrane by FKBP38. *Genes and Cells* 2007;12:709–719.
- Luker GD, Pica CM, Song J, Luker KE, Piwnica-Worms D. Imaging 26S proteasome activity and inhibition in living mice. *Nature Medicine* 2003;9:969–973.
- Paddison PJ, Silva JM, Conklin DS, Schlabach M, Li M, Aruleba S, Balija V, O'Shaughnessy A, Gnoj L, Scobie K, Chang K, Westbrook T, Cleary M, Sachidanandam R, McCombie WR, Elledge SJ, Hannon GJ. A resource for large-scale RNA-interference-based screens in mammals. *Nature* 2004;428:427–431. [PubMed: 15042091]
- Pagano M, Tam SW, Theodoras AM, Beer-Romero P, Del Sal G, Chau V, Yew PR, Draetta GF, Rolfe M. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science* 1995;269:682–685. [PubMed: 7624798]
- Pajonk F, Grumann T, McBride WH. The proteasome inhibitor MG-132 protects hypoxic SiHa cervical carcinoma cells after cyclic hypoxia/reoxygenation from ionizing radiation. *Neoplasia* 2006;8:1037–1041. [PubMed: 17217621]
- Pajonk F, McBride WH. Ionizing radiation affects 26s proteasome function and associated molecular responses, even at low doses. *Radiotherapy and Oncology* 2001;59:203–212.
- Pajonk F, Pajonk K, McBride WH. Inhibition of NF-kappaB, clonogenicity, and radiosensitivity of human cancer cells. *Journal of the National Cancer Institute* 1999;91:1956–1960. [PubMed: 10564680]
- Pajonk F, Riess K, Sommer A, McBride WH. N-acetyl-L-cysteine inhibits 26S proteasome function: implications for effects on NF-kappaB activation. *Free Radicals in Biology and Medicine* 2002;32:536–543.
- Papa L, Gomes E, Rockwell P. Reactive oxygen species induced by proteasome inhibition in neuronal cells mediate mitochondrial dysfunction and a caspase-independent cell death. *Apoptosis* 2007;12:1389–1405. [PubMed: 17415663]

- Pervan M, Iwamoto KS, McBride WH. Proteasome structures affected by ionizing radiation. *Molecular Cancer Research* 2005;3:381–390. [PubMed: 16046549]
- Pervan M, Pajonk F, Sun JR, Withers HR, McBride WH. Molecular pathways that modify tumor radiation response. *American Journal of Clinical Oncology* 2001;24:481–485. [PubMed: 11586100]
- Poppek D, Keck S, Ermak G, Jung T, Stolzing A, Ullrich O, Davies KJ, Grune T. Phosphorylation inhibits turnover of the tau protein by the proteasome: Influence of RCAN1 and oxidative stress. *Biochemical Journal* 2006;400:511–520. [PubMed: 16939415]
- Rasband, WS. Image J. Bethesda, Maryland, USA: U.S. National Institutes of Health; 1997–2008. <http://rsb.info.nih.gov/ij/>, 1997–2008.
- Reinheckel T, Sitte N, Ullrich O, Kuckelkorn U, Davies KJ, Grune T. Comparative resistance of the 20S and 26S proteasome to oxidative stress. *Biochemical Journal* 1998;335(Pt 3):637–642. [PubMed: 9794805]
- Reinheckel T, Ullrich O, Sitte N, Grune T. Differential impairment of 20S and 26S proteasome activities in human hematopoietic K562 cells during oxidative stress. *Archives of Biochemistry and Biophysics* 2000;377:65–68. [PubMed: 10775442]
- Reits EA, Benham AM, Plougastel B, Neeffjes J, Trowsdale J. Dynamics of proteasome distribution in living cells. *EMBO Journal* 1997;16:6087–6094. [PubMed: 9321388]
- Rivett AJ. Intracellular distribution of proteasomes. *Current Opinions in Immunology* 1998;10:110–114.
- Soldatenkov VA, Dritschilo A. Apoptosis of Ewing's sarcoma cells is accompanied by accumulation of ubiquitinated proteins. *Cancer Research* 1997;57:3881–3885. [PubMed: 9307264]
- Sullivan PG, Dragicevic NB, Deng JH, Bai Y, Dimayuga E, Ding Q, Chen Q, Bruce-Keller AJ, Keller JN. Proteasome inhibition alters neural mitochondrial homeostasis and mitochondria turnover. *Journal of Biological Chemistry* 2004;279:20699–20707. [PubMed: 14742431]
- Takami K, Zaleska-Rutczynska Z, Figueroa F, Klein J. Linkage of LMP, TAP, and RING3 with Mhc class I rather than class II genes in the zebrafish. *Journal of Immunology* 1997;159:6052–6060.
- Takeuchi J, Chen H, Coffino P. Proteasome substrate degradation requires association plus extended peptide. *EMBO Journal* 2007;26:123–131. [PubMed: 17170706]
- Tanahashi N, Tsurumi C, Tamura T, Tanaka K. Molecular structure of 20S and 26S proteasomes. *Enzyme Protein* 1993;47:241–251. [PubMed: 7697123]
- Tanaka K, Yoshimura T, Kumatori A, Ichihara A, Ikai A, Nishigai M, Kameyama K, Takagi T. Proteasomes (multi-protease complexes) as 20 S ring-shaped particles in a variety of eukaryotic cells. *Journal of Biological Chemistry* 1988;263:16209–16217. [PubMed: 3141402]
- Thrower JS, Hoffman L, Rechsteiner M, Pickart CM. Recognition of the polyubiquitin proteolytic signal. *EMBO Journal* 2000;19:94–102. [PubMed: 10619848]
- Torres CA, Perez VI. Proteasome modulates mitochondrial function during cellular senescence. *Free Radicals in Biology and Medicine* 2008;44:403–414.
- Unno M, Mizushima T, Morimoto Y, Tomisugi Y, Tanaka K, Yasuoka N, Tsukihara T. The structure of the mammalian 20S proteasome at 2.75 Å resolution. *Structure* 2002;10:609–618. [PubMed: 12015144]
- Varshavsky A. The N-end rule and regulation of apoptosis. *Nature Cell Biology* 2003;5:373–376.
- Varshavsky A. Regulated protein degradation. *Trends in Biochemical Science* 2005;30:283–286.
- Ye Y. The role of the ubiquitin-proteasome system in ER quality control. *Essays in Biochemistry* 2005;41:99–112. [PubMed: 16250900]
- Zhang M, Pickart CM, Coffino P. Determinants of proteasome recognition of ornithine decarboxylase, a ubiquitin-independent substrate. *EMBO Journal* 2003;22:1488–1496. [PubMed: 12660156]

Figure 1.

Time course of MG-132 or 2 Gy induced proteasome inhibition using flow cytometry. (a) PC3-ZsGreen-cODC stable lines were incubated in 5 μ M MG-132 for the given lengths of time and then scored on the FLI window using a flow cytometer. (b) PC3-ZsGreen-cODC stable lines were exposed to 2 Gy gamma irradiation and similarly scored using a flow cytometer. Note the small shift induced by irradiation compared to MG-132 especially.

Figure 2.

Time course response of proteasome inhibition after 2 Gy. (a) Representative confocal microscopy images taken 1.5, 7, and 24 hours post 2 Gy irradiation. Compared to unirradiated controls, there is a marked increase in green fluorescence at all time points. Note also the change in cellular localisation of the fluorescence with time among the irradiated cells. Also shown is the effect of 3 hours in the presence of 5 μ M MG-132; the fluorescence intensity is greater and the cellular localisation is much more homogeneous and diffuse than in the irradiated cells. Samples were kept at 37°C following irradiation until time of imaging. Bar, 50 μ m. To assist in visualisation of the cell and cell nucleus, some representative cells have been overlaid with thin grey lines at the boundaries, (b) Quantitative analysis of time course confocal imagery.

The data are normalised to the number of pixels beyond a value on the scale of 0–255 shades of green that is located on the histogram generated by ImageJ exactly three standard deviations from the mean of the control image. The pixels summed beyond this value give an accurate representation of foci of proteasome inhibition. All points are statistically significant compared to the control. Error bars indicate standard error of the mean for three independent experiments.

Figure 3.

Radiation dose response of proteasome inhibition after 1.5 hours. (a) Representative confocal microscopy images taken at 1.5 h following 0, 0.05, 0.1, or 2 Gy gamma irradiation. Samples were kept at 37°C following irradiation until time of imaging. Bar, 50 μ m. To assist in visualisation of the cell and cell nucleus, some representative cells have been overlaid with thin grey lines at the boundaries. (b) Quantitative analysis of dose response confocal imagery. The data is normalised to the number of pixels beyond a value on the scale of 0–255 shades of green that is located on the histogram generated by ImageJ exactly three standard deviations from the mean of the control image. The pixels summed beyond this value give an accurate

representation of foci of proteasome inhibition. All points are statistically significant compared to the control. Error bars indicate standard error of the mean for three independent experiments.

Figure 4.

Accumulation of ubiquitinated proteins. PC3 cells were treated as designated and ubiquitinated proteins were purified by agarose-immobilised p62-derived ubiquitin-binding domain, separated by SDS-PAGE, and immunoblotted with anti-ubiquitin (a) or with anti-I κ B α antibodies (b). Control and irradiated cells were sampled 30 min post-treatment and cells exposed to MG-132 (5 μ M) were sampled 7 h after addition of the inhibitor. Bar graphs below each blot are densitometry quantifications and are normalised to control values. Error bars indicate standard error of the mean for three independent experiments.