The Proteasome Activator PA200 Regulates Tumor Cell Responsiveness to Glutamine and Resistance to Ionizing Radiation

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Abstract

The cellular response to ionizing radiation (IR) involves a variety of mechanisms to repair damage and maintain cell survival. We previously reported that the proteasome activator PA200 promotes long-term cell survival after IR exposure. The molecular function of PA200 is to enhance proteasome-mediated cleavage after glutamate; however, it is not known how this molecular function promotes survival after IR exposure. Here, we report that upon IR exposure, cellular demand for exogenous glutamine is increased. Cells containing PA200 are capable of surviving this IR-induced glutamine demand, whereas PA200-deficient cells show impaired long-term survival. Additional glutamine supplementation reverses the radiosensitivity of PA200-knockdown cells suggesting impaired glutamine homeostasis in these cells. Indeed, PA200-knockdown cells are unable to maintain intracellular glutamine levels. Furthermore, when extracellular glutamine is limiting, cells that contain PA200 respond by slowing growth, but PA200-knockdown cells and cells in which post-glutamyl proteasome activity is inhibited are nonresponsive and continue rapid growth. This cellular unresponsiveness to nutrient depletion is also reflected at the level of the mTOR substrate ribosomal S6 kinase (S6K). Thus, inability to restrict growth causes PA200-deficient cells to continue growing and eventually die due to lack of available glutamine. Together, these data indicate an important role for PA200 and post-glutamyl proteasome activity in maintaining glutamine homeostasis, which appears to be especially important for long-term survival of tumor cells after radiation exposure, Mol Cancer Res; 10(7); 937–44. ©2012 AACR.

Introduction

The decision of tumor cells to grow or not depends on multiple signals including those from available nutrients, extracellular growth factors, damage, and stress. Stressors such as ionizing radiation (IR) slow cell growth due to cellular responses that attempt to repair DNA damage before replication (reviewed in ref. 1). IR induces reactive oxygen species and double-strand DNA breaks, both of which are sensed by elaborate detection and signaling cascades that lead to the arrest of cell cycle and induction of DNA repair proteins (reviewed in refs. 1–3). Continued growth through cell-cycle checkpoints (i.e., inability to arrest cell cycle) in response to DNA damage is associated with genomic instability and diminished long-term cell survival (4, 5). Thus, defects in proteins involved in sensing or repairing DNA damage are known to impair survival as measured by clonogenic survival assays (4, 6, 7). We have recently reported that depletion of the proteasome activator, PA200, from tumor cells impairs their clonogenic survival after radiation exposure (8). However, the mechanism by which PA200 promotes survival has not yet been reported.

The proteasome activator, PA200, is a heat/armadillo repeat protein that binds to the ends of core (or 20S) proteasomes (9, 10) and responds to IR (8, 11). Core proteasomes contain 3 active sites (chymotryptic, trypsin, and post-glutamyl) to degrade proteins. The caps (i.e., PA200 cap, 19S cap) that bind the ends of the barrel shaped core proteasome regulate the entry of substrates (reviewed in ref. 12). PA200 association enhances proteasome-mediated cleavage of peptides after the amino acid glutamate (post-glutamyl) to degrade proteins. The caps (i.e., PA200 cap, 19S cap) that bind the ends of the barrel shaped core proteasome regulate the entry of substrates (reviewed in ref. 12). PA200 association enhances proteasome-mediated cleavage of peptides after the amino acid glutamate (post-glutamyl) to degrade proteins.
Furthermore, PA200 diminution or specific inhibition of post-glutamyl proteasome activity impaired cell survival after IR exposure (8). Thus, PA200 and hybrid proteasomes, through post-glutamyl proteasome activity, promote survival after radiation exposure. Because glutamate is a precursor for intracellular glutamine generation, we investigated the relationship between radiation and glutamine homeostasis and how PA200 affects resistance to radiation through glutamine homeostasis.

Glutamine is a particularly important amino acid as a precursor for nucleotide, protein, and lipid biosynthesis as well as promoting mTOR activity (13–16). Glutamine is also rapidly converted into glutamate, which can enter the tricarboxylic acid (TCA) cycle for ATP production (14, 17). The central importance of glutamine and glutamate in energy and biosynthetic pathways may explain why tumor cells consume large quantities of glutamine for growth and proliferation (15, 17–19). However, the importance of glutamine homeostasis after exposure of cells to stressors such as IR is not understood. Here, we investigate how PA200 modulates radiation sensitivity through glutamine metabolism.

Materials and Methods

Cell lines, antibodies, and irradiation

HeLa cervical carcinoma and B16.F10 murine melanoma cell lines were grown in RPMI supplemented with 2 mmol/L (final) glutamine and 10% bovine calf serum. BJ human fibroblasts were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 2 mmol/L glutamine (final) and 10% bovine calf serum. Rabbit anti-sera recognizing PA200 has been previously described (20). All other antibodies are detailed in Supplementary Methods. Transfection was conducted using Amxa Nucleofector (Lonza) as detailed in Supplementary Methods. Radiation exposure was conducted using Phillips Mark 1 Cesium irradiator at a dose rate of 0.472 Gy/min as detailed in Supplementary Methods. Different dose rates of irradiation were used because of change of irradiation equipment at Roswell Park Cancer Institute (RPCI; Buffalo, NY) during the course of these studies.

Nutrient depletion

Immediately after transfection, cells were plated at the indicated densities in 6-well dishes (14.4 × 10^4 cells/cm^2) in 3 mL of media. For nutrient-replete conditions, media were replaced daily. Seventy-two hours after transfection, cells were lysed directly in the dish with 1% Triton X-100, 0.5 mmol/L phenylmethylsulfonylfluoride (PMSF), 5 mol/L N-ethylmaleimide (NEM), 1 mmol/L NaF, 5 mmol/L β-glycerophosphate, in TBS.

Results

Exposure of cells to IR enhanced consumption of glutamine from the media compared with nonirradiated cells (Fig. 1A). Because PA200-knockdown cells show impaired survival after IR exposure (8), we hypothesized that it may be due to the inability to survive and grow from this IR-induced glutamine demand. Therefore, we tested whether excess extracellular glutamine could reverse the radiosensitivity that we previously observed in PA200-knockdown cells. We compared clonogenic survival (which is a result of both...
survival and proliferation) of HeLa cells with and without knockdown of PA200 (Fig. 1B), in standard growth media (containing 2 mmol/L glutamine) versus media that had been supplemented with extra glutamine midway (day 7) through the 13-day clonogenic assay. Strikingly, extra glutamine addition alone enhanced survival of PA200-knockdown cells, but not control cells, after radiation exposure (Fig. 1C and D). In the presence of excess glutamine added 7 days after irradiation, PA200-knockdown cells no longer show radiosensitivity but appear radioresistant compared with control siRNA–transfected cells (Fig. 1D). Together, these data suggest that radiation induces an increased glutamine demand in cells to an extent that glutamine in the media become limiting in a long-term 13-day clonogenic survival assay (for PA200-knockdown cells). Consistent with this idea, when colonies were examined on day 7 (rather than day 13), PA200-knockdown cells do not show radiosensitivity but rather show enhanced colony formation compared with control siRNA–transfected cells (Fig. 1E). Thus, 7 days after irradiation, PA200-knockdown cells grow better than control siRNA–transfected cells.

One possibility for why PA200-depleted cells show enhanced growth when measured shortly after radiation (day 7) and why glutamine supplementation restores survival at day 13 after radiation is that PA200-knockdown cells might take up more glutamine from the media, thereby exhausting it sooner than cells that contain PA200. Consistent with this idea, the cell culture media of PA200-knockdown cells are depleted of glutamine more rapidly than control siRNA–transfected cells (Fig. 2A, compare black bar to white bar on day 2 after transfection). However, this increased glutamine uptake did not result in increased growth in typical growth media (which contains 2 mmol/L glutamine). Because typical growth media contain excess (2 mmol/L) glutamine for proliferation (21), we hypothesized that when glutamine conditions were low, the increased glutamine uptake by PA200-knockdown cells might promote enhanced growth. In low-glutamine concentrations,
colony formation (without radiation exposure) of PA200-depleted cells is greater than control siRNA-transfected cells (Fig. 2B). Cell growth is saturated at 0.2 mmol/L or more, where colony formation (on day 13) is comparable between control siRNA–transfected and PA200-knockdown cells (Fig. 2B). Similarly, enhanced growth of PA200-knockdown cells compared with control siRNA–transfected cells in low (0.05 mmol/L)-glutamine conditions is apparent in short-term growth assays (Fig. 2C). Because PA200 enhances post-glutamyl proteasome activity, we tested whether specific inhibition of post-glutamyl proteasome activity caused the same phenotype of enhanced growth in low (0.05 mmol/L) glutamine. Like PA200 inhibition, specific inhibition of post-glutamyl proteasome activity using az-NC-001 (Supplementary Fig. S1A; ref. 22) enhanced short-term growth in low (0.05 mmol/L)-glutamine conditions but not in higher (0.2 mmol/L)-glutamine conditions (Fig. 2D and E). Therefore, PA200-knockdown cells and cells in which post-glutamyl activity is specifically inhibited show enhanced growth after short-term exposure to low-glutamine conditions (even in the absence of irradiation). The enhanced growth of PA200-knockdown cells in the short-term is associated with diminished long-term growth (Supplementary Fig. S1B), consistent with the idea that deficiency in PA200 leads to enhanced growth in nutrient-deprived settings, but eventual death due to inability to restrict growth.

We hypothesized that PA200-knockdown cells grow better (in the short-term) in low-glutamine conditions because control cells limit their growth in response to glutamine limitation. From this perspective, PA200-knockdown cells do not appropriately restrict their growth in low-glutamine conditions. Therefore, we tested whether PA200-knockdown cells could restrict their growth in response to nutrient depletion that occurs over time in culture. We compared a nutrient-depleted setting with a nutrient-replete nutrient-depletion that occurs over time in culture. We replaced daily showed greater cell recovery than those without nutrient replacement (Fig. 3A, black bars). PA200-knockdown cells continued with elevated cell growth even when nutrients were depleted (Fig. 3A, white bars). Restriction of growth in response to nutrient or amino acid deprivation is determined by mTOR (23), which integrates signals from growth factors, nutrient availability, and stressors to determine whether a cell grows or not (24). A direct target of mTOR kinase activity is ribosomal S6 kinase (S6K; refs. 25, 26). Thus, as a second measure to investigate growth restriction in response to nutrient limitation, we used mTOR-mediated phosphorylation of S6K (p-S6K) as a readout. Control siRNA–transfected cells show low levels of p-S6K in nutrient-depleted settings compared with high levels in nutrient-replete conditions suggestive of responsiveness of mTOR activity to nutrient depletion and stimulation (Fig. 3B, lane 2 vs. 1). When nutrients were depleted, PA200-knockdown cells maintain high levels of p-S6K than in control cells (Fig. 3B, lane 3 vs. 1). Furthermore, nutrient replacement does not increase p-S6K in PA200-knockdown cells (Fig. 3B, lane 3 vs. 4). Similar experiments carried out at lower cell density (3.33 × 10^4 cells/cm^2) contained sufficient nutrients such that p-S6K remained high and unchanged, and cell yield was unchanged by media replacement (see Supplementary Fig. S2A). Thus, the effects of PA200 deficiency are observed most dramatically during nutrient deprivation and growth restriction that occurs at higher cell densities. These data show that when PA200 is depleted, p-S6K is nonresponsive to nutrient stress that occurs in nutrient-deprived cultures over time.

If only nutrient-replete conditions are compared, control siRNA–transfected cells are stimulated by nutrients and show higher levels of p-S6K than PA200 siRNA–transfected cells (Fig. 3B, compare lane 2 with 4, respectively). This phenotype of higher p-S6K in control siRNA–transfected cells than in PA200-knockdown cells (in nutrient-replete conditions) is reproduced in human (HeLa, BJ fibroblasts) and mouse (B16.F10 melanoma) cell lines (Fig. 3C). Consistent with blunted responsiveness to nutrients in PA200-knockdown cells, S6K phosphorylation after serum stimulation is also impaired (Fig. 3D).

Because proteasomes degrade proteins, we considered the possibility that lack of nutrient responsiveness in PA200-knockdown cells could be due to degradation or altered levels of components of the mTOR complex. Our results do not support this idea because mTOR, Raptor, and Rictor are all appreciably expressed in both PA200-knockdown and control cells (Fig. 3E). Furthermore, phosphorylated (active) forms of mTOR representing mTORC1 and mTORC2 seem to be present at high levels both in control and PA200 siRNA–transfected cells (Fig. 3E). Because mTOR activity directly correlates with cell size (27), we compared the size of cells with and without PA200 and found no difference (Fig. 3F). Rapamycin, a specific mTOR inhibitor, diminishes cell size of both PA200 and control siRNA–transfected cells, showing that mTOR is likely active and can be inhibited by rapamycin in PA200-knockdown cells (Fig. 3F). Furthermore, rapamycin also inhibited the p-S6K levels in both control and PA200-knockdown cells (Supplementary Fig. S2B). These data show that mTOR components are apparently not degraded in PA200-depleted cells. mTOR function per se is likely intact in PA200-knockdown cells, however, it is the responsiveness of mTOR targets that is altered.

Because PA200 alters pS6K responsiveness and mTOR activity has previously been shown to be regulated in response to DNA damage (28, 29), we examined the effects of PA200 on IR-induced changes in pS6K. In the presence of PA200, IR exposure leads to a dramatic decrease in p-S6K between 1 and 4 hours postirradiation (Figs. 3G and H and 4, and 24 hours after IR). However, p-S6K levels in PA200-depleted cells are not diminished 1 and 4 hours postirradiation, rather they are increased at 4 and 24 hours postirradiation (Fig. 3G and H). These data suggest that IR exposure restrains growth (i.e., lowers p-S6K levels) of control cells, but not PA200-knockdown cells. The enhanced growth of PA200-knockdown cells in glutamine-limiting conditions led us to question whether
PA200-depleted cells are more dependent on an absolute requirement for extracellular glutamine for their survival than cells containing PA200. Therefore, we tested cell growth (3 × 10^4 cells/cm^2) in media completely devoid of both glutamine and its precursor glutamate (Fig. 4A). In this case, PA200-depleted cells showed impaired growth after extended periods without glutamine and glutamate (Fig. 4A). The defect in cell growth of PA200-knockdown cells in glutamine- and glutamate-deficient media was specific as removal of glucose or serum led to decreases in growth that were identical between PA200-knockdown and control cells (Fig. 4A). Thus, PA200-knockdown cells show impaired...
The observation of impaired growth of PA200-depleted cells in the complete absence of extracellular glutamine and glutamate suggests that PA200, which promotes post-glutamyl cleavage by proteasomes, may be required to replenish cells with glutamate and/or glutamine under complete starvation conditions. Consistent with this idea, PA200-depleted cells show diminished intracellular free glutamine levels compared with cells with PA200 (Fig. 4B). This decrease in glutamine is specific as other amino acids such as valine and arginine remained at wild-type levels in PA200-knockdown cells. Although PA200-knockdown cells seem to consume more glutamine from the extracellular media (Fig. 2A), they show diminished intracellular free glutamine. Upon uptake of glutamine into cells, it is rapidly converted to glutamate, of which some is released back into the media. Interestingly, PA200-depleted cells show diminished glutamate release into the media (Fig. 4C, compare black bar with white bar). This diminution can be reversed by introduction of PA200 cDNA that is insensitive to the siRNA (Fig. 4C compare white bar with gray bar). In summary, loss of PA200 results in decreased intracellular glutamine levels and consequently less glutamate is excreted into the media. These data argue that PA200 is required for maintenance of intracellular glutamine homeostasis.

Discussion

Here, we show that IR exposure enhances glutamine consumption and that the radiation sensitivity of PA200-deficient cells is directly related to impaired glutamine homeostasis after IR exposure. Exogenous glutamine supplementation restores resistance of PA200-knockdown cells to IR exposure. PA200-knockdown cells seem to be more dependent on exogenous glutamine to compensate for impaired maintenance of intracellular glutamine levels. We further show that these cells do not restrict growth in conditions of low glutamine and continue attempted growth, which likely leads to their demise in a long-term clonogenic survival assay. The inability to restrict cell growth in nutrient-deprived settings is associated with dysregulated mTOR responsiveness.

Our observation that glutamine is depleted from the supernatant after IR treatment is consistent with microarray data, indicating that the glutamine importers SLC7A6 and SLC7A5 are increased after radiation (30, 31). Furthermore, the glutamine exporter SLC7A7 is diminished after radiation (30, 31). Together, these data suggest that IR induces a greater demand for glutamine, which is compensated by increased uptake of extracellular glutamine.

The demand for glutamine after IR exposure appears to require PA200 because PA200-knockdown HeLa cells exhibit increased radiosensitivity, which is reversed by supplementing cells with additional exogenous glutamine levels. Therefore, glutamine may be a limiting constituent after radiation treatment especially for cells lacking PA200, as these cells do not survive in long-term clonogenic survival assay. Our ability to identify a survival defect in PA200-knockdown cells was only possible because we used a long-term clonogenic assay without feeding (10–14 days). In vitro clonogenic survival of tumor cells after radiation has been reported 7 days (32) after radiation or 10 to 14 days after radiation (8, 33). Because very few cells are plated, they are not expected to deplete the nutrients available in the media and thus media are often not replaced or cultures are not refed during the 7- or 10- to 14-day clonogenic survival assays (in some studies, media are replaced). Because we show that PA200-knockdown cells form more colonies than control siRNA–transfected cells on day 7, but fewer colonies than control cells on day 13 after radiation, we suggest that...
proteins that affect glutamine homeostasis or responsiveness to glutamine can impact long-term clonogenic survival as determined on day 10 to 14 (without feeding).

The increased growth in low-glutamine conditions in the absence of PA200 might indicate an adaptation caused by limiting intracellular glutamine levels. These cells also continue to grow and maintain high mTOR activity (p-S6K levels), whereas control cells slow cell growth and dampen mTOR activity in nutrient-deprived settings. Furthermore, we show that IR exposure elicits a transient decrease in p-S6K in control cells that is not apparent in PA200-knockdown cells. Two previous studies have examined the effects of DNA-damaging agents on mTOR and the mTOR substrate 4EBP1. One report suggests enhancement of mTOR activity after IR exposure (28), whereas another suggests inhibition of mTOR activity (by measuring 4EBP phosphorylation) after etoposide-induced DNA damage (29). Thus, the effect of DNA damage on mTOR appears to be context-dependent. However, a recent report shows that DNA damage–mediated inhibition of S6K phosphorylation requires ATM, liver kinase B1 (LKB1), and S5′-adenosine monophosphate-activated protein kinase (AMPK; ref. 34). How PA200 deficiency affects this pathway of regulation of S6K remains to be elucidated.

Why and how is mTOR potentially dysregulated in PA200-knockdown cells? Nutrient sensing by mTOR is known to be regulated by amino acid availability. mTOR integrates signals from amino acid availability, insulin, growth factors, and stressors (35). A previous study suggests that proteasome activity is required for responsiveness of mTOR and thus S6K to insulin (36). These authors suggested that proteasomes contribute to the homeostasis of intracellular amino acid pools and are therefore necessary to provide the “building block” or “amino acid” signal for mTOR activation. Our observation that PA200-knockdown cells show growth impairment in media completely devoid of glutamine (and glutamate) suggests that PA200 and proteasome catabolic activity may be important to recover intracellular glutamine or glutamate when these amino acids are not available in the extracellular space.

Because we have previously shown increased cellular abundance of PA200–19S hybrid proteasomes after radiation exposure (8), we suggest that specialized proteasomes such as PA200–19S hybrid proteasomes, which shift the peptide specificity of the proteasome, may be required for the generation of free amino acids such as glutamate or glutamine which regulate mTOR signaling. In the absence of PA200 or post-glutamyl proteasome activity to provide intracellular free glutamine, there may be still unknown compensatory mechanisms that increase glutamine uptake and promote sustained mTOR activity, but which are unresponsive to nutrient depletion or stimulation.

Tumor cell survival from radiation appears to be a complex phenomenon that encompasses sensing, repairing, and recovering from cell-cycle checkpoints after DNA damage. In addition to these effects, we reveal here that IR exposure leads to an increased demand for glutamine. Furthermore, we show a specific role for PA200 and glutamine homeostasis in long-term tumor cell survival after radiation exposure. How PA200 impacts tumor resistance to radiation in vivo and the biology of PA200 and glutamine homeostasis after in vivo radiation remains to be explored.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed by the authors.

Acknowledgments

The authors thank Alexei Kisselev (Dartmouth University, Hanover, NH) for the kind gift of the post-glutamyl–specific inhibitor as-NC-001. The authors also thank Amy Stewart, Jennifer Pearce, and Melanie Farraruto for assistance with irradiations.

Grant Support

This work was supported by Investigator Start-Up Funds (to N. Bangia), NIH grant GM-084228 (to M. Schmidt), CA129537 (to T.K. Pandita), CA154320 (to T.K. Pandita), and a National Cancer Institute Cancer Center Support Grant (CA16566) to RPCI.

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Received October 12, 2011; revised March 15, 2012; accepted April 16, 2012; published OnlineFirst May 1, 2012.

References


Correction: The Proteasome Activator PA200 Regulates Tumor Cell Responsiveness to Glutamine and Resistance to Ionizing Radiation

In this article (Mol Cancer Res 2012;9:937–44), which was published in the July, 2012, issue of Molecular Cancer Research (1), the affiliation of the corresponding author, Naveen Bangia, is incorrect.

Dr. Bangia’s current affiliation is The Binding Site, Inc., San Diego, CA. His affiliation at the time that the study was conducted was as follows:

Department of Immunology,
Roswell Park Cancer Institute,
Buffalo, New York.

The publisher regrets this error.

Reference

Molecular Cancer Research

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