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Epigenetic DNA Methylation in Radiation Biology: On the Field or on the Sidelines?

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ABSTRACT

DNA methylation has been studied with regard to chemotherapeutics for a number of years. The radiation field has just begun to look at this in the context of radiotherapy or radiation exposure. So far, the data suggest that radiation induces epigenetic reprogramming which indicates a purposeful response that influences the cell fate or alters the response to future exposure. Further studies may result in discovery of biomarkers for radiotherapy outcome or prediction of the degree of radiation resistance. Past and ongoing development of DNMT1 inhibitors that lead to DNA hypomethylation appear to sensitize many tumor types to radiation and may be an area with long term clinical implications. J. Cell. Biochem. 116: 212–217, 2015.

KEY WORDS: EPIGENETICS; RADIATION; METHYLATION; AZACYTIDINE; DECITABINE; ZEBULARINE

Heritable changes in gene expression that cannot be attributed to changes in the genetic code are termed epigenetic changes. The major forms of epigenetic regulation of gene expression come in the form of DNA methylation, histone modification, and expression of miRNAs. DNA can become methylated on the five position of cytosine in CpG dinucleotides to yield 5-methylcytosine (5-mC). This can occur anywhere in a gene but clustered regions of CpGs called CpG islands are present throughout the genome. The relative hyper- or hypomethylation of CpG islands can affect nearby gene expression (Fig. 1). Generally, hypermethylation tends to result in decreased expression of a nearby gene and hypomethylation tends to make nearby genes permissive for expression. This is not a perfect cause–effect correlation since gene expression is controlled by a great many other mechanisms.

DNA is methylated through the activity of DNA methyltransferase (DNMT) enzymes. There are three such enzymes in mammals which are designated DNMT1, DNMT3a, and DNMT3b. Each has multiple alternative transcripts to which unique functions are yet to be described. DNMT1 is the main enzyme responsible for maintenance of 5-mC patterns on newly synthesized DNA, and is active on hemimethylated DNA. DNMT1 is associated with the replication machinery and performs DNA methylation during DNA synthesis [Hermann et al., 2004]. DNMT3a and DNMT3b are de novo methyltransferases which add methyl groups to bare DNA. DNMT3a and DNMT3b are important for establishing methylation patterns during development.

The mechanism of addition of methyl groups to DNA is well known as described above, however, the removal of methyl groups is not adequately characterized. Loss of 5-mC can occur during DNA replication if new methyl groups are not placed on newly synthesized DNA. This doesn't remove methylation per se, but does result in production of unmethylated or hypomethylated DNA in daughter cells. Likewise, under some mechanisms of DNA repair, new DNA is synthesized to fill in a gap. If this new DNA is not acted upon by DNMT1, then methyl groups are effectively removed.

Reports by one group have recently suggested that DNMT3A and DNMT3B can function as dehydroxymethylases [Chen et al., 2012, 2013]. Under this mechanism, 5-mC is converted to 5-hydroxymethylcytosine (5-hmC) by the TET (ten–eleven translocation proteins) family of proteins, after which DNMT3a/3b remove the hydroxymethyl group, thus leaving unmethylated cytosine. It is unclear if this mechanism has a significant role to play in vivo. Finally, methyl group removal has been suggested to occur through conversion of 5-mC to 5-hmC by TET and then repair of modified bases by the base excision repair pathway.

DNA methylation and demethylation can be a dynamic process with switching occurring on the order of hours or less [Kangaspeska et al., 2008; Metivier et al., 2008]. This suggests that various interventions, such as cancer treatment (radiotherapy or chemotherapy), can alter the methylation landscape and result in changes in the biological response to the current or future treatment. Thus, there are two potential sides to this effect: (1) treatment alters DNA

Conflicts of interest: none.

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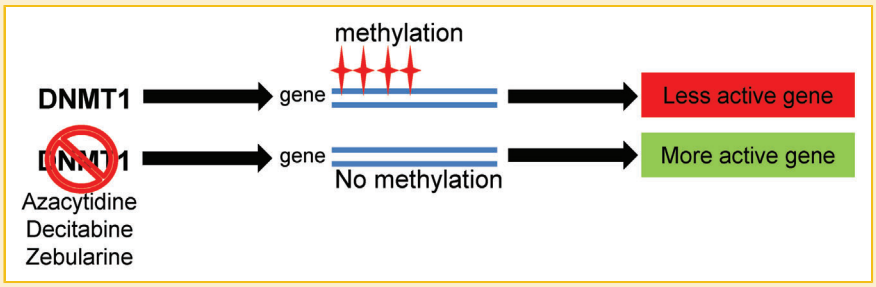

Fig. 1. Methylation of gene promoters leads to gene silencing, while inhibition of DNMT1 leads to hypomethylation and potential re-expression of silenced genes.

methylation and (2) DNA methylation alters response to treatment. These are not entirely independent phenomena since (1) may be the cause of (2). Furthermore, if DNA methylation alters response to treatment, then epigenetic therapies may have an impact on treatment response. These concepts will be reviewed in subsequent sections.

DNA METHYLATION RESPONSES TO RADIATION

DNA methylation changes in response to radiation have been studied for decades. Past work relied on gross measurement of global DNA methylation since more fine analysis was not technically feasible. Most studies showed a global decrease in DNA methylation within a day of radiation exposure [Tawa et al., 1998; Pogribny et al., 2005]. However, changes can be highly specific for sex, tissue, and dose [Pogribny et al., 2004]. Many of these studies were performed in normal cells, tissues, or whole animals and may not reflect what happens in cancer cells.

Studies have now begun to analyze radiation-induced changes in DNA methylation at specific loci using chip technology and pathway analysis to look for patterns that may suggest a unique response. In our own work, we found global changes in DNA methylation following exposure of breast cancer cells (MDA-MB-231) to radiation in vitro which suggested an organized response [Antwih et al., 2013]. Using chip technology [Bibikova et al., 2011] to query 450,000 CpG loci in the genome following exposure to low and high dose radiation, and at time points up to 72 h, gene ontology analysis showed that DNA repair, cell cycle, and apoptosis pathway genes were differentially methylated in a manner consistent with biological responses to radiation at various doses and time points post-irradiation. For example, cell cycle pathway genes were hypomethylated late (24+ hours) after low dose (2 Gy) radiation, but hypermethylated after high dose (6 Gy) radiation. This corresponded with a recovery of G_2/M arrest after 2 Gy, but persistence of a G_2/M arrest after 6 Gy. Similarly, more DNA repair-associated pathways were hypomethylated along with persistence of γH2AX after 6 Gy, than at the lower radiation dose. Finally, apoptosis pathway genes were hypermethylated after 6 Gy, but hypomethylated after 2 Gy. Non-replicative, that is senescent, cells may be more resistant to apoptosis than cells retaining replicative ability.

Interestingly, no global hypomethylation or hypermethylation was observed following radiation treatment which was consistent over the 72 h period post-irradiation. DNMT1 protein levels decreased over 72 h, in agreement with Kalinich et al. [1989]. Thus, a later time point may show progressive global hypomethylation in this model.

Wang et al. [2014] found that low dose acute (0.5 Gy) or chronic (0.5 Gy × 10 days) radiation induced global changes in DNA methylation in mice. After whole body irradiation, they analyzed DNA methylation in multiple tissues 2 h and 1 month post-irradiation. Using MeDIP-on-chip technology (methylated DNA immunoprecipitation), they found 811 regions differentially methylated between acute and chronic groups. Gene ontology analysis of 811 genes was not very informative at the level of "biological process," "cellular component," and "molecular function." KEGG Pathway analysis showed "focal adhesion" and "mTOR signaling pathway" categories with high enrichment scores, among other pathways. Of greater interest was the difference in methylation and gene expression of Rad23b and Ddit3 between acute and chronic radiation exposure, as well as early and delayed time points in the different tissues, particularly lung.

Differential methylation of breast cancer cells (MCF7) following fractionated radiation, with or without a recovery period, was investigated using a 244 k CpG island microarray [Kuhmann et al., 2011]. Locus-specific differential methylation was observed following a regrowth period and methylation changes showed some degree of recovery. No change in radioresistance was found after the recovery period.

In contrast, another study did not find significant changes in DNA methylation following irradiation of normal human cells, including fibroblasts and bronchial epithelial cells in vitro [Lahtz et al., 2012]. Cells were irradiated at 0.1–10 Gy and analyzed 7 days post-exposure and methylation was analyzed using a methylated-CpG island recovery assay (MIRA). This assay contains all human genome CpG islands and all Refseq gene promoters. Only a small number of modest methylation changes were observed which could not be confirmed with follow-up analysis. Recovery of methylation changes may occur over a 2–3 weeks period post-irradiation, as has been observed with some loci studied [Kuhmann et al., 2011].

Clinical studies of DNA methylation during cancer radiotherapy are just beginning to be performed and may provide some additional insight about whether methylation changes have an association with response. Breast cancer biopsies collected before and after radiotherapy and analyzed using the Illumina Infinium 27 k microarray found 82 differentially methylated genes in irradiated versus non-

irradiated samples [Halvorsen et al., 2014]. Inflammatory genes showed the greatest difference in differential methylation. Five genes were chosen for follow-up analysis and their methylation level was associated with clinical response. It is possible that the methylation status of a gene or genes, or a change occurring with radiotherapy, could be used to identify patients expected to have a better or worse response. This might also be used to predict toxicity or guide physicians regarding how aggressive treatment must be to achieve a desired result. The difficulty in acquiring biopsies at multiple time points limits clinical studies and a better approach may be to analyze cell-free DNA in plasma for epigenetic changes during treatment. Many academic radiation oncology departments already collect blood samples from patients at different points during radiotherapy. This may be a resource already available if the right technical skill is brought to bear.

The small number of "before and after" treatment profiling studies utilize models that are quite different from each other, which makes comparison difficult. Primary versus cancer cells, mouse versus human, in vivo versus in vitro context, the time post-treatment analysis is done, and the radiation dosing scheme are all factors which are likely to produce substantially different results. The main question that needs further study is whether a DNA methylation response to radiation alters future cell responses. This can most cleanly be answered in an in vitro system. The greatest impact will be on cancer treatment and the effort should be towards determining cancer cell epigenetic responses to radiation.

EFFECTS OF DNA METHYLATION ON THE RADIATION RESPONSE

Comparison of isogenic cell lines which differ only in their degree of radiation resistance can provide insight into potential epigenetic determinants of radioresistance. This has been studied in the chemotherapy context for cisplatin resistance of ovarian cancer [Yu et al., 2011]. A basket of hypermethylated genes was found and treatment with a demethylating agent led to promoter hypomethylation and increased gene expression. Importantly, there were no data examining whether this altered cisplatin resistance, which greatly diminishes the impact of the study.

Similar studies have not been performed following radiotherapy. It is important that isogenic cell lines be compared or other genetic differences may be responsible for the difference in radioresistance. In one study, a radiosensitive and radioresistant lung cancer cell line were analyzed for DNA methylation determinants of radioresistance [Kim et al., 2010]. However, the cell lines were not isogenic and in fact, the radiosensitive cell line was $p53^{+}$, while the radioresistant cell line was $p53^{-}$. RNAi of a 4-gene set differentially methylated showed modest effects on radiation sensitivity, suggesting that if gene expression were altered by the methylation status of the gene, then radiation sensitivity would be affected, but no experiments were performed to make a direct connection.

A more appropriate study compares one cell line to another that was made radioresistant by repeated exposure to radiation. This was recently performed using a laryngeal cancer cell line [Lee et al., 2014]. However, this study was not designed to find determinants of radioresistance, but rather, determinants of TRAIL resistance. Induction of DR4 hypermethylation by radiation led to TRAIL resistance which could be partially reversed by treatment with a demethylating agent that demethylated the *DR4* gene. A similar study directed specifically at radiation resistance has yet to be published.

The RB1 gene showed consistent hypermethylation over time after 2 Gy in our hands, and this was associated with decreased mRNA expression [Antwih et al., 2013]. Decreased Rb would have the effect of driving cell cycle progression, and a significant proportion of 2 Gy treated cells retained replicative capacity. Treatment with 6 Gy did not show a similar effect on RB1. Also, it has been shown that inactivation of Rb promotes ATM activation [Shamma et al., 2013]. This allows ATM to bind DNMT1, leading to increased DNMT1 ubiquitination and protein degradation. Thus, inactivation of Rb results in decreased DNMT1 protein levels and potentially hypomethylation, including in genes such as Ink4a, Shc2, FoxO6, and Noggin. Other differentially methylated genes affecting the radiation response will be discussed in the context of epigenetic therapies in the next section.

EPIGENETIC THERAPY TO MODULATE RADIATION RESISTANCE OF CANCER

Radiation therapy is a highly effective cancer treatment, however, some cancers exhibit inherent radiation resistance compared to surrounding normal tissues. Radiation can reduce recurrence of cancers such as breast following surgery, but inflammatory and triple-negative breast cancer relapse rates suggest more progress is necessary. Drugs that can sensitize tumors to radiation have been under development for several years. Epigenetic therapies, such as with DNMT1 inhibitors that lead to hypomethylation of DNA, are a new area of investigation for modulating the radiation resistance of tumors.

DNA methylation can be pharmacologically altered using various drugs, some of which are approved for treatment of other diseases such as myelodysplastic syndrome. The most commonly studied drugs for use in combination with radiation have been 5-azacytidine (5AC), 5-aza-2′-deoxycytidine (decitabine), and zebularine. These compounds are nucleoside analogs which inhibit DNMT1 and lead to hypomethylation. 5AC and decitabine (clinically approved) function by incorporation into DNA in place of cytosine during DNA replication. When DNMT1 attempts to add a methyl group, it becomes covalently trapped and is targeted for degradation. Thus, DNMT1 protein is depleted and newly synthesized DNA is deficient in methylation. Zebularine is a nucleoside analog that when incorporated into DNA, forms a covalent complex between DNMT1 and cytidine deaminase, of which it is an inhibitor [Ren et al., 2011]. Toxicity of these drugs are 5AC > decitabine > zebularine. This is partly due to the fact that 5AC can incorporate into mRNA in addition to DNA, while decitabine and zebularine can only incorporate into DNA.

Other compounds are at earlier stages of development and many have not been used in combination with radiation. RG108 is a rationally designed DNMT1 inhibitor that binds directly to the active

pocket of DNMT1, leading to its inhibition [Stresemann et al., 2006]. In this case, DNMT1 is not targeted for degradation. RG108 may show less toxicity due to its specificity. RG108 is also much more stable than the nucleoside analogs, which have short half-lives under physiological conditions. Development of more potent analogs is ongoing [Asgatay et al., 2014].

5-AZACYTIDINE

5AC has been shown to radiosensitize nasopharyngeal [Jiang et al., 2014], colorectal [Hofstetter et al., 2010], and head and neck [Brieger et al., 2012] cancers in vitro in clonogenic assays. Xenograft studies using 5AC have been performed only with nasopharyngeal cancer [Jiang et al., 2014]. Administration of 4 mg/kg twice weekly was tolerable to nude mice and led to significant reductions in tumor size when combined with radiation, versus radiation alone. Mice were treated with 5AC for 2 weeks and a single radiation treatment of 8 Gy was given at week 2. This dose schedule was also sufficient to cause hypomethylation and increased mRNA expression of *RASSF1A*, *RPRM*, and *CDKN2A* genes in vivo. These results are promising, and perhaps surprising, given the short half-life (several hours) of 5AC in vivo [Stresemann and Lyko, 2008].

DECITABINE

Decitabine has been studied in combination with radiotherapy for several cancer types [De Schutter et al., 2009; Patties et al., 2009; Qiu et al., 2009; Kim et al., 2012; Wang et al., 2013]. These in vitro studies showed modest to robust radiosensitization primarily through clonogenic assays. DNMT1 and DNMT3a, but not DNMT3b, levels were reduced after 18 h treatment [Kim et al., 2012]. Gene silencing for selected genes was reversed [De Schutter et al., 2009; Qiu et al., 2009]. In gastric cancer cells, radiosensitization was modest in only two of four cell lines, with the others showing little to no effect [Qiu et al., 2009]. However, selected genes showed increased expression. In a breast cancer study, clonogenic assays showed radiosensitization of MDA-MB-231 cells and MDA-MB-435 cells, however, the latter cell line has been demonstrated to be myeloma, not breast [Rae et al., 2007]. Various methylated genes related to DNA repair (BRCA1, 14–3-3σ, E-cadherin) were hypomethylated following decitabine treatment [Wang et al., 2013].

ZEBULARINE

Combination zebularine and radiation treatment led to a significant tumor growth delay in U251 glioblastoma xenografts, compared to radiation- or zebularine-alone [Dote et al., 2005]. Interestingly, zebularine-alone was as good as radiation-alone in suppressing tumor growth. Zebularine treatment induced expression of RASSF1A, 14–3-3σ, and HIC-1 in xenografts after as few as three doses. In another study, zebularine treatment in vitro caused loss of DNMT1, decreased cell viability, and reduced proliferation in the non-Hodgkins lymphoma cell line MEC1 [Bryan et al., 2014].

MECHANISM OF RADIOSENSITIZATION

The mechanism of radiosensitization for DNMT1 inhibitors is not well understood. Various mechanisms have been put forward, including alteration of cell cycle, apoptotic, and DNA repair pathways. Apoptosis was increased, cells arrested in G_2/M, and γH2AX was increased with exposure to decitabine [De Schutter et al., 2009]. However, only the cell cycle arrest was enhanced in combination with radiation. In gastric cancer cells, decitabine induced a G_2/M arrest, but while an arrest occurred in radiosensitized cells, non-sensitized cells also showed a G_2/M arrest in most cases [Qiu et al., 2009]. However, zebularine was shown to abrogate radiation-induced G_2/M arrest by one group, while another group found zebularine abrogated a radiation-induced G_2/M arrest in one cell line, enhanced a radiation-induced G_2/M arrest in another cell line, and had no effect in combination with radiation in a third [Dote et al., 2005; Kim et al., 2012].

DNA damage signaling as determined by γH2AX was increased for up to 24 h in breast, lung, and glioblastoma cancer cells treated with decitabine or zebularine [Kim et al., 2012; Wang et al., 2013]. This was confirmed in additional cell lines [Dote et al., 2005].

While some results allude to possible mechanisms of radiosensitization, no studies have shown definitive mechanisms. The data regarding cell cycle are inconsistent depending on cell line and do not always correlate with whether a given cell line exhibits radiosensitization. Increased molecular detail may be needed to decipher this potential mechanism. While showing abrogation or enhancement of radiation-induced cell cycle arrest is suggestive, it is unclear whether this is at all related to radiosensitization. Our preliminary data suggest a cell cycle mechanism, but a confounding factor may be differences in the response of various cell lines to radiation.

More consistent data has been reported regarding DNA repair, but most data utilizes only the γH2AX assay. Additional measures of DNA repair capacity are necessary to increase the confidence in this as a mechanism of radiosensitization.

There are little data regarding an apoptotic mechanism and some of the data reports cell viability, not apoptosis. Given that apoptosis is not a significant mechanism of cell death from irradiation of solid tumors and that the clonogenic assay measures clonogenic survival and provides no information regarding cell viability or true death, it is also possible that DNMT1 inhibitors enhance radiation-induced senescence. More careful study of apoptosis or other mechanisms of replicative death are needed.

CONCLUSION

Emerging data suggests that radiation treatment causes differential DNA methylation throughout the genome. DNA methylation changes may constitute a 'reprogramming' of the epigenetic landscape that leads to an altered cellular response to further radiation, that is, increased or decreased resistance, or may partly control cell fate in response to the initial irradiation. However, it cannot be excluded that the epigenetic reprogramming is a result of a change in cell state induced by radiation such as senescence or changes to the cell cycle. In other words, is epigenetic reprogramming responsible for the change in cell state, or is it a consequence of the change in cell state?

It is likely that the methylation status of one or more genes will be found to predict radiotherapy outcome in much the same way that DNA methylation of the MGMT gene has been shown to predict

resistance and outcome to temozolomide treatment in glioblastoma [Hegi et al., 2005]. It will be important in future studies to carefully consider the model system, particularly when comparing radioresistant and radiosensitive cell lines. Clinical studies will be important to validate this information. Development of efficient techniques to analyze tumor DNA in blood for epigenetic profiles will contribute to this effort.

Much of the data regarding DNMT1 inhibitors as radiosensitizers are of limited value. Clonogenic assays have shown modest radiosensitization in many cases and robust radiosensitization in others. Little careful work beyond clonogenic assays has been done to advance the field. The two xenograft studies that have been published show more promising results than the clonogenic assays would predict and suggests further in vivo experiments should be performed. The mechanism of radiosensitization is elusive, but may center on cell cycle or DNA repair. More complex experiments are needed to analyze this further. It is this author′s opinion that radiosensitization may occur with some cell lines, but radioprotection may occur in others (unpublished data). It is encouraged that both cases be presented for publication. There may be distinct molecular or genetic reasons for such differing results. It is possible that DNMT1 inhibitors may be useful as radiosensitizers for a subset of tumors or tumor types.

In conclusion, radiation epigenetics is an emerging area of research that may provide further insights into the radiation response. DNA methylation reprogramming may lead to an altered cell response to subsequent radiation. Radiosensitizers based on DNMT1 inhibitors require further study, but in vivo experiments are promising.

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