Review Article

Utilization of Peripheral Blood B Cells to Determine the Role of gDNA Methylation in the Dysregulation of Cell Division/DNA Replication in Diabetes Mellitus

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Abstract

This review focuses on approaches to understand the underlying mechanisms of Diabetes mellitus (DM) associated with the long term complications of this disease. The long term complications of DM are related to the phenomenon of Metabolic Memory (MM) as discussed in this review. It is proposed that peripheral blood B cells can be utilized to analyze methylated gDNA-based mechanisms underlying the relationship of hyperglycemia to the induction of tissue dysfunctions related to impaired cell division with special reference to DNA replication genes that are common among mammalian cells. It should be noted that dysfunction in cell division and the DNA replication machinery affects a wide spectrum of tissues in patients with DM and subsequent MM. In this regard, rationale for the use of B cells to approach this problem is discussed. It is proposed that such an approach will provide epigenetic data pertaining to 1) methylated gDNA changes in DM/MM, 2) concomitant gene expression changes associated with these methylation changes, and 3) functional changes that can establish a link between DNA methylation and impaired cell division/ DNA replication in the peripheral blood B cell of patients with diabetes.

Keywords: Diabetes; Secondary complications in diabetes; Metabolic memory; Peripheral blood; Blood B cells; Epigenetics; gDNA methylation; Bioinformatics; Transcription factor binding

Abbreviations

DM: Diabetes Mellitus; gDNA: Genomic DNA; MM: Metabolic Memory; HG: Hyperglycemia; ROS: Reactive Oxygen Species; AGE: Advanced Glycation End Products; T1 DM and T2 DM: Type 1 and Type 2 diabetes; MRs: Methylated Regions; FACS: Fluorescent Activated Cell Sorting; **CTGF:** Connective Factor Growth Factor; TSS: Transcription Start Site; ChIP: Chromatin Immuno-Precipitation; CpG: 5'—C—phosphate—G—3' with C and G representing Cytosine and Guanine, respectively

Introduction

Overview of DM and MM as related to epigenetic processes and peripheral blood B cells

Diabetes mellitus (DM) will affect over 400 million worldwide by 2030 using current epidemiological methodologies [1]. Diabetes mellitus is classified as a disease of metabolic dysregulation [2,3] leading to long term complications affecting a wide variety of tissues and resulting in multiple pathologies such as diabetic retinopathy, nephropathy, altered angiogenesis, and impaired wound healing, to name only a few [2,3]. Evidence from the bench [4,10] and from large scale clinical trials [11-25] reveal that complications from the onset of Hyperglycemia (HG) progress unimpeded via the phenomenon of "Metabolic Memory" (MM) even when glycemic control is pharmaceutically achieved [11-25]. This clinical finding applies to both Type 1 and Type 2 diabetes (T1 DM and T2 DM). The underlying molecular mechanisms of hyperglycemic complications

and MM have been proposed to include: 1) the involvement of excess reactive oxygen species, 2) the involvement of advanced glycation end products, and 3) alterations in tissue-wide gene expression patterns [2,3]. It should be noted, however; that the heritable nature of metabolic memory [26,27] suggests a role for the epigenome. The epigenome comprises all chromatin modifying processes (including DNA methylation and histone modifications) that allow cells and organisms to rapidly respond to changing environmental stimuli [28-30]. These processes not only allow for immediate adaptation but also allow the cell to "memorize" these encounters [28-30]. The underlying molecular mechanism(s) of MM have been examined using both animal model approaches and *in vitro* based studies [4-10]. Such studies have established that the initial hyperglycemic episode(s) results in permanent aberrant gene expression in DM target tissues. With the majority of epigenetic research focusing on histone modifications [31-40] and microRNA mechanisms [41-46] much less is known about the role of HG-induced persistent gDNA methylation changes that occur in both Type 1 and Type 2 DM. Using a DM/MM animal model, our laboratory has previously reported that HG induces aberrant gDNA methylation with concomitant altered gene expression patterns that correlate with persistent diabetic complications [8,47]. The role of HG-induced gDNA methylation changes in cells, as related to persistent MM dysfunction, remains unclear. As a means to understanding how epigenetic mechanisms regulate the process of metabolic memory within cells, previous studies have utilized the lymphocyte fraction obtained from patients with diabetes [36-38,48]. This approach creates complications in

the interpretation of the results because it reflects pools of cells that likely display different gDNA methylation patters (e.g. T-cells versus B-cells). To eliminate this problem we propose that one focus on a specific lymphocyte subtype, namely the peripheral blood B cell. As explained below, there are advantages to selecting the B cell over other lymphocytes. In broad terms, this cell enables one to examine the problem of cell division and DNA replication in either Type 1 or Type 2 patients in a less invasive manner than is required for other tissues of the body. Given the conserved nature of cell division/DNA replication among mammalian cells, such an approach could shed light on a process that underlies a broad range of tissue dysfunctions related to diabetic pathologies (e.g. retinopathy, nephropathology, altered angiogenesis, and impaired wound healing) in long term DM [2,3].

Body of the Review

A proposed experimental approach to ascertain the role of changes in methylated gDNA patterns to the process of altered cell division/DNA replication in DM

Given the impact of DM on society, it is important to decipher the molecular mechanisms of the long term complications of DM as related to the epigenome. One experimental approach to this problem could center around testing the hypothesis: "*Persistent altered division of B cells (CD19+/CD20+) in the long term DM patient arises in part from hyperglycemia-induced aberrant gDNA methylation of genes related to cell division/DNA replication in the distal and proximal "Methylated Regions" (MRs) of these genes*". The use of Blood B cells has advantages for approaching this problem for a number of reasons to include: 1) cell division/DNA replication processes are essential to the B cell life cycle and as part of the peripheral blood are an easily accessible cell type from Control and T1 or T2 DM patients; 2) B cells have been shown to have problems with cell division/DNA replication in DM that is inherit to the cell and separate from cellular signaling pathways [49-54]; 3) recent work has shown that B cells of DM patients have alterations in their gDNA methylation patterns; although the extent of these changes across the entire genome has not been determined as of yet [48]; and 4) it should be noted that B cells do have an important role in the generation of diabetes and are important to our understanding of the basic pathological mechanisms of this disease [55].

The focus on cell division/DNA replication dysfunction induced by gDNA methylation in DM creates challenges in regard to obtaining cells in sufficient numbers for gDNA methylation sequence analysis without the need for cell culture as a means to expand cell number prior to epigenetic analysis given that cell culturing can itself induce epigenetic changes in cells [56]. Problems pertaining to isolation procedures would of course apply to both healthy (Control) individuals and individuals with either T1 or T2 diabetes. In this approach, cell isolation from patients is simplified because B cells (in both healthy and DM patients) are found in high numbers in the peripheral blood and are readily purified by FACS. It should be noted that hyperglycemia is the primary trigger for gDNA-methylation changes and therefore because hyperglycemia [to some extent] occurs in all individuals with DM, it applies to both T1 and T2 patients with the disease. In this approach, it should be noted that B Cells (CD19+/CD20+) are used as a paradigm model

for cell division/DNA replication processes common to most cells of the body when one notes the involvement of a common tool-kit of genes for the replication machinery of the nucleus (e.g. such members as DMNT1, MCMs, ORCs, etc) In this approach, B cells can be obtained from the peripheral blood of healthy [Control] and diabetic patients [Type 2 or 1] to identify persistent aberrant gDNA methylation with concomitant gene expression pattern changes that regulate cell division.DNA replication in diabetes. In this regard, previous studies have established that cell division is altered in B cells of diabetic patients [49-54] and very recent studies have established that gDNA methylation changes occur in the B cells following hyperglycemic episodes in patients with diabetes [48]. Bioinformatics analysis of global gDNA single nucleotide bisulfate sequencing in combination with concomitant gene expression analysis would allow the identification of gene-associated MRs that are either distal or proximal relative to the gene's transcription start site and that correlate with misexpression of their transcripts. Methylated Regions have been shown to have an important role in the regulation of gene expression and have also been shown to have some role in epigenetic changes within the genome [57-60]. They have been shown to be as far upstream as 30kb of the Transcription Start Site (TSS) [57,58] as well as in a more proximal position near the TSS [61]. Recent published studies by our laboratory have identified MRs as far upstream as 6-13kb from the TSS in the DM and MM states following initial hyperglycemia [62]. It should be noted that methylation changes can also occur within the gene proper [63]. The positioning of methylated regions (MRs) in the genome in such unpredictable regions relative to the TSS necessitates the use of global gDNA single nucleotide bisulfite sequencing to identify them via bioinformatics analysis. Their role in the gDNA-methylation changes seen in diabetes is not understood; although, recent data from our laboratory indicates they appear to have a role in the persistence of tissue dysfunctions in Metabolic Memory [62]. As indicated above, gDNA methylation changes within the MRs of these genes can be analyzed in terms of impairment of Transcription Factor (TF) binding to their respective DNA binding sites (identified through bioinformatics analysis of methylated gDNA sequence analysis data) via ChIP analysis. This would directly establish a link between gDNA methylation and altered gene expression because of alterations in TF binding to their DNA binding sites.

Mechanisms related to epigenetics in the onset and propagation of DM, with particular reference to cell division/DNA replication processes

The role of epigenetics in diabetes is an important and expanding area of study. In this context it is important to note that the role of gDNA methylation in DM (as opposed to histone modifications and microRNA function) is much less understood or studied. This is particularly true for the role of epigenetic Methylated Regions in the process of hyperglycemia-induced gDNA methylation changes. Methylated Regions of gDNA have been implicated not only in the regulation of gene expression, but also in the overall control of epigenetic changes in the cell [57-60]. As a general overview, epigenomes consist of all the chromatin modifications for a given cell type and are responsible for a cell's unique gene expression pattern. These chromosome modifications support cell differentiation and change throughout development [64-66]. In addition, they are

responsive to external conditions, are altered in disease [67], and are mitotically stably inherited [8,26,27]. In general, epigenetic mechanisms include: 1) post-translational histone modifications, 2) non-canonical histone variant inclusion in octomers, 3) chromatin access changes through gDNA methylation, and 4) gene expression control through non-coding microRNAs [68-71]. Together, these processes allow cells to quickly respond to changing environmental conditions [28,29] and all cells to "memorize" these encounters once the stimulus is removed [28,29]. Therefore, because gene expression changes resulting from epigenetic processes are stable in the absence of the signal that initiated them and are heritable through cell division; they have important in terms of a potential mechanism(s) underlying metabolic memory (MM). Advances have been made towards understanding the roles that histone modifications [31-34,36-40,72] and microRNAs [41-46,73] play in the metabolic memory phenomenon; however, as indicated, much less has been documented regarding the role of gDNA methylation [8,74-76]; especially as related to diabetes [8]. In over-view, gDNA methylation occurs predominantly as 5-methyl-cytosine [5mC]; mostly in the context of CpG dinucleotides. In mammalian genomes, these dinucleotides are clustered into regions (in order of decreasing CpG density) termed islands, shores, shelfs, and open seas [77,78]. Multiple roles for gDNA methylation have been proposed, to include: gene silencing, silencing of transposable elements, developmental regulation of transcription, cell cycle control, and differentiation have been documented [79-83]. It was previously thought that hypermethylation of CpG islands in promoter regions acted to inhibit promoter activity by maintaining chromatin in a stably repressed state that caused changes in gene expression patterns. In contrast to this, more recent studies indicate that while this is correct for some loci; the majority of tissue specific expression and cancer-induced aberrant expression is governed by variations in the shore regions [78]. Additionally; genome wide gDNA methylation analyses have indicated that methylation in the "bodies" of active genes is significantly higher than those of inactive genes [84,85]. This appears to be highly conserved and may function to suppress inappropriate transcription, regulate mRNA splicing, modulate elongation, and regulate tissue specific alternative promoter usage [86-90]. Due to its critical role in gene expression, altered gDNA methylation is associated with several human diseases including many cancers [91-95]. In addition, changes in "normal" gDNA methylation are correlated with many aspects of diabetes to include: susceptibility to DM [96-98], insulin resistance [99], diabetes complication development [99], and early detection [100-102]. Recently, a comprehensive genomic gDNA methylation profiling of T2 diabetic islets revealed that CpG loci displayed a hypomethylation phenotype and this finding may provide insight regarding diabetic islets and disease pathogenesis [103]. The first report demonstrating a cause and effect relationship between hyperglycemia and altered gDNA methylation related to genomic hypomethylation within the liver of T1 diabetic rats as early as 2 weeks post hyperglycemia onset [76]. Using primary aortic endothelial cells exposed to high glucose [24 hr] under *in vitro* conditions, Pirola et al. performed comprehensive analysis of both histone acetylation and gDNA methylation [75]. In their study, these investigators observed significant alterations in gDNA methylation patterns and showed that induced methylation changes localized to regions within five kilobases of transcriptional start sites. They also found broad changes to H3K9/K14 acetylation

and reported that regionalized hyper-acetylation correlated with gDNA hypomethylation and hyperglycemia-induced gene induction. These studies were limited to *in vitro* conditions and did not examine results from a prolonged hyperglycemic state or the metabolic memory state. In total, such studies establish a relationship between gDNA methylation and DM.

To provide supporting data that gDNA-methylation changes occur in the long-term diabetic state (described earlier as the MM state), our laboratory has recently reported, using a DM/MM animal model [8], that genes fundamental to cell division have altered methylation patterns in DM and this alteration continues into MM [62]. The functional groups that were found in the DM state and persisted into the MM state were identified by bioinformatics analysis to represent the DNA replication and DNA metabolism groups with up-regulation of the apex1, mcm2, mcm4, orc3, lig1, and dnmt1 genes of these groups [genes fundamental to DNA replication and cell proliferation] [62]. Methylated Regions of gDNA were reported as far as 6-13 kb upstream of the transcription start site for a subset of functionally important genes [e.g.dnmt1, mcm2, and orc3] within these groups. As indicated, these genes are fundamental to the cell division/DNA replication processes. It should be noted, that alterations in the human MCM2 gene has also been reported for patients with diabetes [104]. This indicates interesting parallels between the DM/MM animal model studies and the human diabetic condition; thereby providing some validity to the use of the DM/MM animal model. This data establishes a tie between gDNA methylation changes and gene expression changes in transcripts related to the control of DNA replication and thereby, cell division.

Lastly, MRs are associated with in silica identified Transcription Factor (TF) binding sites whose methylation is changed in the DM and MM state as studied in the DM/MM animal model by our laboratory (unpublished data). Published studies with mammalian cells have shown that such methylation changes are known to perturb TF binding to their respective DNA binding sites [105]. Methylation of TF binding sites in the proximal gene region of the human CTGF gene (involved in the regulation of endothelial cell division) has been reported for T2 DM patients [61] and also in our DM/MM animal model (unpublished data); again showing parallels in the process of gDNA-methylation changes found in our DM/MM animal model with that of patients with DM. The clinical findings regarding CTGF were discussed in terms of the long term complications seen in T2 DM patients; suggesting that alterations in gene expression of CTGF triggers dysfunction in processes targeted by this gene. Extensive work by others has established that CTGF is critical in the regulation of endothelial cell proliferation as it relates to angiogenesis [106-109] and alterations in blood vessel growth [both angiogenesis and neovascularization] are known to be altered in diabetes [11,110-112]. This establishes at least one human gene related to cell division regulation that is altered in DM via gDNA methylation processes. In line with these studies, a recent report by Ollikainen et al. [106] indicates that lymphocytes and leukocytes of T2 DM patients (and pre-condition patients) also exhibit de-methylation of proximal promoters in human genes. It should be noted that the studies of Ollikainen et al. [106] describe hypomethylation patterns for a wide variety of genes. In aggregate, these studies establish relationships between gDNA methylation and concomitant gene expression

changes associated with tissue dysfunction and impaired cell division in the diabetic patient. The application of these questions utilizing the B cell as outlined in this review should expand our understanding of the relationship of gDNA methylation to impaired cell division.

Summary and Conclusion

This review has focused on the problem of epigenetic mechanisms underlying DM and MM. It has discussed this in terms of the long term deficits observed in fundamental cellular processes such as cell division/DNA replication. Based on current literature, the review has described experiments and related literature to analyze methylated gDNA-based mechanisms to explain, in part, the relationship of hyperglycemia to the induction of tissue dysfunctions related to impaired cell division/DNA replication using the peripheral blood B cell. In this context, cell division/DNA replication processes are affected in a wide spectrum of tissues in DM. It proposes an experimental approach to this problem utilizing the human blood B cell as a paradigm for fundamental aspects of the cell division processes (e.g. DNA replication). It was noted that unlike many tissues, peripheral blood B cells can be easily obtained from both control and DM patients and it was suggested that such an approach will provide, in total, a broad spectrum of pertinent information related to: 1) epigenetic-related sequence data, 2) gene expression data, and 3) functional studies that will establish a link between gDNA methylation and subsequent alterations in gene expression regulation that can lead to impaired cell division/DNA replication.

Acknowledgment

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