Pathophysiological role of enhanced bone marrow adipogenesis in diabetic complications

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Abbreviations: AGE; advanced glycation end product; BADGE; bisphenol-A-diglycidyl ether; C/EBP; CCAAT/enhancer-binding protein; DAG; diacylglycerol; ERK1/2; extracellular signal response kinase; FABP4; fatty acid binding protein-4; FADH2; flavin adenine dinucleotide; FoxO1; forkhead box protein O1; GAPDH; glyceraldehyde-3 phosphate; GSK3β; glycogen synthase kinase 3β; IGF-1; insulin-like growth factor-1; IP3; inositol triphosphate; LEF/TCF; lymphoid enhancer factor/T cell factor; MPC; mesenchymal progenitor cell; mTOR; mammalian target of rapamycin; NADH; nicotinamide adenine dinucleotide; PI3K; phosphatidylinositol-3-kinase; PKA; protein kinase A; PKB; protein kinase B; PKC; protein kinase C; PLC; phospholipase C; PPAR; peroxisome proliferator-activated receptor; RAGE; receptor for advanced glycation end product; ROS; reactive oxygen species; Runx2; runt-related transcription factor 2; SETB1; SET domain bifurcated-1; TCA; tricarboxylic acid; TNF-α; tumor necrosis factor-α; TZD; thiazolidinedione derivatives; Wnt; wingless-type MMTV integration site family

Diabetes leads to complications in select organ systems primarily by disrupting the vasculature of the target organs. These complications include both micro- (cardiomyopathy, retinopathy, nephropathy, and neuropathy) and macro- (atherosclerosis) angiopathies. Bone marrow angiopathy is also evident in both experimental models of the disease as well as in human diabetes. In addition to vascular disruption, bone loss and increased marrow adiposity have become hallmarks of the diabetic bone phenotype. Emerging evidence now implicates enhanced marrow adipogenesis and changes to cellular makeup of the marrow in a novel mechanistic link between various secondary complications of diabetes. In this review, we explore the mechanisms of enhanced marrow adipogenesis in diabetes and the link between changes to marrow cellular composition, and disruption and depletion of reparative stem cells.

Diabetes and Its Complications

Diabetes is an incredibly prevalent disease, afflicting an estimated 220 million people in North America and 347 million people worldwide. Prevalence estimates have increased sharply since 1980 and are predicted to continue rising. As diabetes is a significant cause of morbidity and mortality, the economic burden is truly staggering and estimated to reach $17 billion a year by 2020 in Canada, and $116 billion in the United States. The main concern here is that nearly three-fourths of all diabetic people worldwide. Prevalence estimates have increased sharply by 2020 in Canada, and $116 billion in the United States. One of the earliest defects apparent in target organs of diabetic complications is a diminished capacity for vasodilation due to the unbalanced production of vasodilators and vasoconstrictors. One of the earliest defects apparent in target organs of diabetic complications is a diminished capacity for vasodilation due to the unbalanced production of vasodilators and vasoconstrictors. This functional alteration is accompanied by sustained structural remodelling of the vessels in target organs manifesting as retinopathy, nephropathy, cardiomyopathy, and accelerated atherosclerosis. Initiation as well as the progression of these complications also entails an impaired repair/regenerative mechanism. Vascular repair is largely dependent on the proliferation, mobilization and differentiation of bone marrow-derived progenitor cells. The angiogenic potential (reparative function) of these precursor cells is diminished in vasculopathies and may be resultant from diabetes-induced changes to the cellular composition of the marrow where these stem/progenitor cells reside.

Diabetic Marrow Dysfunction: Consequences of Enhanced Adipogenesis and Impaired Osteoblast-Genesis

Bone marrow is a rich source of stem cells. At least two different stem cell populations reside in the marrow: hematopoietic stem cells and multipotential stem cells (also known as mesenchymal/mesodermal stem cells, mesenchymal/marrow stromal cells; MSCs). Both of these stem cell types consist of a hierarchy of cells. MSCs are believed to give rise to endothelial cells, adipocytes, osteoblasts, and chondrocytes.
mesenchymal progenitor cells (MPCs; cells restricted to the mesenchymal lineage), adipocytes and osteoblasts. MSC progeny also create a cellular environment to maintain stem cell self-renewal in the marrow (Fig. 1).

Long-standing diabetes leads to cellular changes in the bone marrow, the functional significance of which is just being realized. These cellular changes include enhanced adipogenesis of MPCs as observed in both type 1 and 2 models of diabetes. In the insulinedeficient form of the disease, this leads to diminished bone density, with human studies and streptozotocin-induced diabetic animal models noting a decrease in trabecular bone mass and a reciprocal increase in the adiposity of the marrow. In the insulin-deficient form of the disease, this leads to diminished bone density, with human studies and streptozotocin-induced diabetic animal models noting a decrease in trabecular bone mass and a reciprocal increase in the adiposity of the marrow. Alternatively, analyses of bones of type 2 diabetics have generally observed unchanged or increased bone mineral density, though clinically, both diabetic populations have a substantially increased risk of fractures in comparison to non-diabetics. Additionally, there is increasing evidence that some diabetic medications may negatively impact bone density and marrow adiposity. Diabetes also induces microvascular remodeling in the bone marrow manifesting as impaired angiogenic ability, vascular endothelial cell dysfunction, and a reduction in stem cell number. These findings suggest that disruption of the bone marrow microenvironment, enhanced adipogenesis/suppressed osteoblastogenesis, may be responsible for detrimental effects on stem cell function and differentiation. If true, this provides a novel mechanistic link to impairment of endogenous repair in diabetes (Fig. 2). Indeed, both type 1 and type 2 diabetes is associated with lower circulating number of endothelial progenitor cells (cells that play a critical role in vascular regeneration) when compared with healthy subjects. Furthermore, the number of endothelial progenitor cells correlates with glycemic control. There are a number of possible mechanisms at play here: (1) diabetes may cause depletion of resident stem/progenitor cells in the marrow through alteration of the marrow stem cell environment, (2) diabetes may alter the mobilization of stem/progenitor cells, and (3) high levels of glucose in the circulation may reduce the number of cells that have mobilized. In fact, there is experimental evidence for all three possibilities. We and others have recently shown that diabetes leads to reduced number of stem cells in the bone marrow. These stem cells can be distinguished from hematopoietic stem cells by their ability to differentiate into endothelial and mural lineages and to regenerate functional vessels. Studies have also shown that diabetes leads to reduced mobilization of stem cells from the marrow. A number of signaling mechanisms have been identified underlying this abnormality. And finally, we have shown that high levels of glucose decrease endothelial and mesenchymal progenitor cell numbers acutely (within 24 h of culture). However, cells recover from glucose toxicity with sustained exposure. In addition, the differentiation capacity of blood and bone marrow-derived stem cells to produce endothelial and mesenchymal progenitor cells is not altered by the presence of high levels of glucose. Experimental evidence also shows that stem/progenitor cells isolated from diabetic mice are able to restore vascular homeostasis.

Taken together, these studies suggest that changing the cellular microenvironment in the marrow directly leads to dysfunction and reduction of stem cells in diabetes. The aim of this review is to elucidate the mechanisms underlying the increase in bone marrow adipogenesis observed in diabetes and examine the bidirectional relationship between bone adiposity and disease progression.

Mechanisms of Enhanced Marrow Adipogenesis in Diabetes

There is a wealth of knowledge on the process and factors involved in adipogenesis. The current understanding of adipogenesis has largely emerged from in vitro studies using cell lines such as the preadipocyte 3T3-L1 and 3T3-F442A cells. Although recently, studies conducted in human cells have also emerged. The process of differentiation in murine cell lines appears to be similar to the signaling cascade that drives adipogenesis in human bone marrow cells, with the principle actors being peroxisome proliferator-activated receptor γ (PPARγ) and the CCAAT/enhancer-binding protein (C/EBPα, β, and δ) transcription factors. It should be noted that there are reports of differences between murine cell lines and human MSC/MPCs. For example, Yu and colleagues suggested that human marrow cells primarily express PPARγ1 isoform upon differentiation with PPARγ2 increases being noted at later time point, which is believed to be in contrast to murine cells. PPARγ2 isoform does appear to be the minor species, comprising only 15% of all PPARγ expression within adipose tissue, although it has been shown to be the predominant isoform in regulating adipogenesis. Furthermore, knocking down the expression of C/EBPα prevents PPARγ2 induction and adipogenesis in human marrow cells. The expression profile during terminal adipogenic differentiation in human MSCs
is also similar to that of 3T3-L1 preadipocytes, characterized by early expression of C/EBP\(\text{\(b\)}\) and C/EBP\(\text{\(d\)}\) and followed by C/EBP\(\text{\(a\)}\) and PPAR\(\text{\(g\)}\) expression.\(^{42}\) In addition, we have shown specific induction of PPAR\(\text{\(g\)}\)\(_2\) in human marrow MPCs following addition of adipogenic differentiation media and the levels parallel C/EBP\(\text{\(a\)}\).\(^{36,37}\) Therefore, examining the expression of these transcription factors offer insight into paracrine factors regulating adipogenesis in diabetes.

A vast number of factors have been shown to modulate adipogenesis. Some of these modulating factors that are pertinent to the diabetic context include insulin,\(^{47,48}\) insulin-like growth factor-1 (IGF-1),\(^{49}\) extracellular proteins including collagen and fibronectin,\(^{50,51}\) and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)).\(^{52,53}\) Shifts in the expression or function of these and other effectors disrupt the homeostatic balance between adipogenic and osteogenic differentiation of MPCs.\(^{54-59}\) While the general consensus is that diabetic hyperglycemia is associated with increased adipogenesis in the marrow, inhibition of fat cell formation and promotion of osteoblastic differentiation following the administration of exogenous glucose has also been reported.\(^{60,61}\) Shilpa and colleagues found that culturing 3T3-L1 preadipocytes in extremely high glucose levels of 105 mM resulted in diminished adipogenesis, with downregulation of PPAR\(\text{\(g\)}\) and C/EBP\(\text{\(a\)}\) relative to cells cultured in 25 mM glucose concentration.\(^{61}\) The extreme hyperglycemic conditions emulated by the 105 mM glucose condition was found to increase cellular stress, leading to the induction of inflammatory cytokines, such as TNF-\(\alpha\), known to inhibit adipocyte differentiation and potentially dedifferentiation.\(^{61-63}\) This glucose level was considerably greater than the 25 mM concentration used to mimic hyperglycemia in most other studies, which may account for contrasting results.\(^{36,63}\)

The enhanced adiposity of the bone marrow observed in diabetes models and human diabetes appears to be a multifactorial consequence of augmented insulin signaling, hyperlipidemia, elevated blood glucose levels, and heightened oxidative stress. Recently however, novel signaling mechanisms have been highlighted that enhance adipogenic differentiation.

**PI3K-PKB pathway**

High levels of blood glucose have been demonstrated to increase adipocyte formation, lipid accumulation, and the expression of PPAR\(\text{\(g\)}\) in MPCs.\(^{64}\) It has been suggested that hyperglycemia mediates its effects through changes in post-receptor insulin signaling, which may be implicated in the development of insulin resistance.\(^{64}\) High levels of glucose increases the activity of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and the subsequent phosphorylation of protein kinase B (PKB), both of which are involved in the insulin signaling cascade. PKB-facilitated derepression of the \(ppary\) gene though forkhead box protein O1 (FoxO1) nuclear export leads to the induction of PPAR\(\text{\(g\)}\) and C/EBP\(\text{\(a\)}\) expression, resulting in increased adiposity of the bone marrow.\(^{64-66}\) PKB induced by hyperglycemia is also able to activate mammalian target of rapamycin (mTOR), which leads to increased expression of C/EBP\(\text{\(a\)}\), and other adipocyte-specific factors in pre-adipocytes, as well as muscle satellite cells, leading to the formation of intramuscular adipose depots.\(^{67-69}\)

**Reactive oxygen species**

The cellular production of reactive oxygen species (ROS) has been shown to be elevated in diabetic patients, largely due to increased glucose levels and metabolism.\(^{70-73}\) The predominant mechanism underlying the heightened oxidative stress in diabetes involves dysfunction of the mitochondrial electron transport system.\(^{74}\) In hyperglycemic cells, more glucose becomes oxidized through the tricarboxylic acid (TCA) cycle, which results in an increased number of electron donors, NADH and FADH\(_2\), being fed into the electron transport chain. This leads to an increase in the mitochondrial membrane voltage gradient until a specific
threshold limit is reached, at which point further electron transfer inside complex III is halted, causing a backlog of electrons in coenzyme Q. Coenzyme Q dissipates this excess negative charge through the donation of single electrons to molecular oxygen, leading to the formation of superoxide.

Superoxide inhibits the action of glycolytic enzyme glyceraldehyde-3 phosphate (GAPDH), which leads to the activation of the advanced glycation end product (AGE) pathway that has been shown to be increasingly stimulated in diabetes.\textsuperscript{74-76} AGEs are proteins or lipids that become glycosylated following exposure to sugars and accelerate cellular oxidative damage, and have been implicated in both micro- and macro-vascular diabetic complications.\textsuperscript{72,77,78} Binding of AGE and their receptors, known as RAGE, have been associated with reduced bone formation by osteoblasts and diminished matrix mineralization, in addition to impaired osteoblastogenesis.\textsuperscript{79,81} AGE-RAGE interactions have also been identified as promoting the apoptosis of osteoblasts and MPCs, contributing to the depletion of the stem cell niche.\textsuperscript{82,83}

Oxidative stress induced by hyperglycemia has also been found to stimulate the PI3K/PKB pathway, which acts to inhibit osteoblastic maturation and stimulate adipogenesis.\textsuperscript{84} Osteoblasts exposed to ROS resulting from a high glucose environment demonstrate decreased expression of runt-related transcription factor 2 (Runx2) and osteocalcin, with a concomitant increase in the abundance of the adipogenesis-related factors PPAR\textsubscript{γ}, adipin, and fatty acid binding protein-4 (FABP4).\textsuperscript{84} ROS is also able to prevent the mineralization of osteoblasts and enhance their accumulation of lipid droplets.

**Non-canonical Wnt-PKC pathway**

Perhaps the best-studied system in adipogenesis is the wingless-type MMTV integration site family (Wnt)-mediated signaling pathway.\textsuperscript{85} In humans, the Wnt family is comprised of 19 secreted glycoproteins that affect the differentiation and development of many cell types through autocrine and paracrine processes.\textsuperscript{86,87} It has been well accepted that activation of the Wnt pathway (β-catenin signaling) constrains progenitor cells to differentiate into osteoblast or myoblasts and prevents development along the adipocytic lineage.\textsuperscript{85} It is believed that endogenous production of Wnt ligands act to curb the terminal differentiation of preadipocytes and attempt to maintain a stem cell-like phenotype. Crosstalk also appears to exist between the canonical Wnt system and PPAR\textsubscript{γ}. When induced, PPAR\textsubscript{γ} binds the Lymphoid enhancer factor/T cell factor (LEF/TCF)-binding domain of β-catenin and facilitates its phosphorylation by glycogen synthase kinase 3B (GSK3β), directing the factor to the proteasome for degradation.\textsuperscript{88-91} Following the induction of differentiation within preadipocytes, levels of β-catenin remain elevated, until the expression of PPAR\textsubscript{γ} is heightened, resulting in the post-transcriptional downregulation of β-catenin and terminal differentiation.\textsuperscript{92}

Of the several non-canonical Wnt signaling cascades, the Wnt/Ca\textsuperscript{2+} pathway is presumed to be the most relevant in the regulation of adipogenesis. Interactions between specific members of the Wnt and Wnt receptor subtypes result in the activation of phospholipase C (PLC)\textsuperscript{87,93} PLC then leads to the generation of diacylglycerol (DAG) and inositol triphosphate (Ins[1,4,5]P\textsubscript{3}). Release of intracellular calcium activates protein kinase C (PKC), ultimately leading to phosphorylation of SETB1 (SET domain bifurcated-1) histone methyltransferase. This leads to the creation of a co-repressor complex that inhibits PPAR\textsubscript{γ} through H3-K9 histone methylation and directs the progenitor cell toward osteoblastogenesis through upregulated expression of Runx2, which is requisite for bone cell maturation.\textsuperscript{85,94} Interestingly, depending on the distinct isoform activated, PKC may have either a positive or negative influence on adipogenesis. PKC isoforms -α, -δ, and -μ are suspected to inhibit maturation.\textsuperscript{95} The initiation of adipogenesis appears to be reliant on PKC-B1 and PKC-γ is believed to be necessary for clonal expansion.\textsuperscript{95,96} PKC-ε is presumed to be critical for pre-adipocyte commitment and the final acquisition of the adipocytic phenotype, though the mechanisms leading to the effects of these three positive modulators are not yet understood.\textsuperscript{97,98}

We have recently shown that non-canonical Wnt11 is induced by hyperglycemia in MPCs and enhances the adipocytic differentiation.\textsuperscript{36} While the mechanism remains to be fully elucidated, a current hypothesis is that, through a non-canonical pathway, hyperglycemia induces a switch in Wnt11 signaling that differentially activates the various isoforms of PKC, specifically inducing the phosphorylation and consequent activation of PKC. PKC-ε is translocated from the cytoplasm to the nucleus where it is expressed in spatiotemporal symmetry with C/EBPβ, indicative of a potential interaction.\textsuperscript{36,95} Through a currently unknown process likely involving the phosphorylation and regulation of key nuclear adipogenic factors, PKC-ε activation results in the acceleration of adipogenic differentiation.

**Hyper- and hypo-insulinemia**

Insulin is one of the factors commonly used to stimulate adipogenic differentiation in cell culture systems, and in vivo models of insulin receptor knockout display impaired adipogenic differentiation and lipid storage capacity.\textsuperscript{99,101} A hyperinsulinemic state is frequently observed in the development of type 2 diabetes as pancreatic production of insulin surges in an attempt to counteract the ever-increasing resistance of peripheral tissues.\textsuperscript{102,103} Hyperinsulinemia may be capable of inducing the adipogenesis of cells within the marrow stem cell niche through a signaling cascade involving PKB and mTOR, culminating with activation of C/EBPα and PPARγ.\textsuperscript{104} Conversely, hypoinsulinemia, a hallmark of type 1 diabetes and an eventual occurrence following β-cell failure in type 2 diabetics, may also indirectly lead to enhanced adipogenesis. Insulin receptor knockout mice display a 2-fold upregulation of the IGF-1 receptor through a yet unknown mechanism.\textsuperscript{103} Both the IGF and insulin signaling systems converge on a common pathway involving PKB, which may grant IGF partial control of adipogenic differentiation under hypoinsulinemic conditions.\textsuperscript{105} When combined with the administration of exogenous insulin therapies, the overexpression of IGF-1 receptor may lead to disproportionate fat cell development.
Hyperlipidemia

A large proportion of diabetics are subject to hyperlipidemia, particularly if their condition is poorly controlled. A study of diabetic mice has observed elevations in the relative quantities of plasma di- and tri-unsaturated fatty acids compared with saturated fats. Fatty acids, particularly polyunsaturated fatty acids, have been identified as agonists for PPARγ, and although they possess a relatively low affinity, the substantial elevation of serum lipids in diabetes may be sufficient for activation. Dyslipidemia may prohibit the efficient maturation of osteoblast-like cells and is capable of inducing the trans-differentiation of osteoblast-like cells into adipocytes, further attenuating the density of the bone marrow.

Diabetic Medications

Another potential contributor to the diminished bone integrity seen in diabetes may be the effects of anti-diabetic medications. While insulin-sensitizing agents are crucial to the maintenance of normoglycemia and avoidance of life-threatening complications, they also have a chronic effect on the bone marrow. The current therapy in the treatment of type 2 diabetes involves metformin, which suppresses hepatic gluconeogenesis to moderate blood glucose levels and increase insulin sensitivity. Thiazolidinedione derivatives (TZDs), also known as glitazones, are a group of medications that act to improve insulin responsiveness within target tissues, concomitantly augmenting hyperglycemia and hyperlipidemia. TZDs have also been implicated in diabetic bone loss, with a significantly increased risk of fractures and osteoporosis while on these medications being well-documented. The primary mechanism of action of TZDs is through the direct induction and activation of PPARγ, leading to improved insulin sensitivity throughout the body via an unknown mechanism. The efficacy of TZDs in rectifying systemic insulin resistance through PPARγ knockout systems, embryonic stem cells spontaneously differentiated into osteoblasts, while adipogenesis was inhibited. In vivo, PPARγ haploinsufficient mice also display heightened levels of osteoblastogenesis, leading to increased bone mass. Conversely, deletion of β-catenin in osterix-expressing cells (early osteoblast lineage) leads to a striking reduction in bone mass and an increase in bone marrow adiposity. These studies suggest that one mechanism of diabetic bone phenotype may be depletion of available progenitor cells through commitment to one lineage. Treatment of diabetic mice with the PPARγ antagonist bisphenol-A-diglycidyl ether (BADGE) inhibits adipogenesis without suppression of osteoblast markers and consequent bone loss. The mechanism of this disconnect is not fully clear but it may be related to duration of BADGE treatment. In fact, acute exposure of BADGE has been shown to be ineffective in reducing osteocalcin levels in cultured osteoblasts, whereas chronic treatment significantly suppresses the levels. Osteocalcin has also been observed to decrease in diabetes, with changes in factors involved in earlier differentiation, such as Runx2, occasionally being observed. The changes to osteoblastic gene expression are mediated through activation of...
protein kinase A (PKA) and extracellular signal response kinase (ERK1/2) signaling mechanisms, which attenuate osteoblast differentiation. 145

In diabetes, both acute and chronic hyperglycemia induces osmotic changes in cells as they adapt to the heightened colloid pressure by reducing their volume and augmenting gene expression. 143, 146 Acute in vitro exposure of osteoblast precursor cells to high glucose levels and hyperosmolarity resulted in increased expression of collagen I, along with downregulation of osteocalcin mRNA. 146 With sustained exposure, high levels of glucose are able to induce the upregulation of alkaline phosphatase, along with diminishing production of osteocalcin. The mRNA levels of PPARγ were also found to be increased nearly 2-fold in pre-osteoblasts challenged with high levels of glucose. 143, 145 In vivo studies have shown similar findings in the bones of diabetic mice, reporting a 40% reduction in osteocalcin expression, though markers of early osteoblast development, such as Runx2, remain unchanged. 16, 17 Adipogenic genes PPARγ, resistin, and FABP4 were all found to be significantly upregulated, with a 3-fold increase in the quantity of marrow adipocytes being observed. This suggests that an elevated serum glucose level impairs the later stages of osteoblastogenesis, while promoting the expression of markers of the adipogenic phenotype.

Upon enhanced adipogenesis, the interaction between adipocytes and osteoblasts takes on another layer of complexity. Adipocytes secrete numerous proteins collectively referred to as adipokines, which include adiponectin, leptin, resistin, and tumor necrosis factor-α (TNF-α), among others. 147 Paradoxically, adiponectin which is secreted nearly exclusively by adipocytes has been found to prevent adipogenic differentiation in bone marrow cultures and increase trabecular bone mass by promoting osteoblastogenesis and repressing osteoclast formation. 148-151 Unlike adiponectin, the expression of leptin increases concurrently with adiposity and appears to be unaffected by diabetes. 152-154 Leptin appears to have contradictory effects on bone, activating the sympathetic nervous system to accelerate bone loss, as well as stimulating the osteogenic differentiation of marrow MPCs, with the net outcome dependent on its concentration. 155-160 Lastly, TNF-α, has also been identified as having detrimental effects on the skeleton. A positive correlation has been found between the expression of TNF-α by adipocytes and both obesity and insulin resistance. 161 Local TNF-α signaling leads to enhanced differentiation of osteoclast precursors and increased bone resorption. 162, 163 We are just starting to understand how these adipokines are altered in diabetes and the subsequent effect of this alteration both systemically and in the marrow. This field of research will bring upon a new era in our understanding of chronic diabetic complications.

Concluding Remarks

With the incidence of diabetes on a rise, it is imperative that we understand the mechanisms of secondary diabetic complications. Sustained hyperglycemia has been demonstrated to lead to increased adiposity of the bone marrow, with a concomitant escalation in the risk of fractures and may potentially be the cause of reduced stem cells for endogenous vascular repair. While the basic process underlying adipogenesis is well-elicited, the specific factors promoting and inhibiting the C/EBP-PPAR signaling pathway are numerous and complex, with their potential roles in preventing diabetes-induced bone marrow adipogenesis relatively unexplored. The exploitation of marrow adipose biology may soon become a central treatment strategy in diabetes, precluding complications or preventing the disease itself.

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