

Molecular interactions in extracellular matrix of tendon

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1. ABSTRACT

Tendon is a poorly vascularized and highly specialized connective tissue containing few scattered cells that play an important role in the musculoskeletal apparatus by resisting mechanical stress. Because of the slow rate of the metabolism of its molecular components, the tendon gradually loses its mechanical properties and may rupture upon an array of physical activities. In this report, we discuss the molecular changes involved in the extracellular matrix-tendon interactions leading to tissue degeneration and rupture.

2. INTRODUCTION

Cells are closely connected to each other in all tissues and their cross-talk allows appropriate biological tissue function. Cells are organized in specialized structures responsible for particular mechanical properties, size and shape. The extracellular matrix (ECM) is a specialized environment surrounding all structures, and interacts with various cells. The ECM plays a pivotal role in maintaining efficient tissue function and organ development. However, it undergoes several changes during the pathological process, and impact the biomechanical behaviour of the

tendon. The importance of the ECM is evidenced by the interaction of hydrophilic and hydrophobic molecules to confer specific characteristics to the intercellular milieu; this feature determines the mechanical tissue properties of bone tissue or an elastic organ such as lung. Although the ECM of these tissues contains fibrillar collagen type I mainly, the bone ECM composition and organization provide rigid mechanical properties to the tissue. In contrast, the ECM of an elastic organ, such as lung, has a different molecular composition that determines its specific mechanical properties. Thus, the mechanical properties of a tissue depend on the structural organization and the molecular composition of the ECM. However, ECM composition changes during the development stages of the same tissue. As an example, glycosaminoglycan (GAG) cartilage composition changes dramatically during aging in all mammals, suggesting that GAG can be used as a marker of cartilage aging. Moreover, the number of different molecules is an additional variable to consider in defining a specific tissue.

Tendons and ligaments are dense, regularly arranged, connective tissues that support joint movement (1). The mechanical properties of tendon ECM provide a functional link between skeletal muscle fibers and the bone, playing a critical role in force displacement. Contractile filaments in skeletal muscle react to force development whereas the adjacent tendon tissue shows a passive structure allowing joint movement (2-5). Signals from mechanical movement can trigger a cascade of molecular events, including gene expression, transcription, translation and posttranslational modifications, leading to changes in tendon proteins (6). However, these events are poorly understood in tendon ECM compared to other tissues.

In addition to describing the structural and functional properties of tendon ECM, this review will discuss the gene expression changes that drive a healthy tendon to rupture.

3. ECM IN TENDON

Synthesis of the components of the tendon occurs in fibroblasts. Studies using isolated tendon fibroblasts showed that fibroblasts respond to mechanical stress, and release several components of the ECM (7). Cells in a healthy tendon are associated through gap junctions as revealed by immunolabeling for connexin32 and connexin43 (8-9). In the tendon, the architectural interconnection of fibroblasts forms a three-dimensional network surrounding the collagen fibrils, which provides a basis for cell-to-cell interaction. Under mechanical cyclic loading, tendon cells upregulate collagen and gap junction production, whereas pharmacological inhibition of the gap junction abolishes this response (10). The ECM encompasses various molecules of which collagen fibrils and proteoglycans (PGs) are the major components. In addition to PGs, the hydrophilic ECM includes a variety of other proteins such as noncollagen glycoproteins (11-12). ECM integrity is necessary for tendon function during muscle-tendon complex force transmission and tendon fibrillar arrangements (13-14).

Matrix tensile strength depends on intra- and inter-molecular crosslinks, orientation, density, and length of both collagen fibrils and fibers, indicating that the integrity at molecular level is the critical part of the architecture (15). However, very little is known about the growth of connective tissue cells under mechanical loading as well as the expression and synthesis of specific ECM proteins (7).

3.1. Tendon components

Tendons consist of 55–70% water with a systematic and densely packed connective tissue (16). Collagen is its major component (60-85% of tendon dry weight), organized into fibrils, fibers, fiber bundles and fascicles, and it forms 20-25% of ECM proteins. The predominant form, type I collagen (60%), is arranged in tensile-resistant fibers. It is composed of two $\alpha 1$ and one $\alpha 2$ chains, which are products of separate genes rather than a posttranslational modification of a single molecule. Other ECM molecules are also present in the tendon, including collagen types III, IV, V and VI (less than 10%) (17), PGs and other glycoproteins (18). Collagen synthesis/degradation is measured indirectly by determining the collagen peptides produced during the different steps of collagen biosynthesis and catabolism. Pro-collagens contain amino terminal and carboxy terminal extension peptides at the respective ends of the collagen molecule. After secretion, the amino-propeptides are cleaved by specific proteinases and the collagens self-assemble into fibrils or other molecular structures. While pro-collagen release and turnover have been extensively studied in the bone, these mechanisms are not well explored in the tendon (19).

The non fibrous component of the tendon is characterized by PGs, namely small leucine-rich proteins of which decorin (up to 1%) is the main molecule (11). Also, tendon tissue contains other small leucine-rich PGs such as fibromodulin, biglycan (up to 0.5%) and lumican, together with cartilage oligomeric matrix protein (COMP, less than 1%), osteoadherin, tenascin-C, proline arginine-rich and leucine-rich repeated proteins, optican, keratocan, epiphykan, syndican, perlecan, agrin, fibronectin, laminin, versican and aggrecan (20-23). PGs contain sulphated polysaccharide chains or glycosaminoglycan (GAG), macromolecules that are widely distributed in nature showing great variability in structure and function. PGs are vital in tissue structure maintenance and differentiation, especially articular cartilage. Like the majority of polysaccharide chains, PG functional peculiarities depend on their structural complexity. They contain a core protein coded by a single gene and at least one sulphated polysaccharide chain, GAG. GAGs are covalently linked to the core protein and show a wide variety of chemical species and structures. For these reasons, GAGs are classified as galactosaminoglycuronan (dermatan sulphate and chondroitin sulphate) and glucosaminoglycuronan (heparansulphate and heparin). Glucuronic acid covalently linked to a mono- or di-sulphated hexosamine constitutes the disaccharide units. Hyaluronan (HA) is the only GAG without core protein and sulphated hexosamine. The core proteins have been studied in depth and their gene

sequence, splicing variants and crystallographic properties have been characterized, emphasizing the vital functional role of PG core protein (24-27). PGs seem to interact with several ECM molecules, primarily growth factors, in healthy and pathological conditions, playing an important role in tissue metabolism.

3.2. Metabolism of collagen and other extracellular matrix proteins

3.2.1. Structures

Collagen is synthesized and secreted by fibroblasts, and undergoes co- and post-translational modifications, which contribute to the quality and stability of the collagen molecule. In the cell, the C-propeptide domains of polypeptide alpha-chains fold and the trimerization process leads to the formation of fibrillar collagen types. The gene coding pro-collagen chains encompasses large intronic and exonic DNA sequences, which requires extensive RNA processing for protein synthesis (28). Pro-collagens are transferred from ER to the extracellular space through the Golgi apparatus and contain amino-terminal and carboxy-terminal extension peptides at the respective ends of the collagen molecule (29). Pro-collagen requires peculiar transport within the Golgi apparatus, being larger than the conventional transport vesicles (30). After secretion into the extracellular space, the amino-propeptides are cleaved by specific proteinases and the collagens self-assemble into fibrils or other molecular structures (31). Following the transcription of genes coding for collagen type I formation, the initially synthesized pro-alpha-chains undergo posttranslational reactions. At the outset hydroxylation converts residues to 4-hydroxyproline or 3-hydroxyproline by three different hydroxylases (32). Hydroxylases are specific enzymes that act on non-helical substrates. Therefore they do not affect collagen or collagen-like peptides that are triple helical structures. Newly synthesized collagen polypeptides are glycosylated and this process ends before the collagen is folded into a triple helix structure. The intracellular processing leads to the synthesis of both intrachain and interchain disulfide bonds (33), and secretion of pro-collagen. The three polypeptide chains form a triple-helical structure, and the alpha-chains, forming the structure, are composed of repeating amino acid sequences Gly-X-Y, where the glycine residue enables the three alpha-chains to coil the polypeptide chain. Proline and 4-hydroxyproline residues appear frequently at the X- and Y-positions, respectively, and promote the formation of intermolecular cross-links. The stability and quality of the collagen molecule is largely based on the intra- and inter-molecular cross-links. A unique feature of collagen is that 4-hydroxyproline formation is catalyzed by P-4-H, indicating that it is a biomarker for evaluating collagen content. P-4-H activity is generally associated with collagen biosynthesis and has been used to estimate changes in the rate of collagen biosynthesis in various experimental and physiological conditions (34). Pro-collagen C-proteinase is identical to bone morphometric protein (BMP-1) (35). A more recent report described both intra- and extracellular pools of active procollagen C-proteinase (BMP-1) in embryonic chicken tendon fibroblasts, whereas the N-proteinase (called ADAMTS-2) is located in close

proximity to or within the plasma membrane (36). The block of C-proteinases stops the conversion of extracellular pro-collagen to collagen inducing an intracellular procollagen accumulation in post-Golgi.

Fibril segments are prerequisites for fibril formation, acting as primers and have been shown to increase in length gradually increasing diameter (37-40). Collagen molecules can be either unipolar or bipolar and fuse end to end (41-43). Although, cross-link deficient tendons are less resistant to loading and that tendon elongation depends on molecular cross-links within the collagen fibrils, the role of fibrillar structure in resistance to loading is not clear (44-46).

PGs play a critical role in fibrillogenesis and when decorin/fibronectin binding is inhibited, tendon length increases (47-48). Pyridinoline (Pyr), a cross-link component of the ECM of mature collagen fibers, is critical to the formation of collagen structures (49). The ratio between Pyr and collagen is particularly high in tendon indicating that this parameter could be useful in the study of tendon metabolism.

3.2.2. Glycosaminoglycan

The GAG sulphated groups providing negative charges for PGs are critical for macromolecule function. While HA synthesis occurs in the cytoplasm, and HA synthases (HAS 1, 2 and 3) are located on the cell membrane, sulphated GAGs polymerization takes place in the Golgi apparatus where several polymerases cooperate with sulphotransferases to build polysaccharide chains. Sulphotransferases transfer a sulphate group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to a growing chain in the Golgi apparatus after the action of the PAPS transporter. The enzymatic machinery operates in the Golgi membranes to facilitate the biosynthesis of sulphated sugar chains, similar to the process of HS/heparin biosynthesis where glucuronyl C5-epimerase interacts with heparansulphate (HS) 2-O-sulphotransferase (HS2ST) (50). Glycan sulphation by Golgi-resident sulphotransferases is now recognized as a major regulatory mechanism. Glycan sulphation is known to affect glycoprotein hormone pharmacokinetics, growth factor and cytokine activity, and viral and bacterial adhesion (51-55). While the PGs and water provide space and lubricating properties for the tendon, the precise role of the small non aggregating leucine-rich PGs remains unclear. The PGs also seem to play an important role in fibril fusion, as do fibrillin molecules aligning along fibrils (56).

3.2.3. Turn-over

Carboxypeptide cleavage allows indirect determination of collagen type I formation. Development of assays to determine markers for synthesis of type I collagen (the COOH-terminal propeptide of type I collagen (PICP)) and degradation (the COOH-terminal telopeptide region of type I collagen (ICTP)) has offered the possibility to study the effect of mechanical stress on collagen type I turnover (57). The microdialysis technique demonstrated that acute exercise induces changes in metabolic and inflammatory activity in the peritendinous region (19). In addition, acute

exercise induced a higher type I collagen formation in the recovery process, suggesting that acute physical loading leads to adaptations in human non-bone-related collagen. Like in the blood, values for P1CP did not change significantly over the training period, suggesting that the increased collagen type I formation occurs locally in tendon, rather than reflecting a general increase in the formation of collagen type I in all tendons (58). The stimulation of synthesis and degradation of collagen in tendon in response to exercise is associated with the events occurring after muscle exercise (59). An increased collagen turnover was observed in response to training and a net synthesis of collagen type I occurs in tendon with prolonged training. Using magnetic resonance imaging (MRI), the Achilles tendon cross-sectional area was enlarged in trained individuals compared to untrained controls (60).

Collagen turnover in the tendon is comparable to that in the bone (61-62). Therefore, the load leads to a higher increase in tendon collagen type I synthesis than the increase of muscle type IV collagen synthesis. Similar results were obtained measuring other non-collagen tendon proteins synthesized after loading, indicating that the tissue response involves all ECM proteins (7, 23, 63). On the other hand, immobilization leads to reduced collagen synthesis, and subsequent decrease in the enzyme activities of collagen biosynthesis in both skeletal muscle and tendon (64-65), suggesting that collagen biosynthesis decreases with inactivity (66). Gene expression experiments suggest that the rate of collagen expression during immobilization is partially downregulated at the pre-translational level (67). An early decrease of mRNA expression for collagen type I α 1, I α 2, and I β 1 was evident after 3 days of immobilization (68).

3.2.4. ECM

ECM protein balance is regulated by synthesis and degradation of ECM components (69-71). Several proteolytic enzymes are involved in matrix degradation, metalloproteases (MMPs) being the most important molecules. MMPs are a family of zinc- and calcium-dependent endopeptidases involved in remodeling the normal ECM connective tissue. Collagen degradation is a critical step in turnover and remodeling of connective tissue as well as tendon healing, and it is initiated extracellularly by MMPs (or matrixins) present mainly as latent proMMPs (72-74). Using rat model, MMP-9 and MMP-13 were involved in collagen degradation only, whereas MMP-2, MMP-3 and MMP-14 were implicated in both collagen degradation and remodeling (75). The endogenous activity of MMPs is normally inhibited by the endogenous tissue metalloprotease inhibitors (TIMPs). The metabolic balance between the activities of endogenous MMPs and TIMPs is responsible for the normal remodeling of tendon. However, excess of MMP activity can lead to progressive degeneration and weakening of the tendon's ECM. Erlichman and co-workers demonstrated that MMPs act extracellularly without any involvement in the major intracellular lysosomal proteolytic machinery (76). Tendon collagen degradation is described as a consequence of

inflammatory processes, tissue damage or development (77-79).

Collagenases (MMP-1 and MMP-8), degrading collagen type I α 1 and I α 2, are the most important enzymes for tendon metabolism. Gelatinases (MMP-2 and MMP-9) can digest non fibrillar collagen and other ECM compounds (80). However, MMP-2 can also degrade type I collagen, most likely in a two-step fashion (81-82). MMPs are present in the musculotendinous apparatus, being produced from fibroblasts of the endotenon and intramuscular matrix. The immobilization leads to an increase in MMP gene expression at both pre- and post-translational levels, suggesting accelerated collagen breakdown. Recent experimental evidence showed that acute exercise induced an elevated interstitial accumulation of MMP-2 and MMP-9 in human peritendinous tissue supporting the idea that MMPs (and their inhibitors) play a pivotal role in ECM adaptation to exercise in tendon tissue (83). Furthermore, MMP-1 expression can be modulated by growth factors, inflammatory cytokines and cytoskeleton-disrupting drugs like cytochalasin D, and MMP-1 gene expression can be stimulated by an IL-1 autocrine feedback loop (84-85).

Four different TIMP molecules have been identified so far (86). TIMP-1 and TIMP-2 are the most common and inhibit the activities of all MMPs with a preference for MMP-9 and MMP-2, respectively. TIMP-2 is not only involved in MMP2 inhibition, but acts as a docking element exposing pro-MMP-2 to MT1-MMP that cleaves the pro-peptide to activate MMP2 (87). After exercise, TIMP expression is upregulated at the pre- and post-translational levels, suggesting an increase in collagen degradation coupled with collagen synthesis (88-89). These findings indicate that concomitant stimulation and inhibition of ECM degradation occurs during exercise suggesting that TIMPs regulate ECM degradation.

4. TENDON NUTRIENTS SUPPLY

Compared with muscle, tendons have a poorly developed vasculature, and the area occupied by vessels represents 1-2% of the entire ECM (90). The vessels mainly stem from the epitendon where they run longitudinally into the endotenon (91). Feeding arteries and arterioles may come from the perimysium at the musculotendinous junction and vessels from the tendon bone junction (92). However, how blood flows in different conditions of tendon remains poorly understood. For example, rabbit tendons have 1/3 of muscle blood flow during inactivity, whereas blood flow increases both in ligaments and tendons during activity and tissue healing (93). Different human models have shown a blood flow increase up to sevenfold within and around tendon during exercise in young, middle aged and elderly subjects. Tendon blood flow is regulated independently, implying a refined balance in different mechanical situations (94-98). However, the role of ECM in regulating blood flow and vessel diameter is not clear. An increase in adenosine concentration, intensity-dependent, was observed in the muscle, but tendon modifications were not significant and

unrelated to intensity (99). Furthermore, bradykinin concentrations showed parallel increase in the both tissues during exercise, and its maximal response was recorded at low exercise loads (99). These two substances are involved in skeletal muscle and tendon blood flow regulation during exercise. Whether bradykinin has a vasodilatory effect on the vasculature, directly or indirectly *via* release of other substances such as nitric oxide (NO), prostaglandins or endothelium derived hyperpolarizing factor (EDHF), is yet to be established (100-103).

There is no evidence that the blood flow increase is sufficient to meet the oxidative needs of the tendon during exercise (94,104,105). There is a correlation between increasing tendon blood flow and declining oxygen tissue saturation, indicating that the estimated oxygen uptake in human tendon increases significantly during exercise compared to the resting state (106).

5. FUNCTIONAL ROLE OF ECM IN TENDON

Together with muscles, tendons act to transmit the contraction force (107-109). Although several ECM molecules, in addition to collagen, have been located in tendon and muscle, little is known about their functional role. The use of knock-out (KO) mice models helped elucidate the role of ECM molecules in the muscle and tendon. A mix of alterations was shown using these models in various connective tissues such as the development of severe osteogenesis imperfecta in type I collagen KO mice (110). The lack of laminin, collagen type VI and integrins altering the basal membrane structures, induces muscle dystrophy and myopathy (111). Irregular collagen fibrils were observed in tendon structure using fibromodulin-null mice models to study proteoglycan defects, whereas no changes were detected in bone or cartilage (112). Mice KO for biglycan and fibromodulin showed ectopic tendon ossification (113). Interestingly, mice lacking COMP showed no clear musculotendinous abnormalities, whereas COMP deficiency in humans causes skeletal dysplasia (114).

PGs play a critical role in the ECM molecules interaction (115-117). Decorin KO prevents any lateral fusion of collagen fibrils, indicating its involvement in collagen fibril formation and diameter control (47,115). The inhibition of decorin results in larger collagen fibrils and increased mechanical properties in healing ligaments (118). The role of decorin and the fibromodulin or lumican are not fully understood (119-121). In chicken embryonic tendon, small leucine-rich PGs like decorin are bound to collagen even prior collagen fibril assembly, suggesting complex effect of these PGs in collagen synthesis (41).

It is not surprising that the PG composition in tendon can change in different physiological conditions. Aggrecan, the large chondroitin sulfate PG, is largely upregulated after tendon mechanical loading, whereas decorin only responds to tensile loading (63,122). This could explain why type II collagen is found in tendon regions subjected to compressive forces (9,123,124). Several observations confirm these findings, including the

increase in larger weight of PGs in tendons in response to compressive stress (125-126). Thus this data illustrate the differentiated response to tensile and compressive loading, respectively, on collagen and ECM PGs (127-128).

6. FROM GENE EXPRESSION TO TENDON RUPTURE

Spontaneous tendon rupture and chronic tendon pain are common events. However, the underlying pathological process is not well understood and hence current treatment is not effective in all cases. A genetic component has been implicated in several tendinopathies that could explain why there is an increased risk of contralateral rupture of the Achilles tendon in subjects with previous rupture (129). Tendinopathy may develop due to the interaction between a particular genetic configuration and the various intrinsic and extrinsic factors affecting tendon health (130). Histopathological studies have revealed that inflammatory processes were substantially absent, as was cell infiltration. Nevertheless, many ECM degeneration aspects were evident including loss of collagen fiber organization, decreased fibril diameter, changes in cell density (both increased and decreased), cell rounding, GAG accumulation, lipid accumulation and calcification (131-133). Although similar alterations were found in normal tendons, they are generally less severe, and it is assumed that ECM degeneration precedes the onset of clinical symptoms (134). Collagen total content was decreased whereas collagen type III showed a relative increase in ruptured tendons (135). These collagen changes are coupled with an increase in crosslinks related to gradual accumulation of type III collagen over a relatively long time period, allowing maturation of collagen crosslinks and stabilization of the ECM (136). As a result, the gradual incorporation of type III collagen into the main fibre bundles is consistent with a reduction in average fibril diameter (137). These molecular alterations reduce the tendon's capacity to resist stress, and are involved in tendon rupture (138). In addition, other biochemical studies have shown an increase in the amount of HA and various PGs in degenerated tendons (17,139). Increased hydrophilic polymer such as HA induces a high water concentration. Molecular approaches such as PCR and microarray analyses have provided relevant information on gene expression in tendinopathy (140-142). Ireland and coworkers found 23 genes upregulated and 17 genes downregulated in degenerated Achilles tendon (140). Proteomic Validation studies are underway to verify the presence or absence of the corresponding proteins. Interestingly, no changes in the expression of cytokines and their receptors were observed, indicating the absence of any ongoing inflammatory process, as confirmed by analysis of the fluids surrounding painful tendons (143). Alteration of ECM at molecular level is the key element of tendon pathology, and in this context the expression of proteolytic activities and their effects on tendon ECM turnover are critical. While the precise role of MMPs in tendinopathy remains to be determined, several MMPs have been identified in acute tendon injuries, showing different timing and location of expression (144). The expression levels of MMP-9 and MMP-13 were high one and two weeks after

injury, whereas MMP-2, MMP-3 and MMP-14 were highly expressed after four weeks (75). It appears that MMP-9 and MMP-13 are involved in collagen degradation during the initial inflammatory phase, whereas MMP-2, MMP-3 and MMP-14 play a critical role in scar tissue remodelling. The absence of MMP-1 and MMP-8 expression was associated with absence of inflammation. In addition, while MMP-9 and MMP-13 were detected only in ruptured tendons, small but variable increase in MMP-2 and MMP-14 was observed in degenerated tendons (140-143). MMP-3 activity showed the greatest difference between normal and pathological tendon specimens (140-143). In fact, a remarkable loss of MMP-3 activity has been reported in tendinopathy which may cause an increase in PGs. However, PG degradation is attributed to aggrecanases, and it is still unclear how the decrease of MMP-3 could be associated with an increase in PG content (80). Because PG levels are increased in degenerated tendon lesions (unlike osteoarthritis), it would be interesting to see whether this is due to enhanced synthesis or decreased degradation of PGs.

Tendons are frequently affected by chronic pain or rupture, and many causative factors implicated in their pathology remained unexplored until recently. Tendinopathy is mainly associated with degeneration, which is thought to be an active, cell-mediated process involving an increased turnover and remodelling of the tendon ECM (145). Neuropeptides and other factors released by stimulated cells or nerve endings in or around the tendon might influence matrix turnover and could provide new targets for therapeutic intervention.

7. PERSPECTIVES

This review focuses on the critical role of ECM, namely collagen, in the tendon-muscle interaction and adaptation to mechanical loading, and the effect of these stimuli on ECM composition at molecular level. In contrast to the view of the ECM as a static and inert portion of the tendon, several studies have introduced the concept that ECM is a dynamic structure that adapts to a variety of functional demands. Further studies could clarify the relation between the different ECM molecules and the balance between catabolic and anabolic processes.

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Abbreviations: Extracellular Matrix (ECM), glycosaminoglycan (GAG), proteoglycans (PGs), Cartilage oligomeric matrix protein (COMP), Hyaluronan (HA), endoplasmatic reticulum (ER), prolyl-4-hydroxylase (P-4-H), galactosylhydroxy-lysyl-glucosyltransferase (GGT), bone morphogenetic protein (BMP-1), pyridinoline (Pyr), HA synthases (HAS), 3'-phosphoadenosine 5'-phosphosulfate (PAPS), HS 2-O-sulphotransferase (HS2ST), propeptide of type I collagen (PICP), telopeptide region of type I collagen (ICTP), Magnetic Resonance Imaging (MRI), Matrix Metalloproteases (MMPs), Tissue Inhibitors of Metalloproteases (TIMPs), Membrane type-1 matrix metalloprotease (MT1-MMP), nitric oxide (NO), endothelium derived hyperpolarizing factor (EDHF), knock – out (KO)

Key Words: Tendon, Extracellular matrix, Gene expression, Collagen, Matrix Metalloproteases, Tissue inhibitor of Matrix Metalloproteases, Tendinopathy, Review

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