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The contribution of Ig-superfamily and MARVEL D tight junction proteins to cancer pathobiology

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Abstract

The epithelial linings of eukaryotic organs form dynamically-regulated selectively-permeable barriers that control the movement of substances into (and out of) mucosal tissues. The principal structural determinants of epithelial barrier function are intercellular tight junctions (TJs), multi-protein complexes composed of claudin and non-claudin transmembrane proteins in addition to cytosolic linker proteins. As well as their crucial roles in barrier function, it is now well recognized that TJ proteins coordinate a variety of signaling and trafficking functions regulating physiological events such as cell differentiation, proliferation, migration and polarity. Accordingly, dysregulations in TJ protein expression or function are increasingly being linked to several pathophysiologies including cancer. To date, claudins have received the most attention as putative contributors to cancer initiation or progression. However the contribution of non-claudin transmembrane TJ proteins (including select immunoglobulin superfamily members, nectins, occludin and Marvel D family members) to the pathophysiology of cancer remains incompletely understood. Therefore the focus of this review is to collate recently-published evidence that supports or discounts a role for non-claudin transmembrane TJ proteins in cancer, and to speculate upon the feasibility of these molecules as prognostic biomarkers or therapeutic targets in cancer.

Introduction

The development of multicellular organisms starts from single cells in a highly regulated process during which individual cells recognize their neighbours and start to form intercellular junctions. Correct spatial and temporal establishment of cell-cell contacts is critical for developmental and physiological functions including morphogenesis, differentiation, proliferation and migration (1). Cell-cell contacts within epithelial or endothelial barriers are comprised of spatially distinct junctional complexes with distinct functions: tight junctions (TJs), adherens junctions and desmosomes. Within these multi-protein complexes, most adhesion molecules belong to four protein families: integrins, selectins, cadherins and the immunoglobulin superfamily (IgSF). The focus of the review is on transmembrane TJ proteins other than those in the claudin family (which is the topic of another article in this series). Emerging revelations about alterations in TJ structure / function and their contributions to carcinogenesis (2) make it a timely opportunity to review the state of the field in the context of cancer.

Junctional adhesion molecules (JAMs):

JAMs are transmembrane TJ proteins that belong to the IgSF of proteins. The IgSF counts among its members the JAM family of JAM-A, -B, –C, JAM-4 and JAM-L but also the structurally-related TJ proteins Coxsackievirus-Adenovirus Receptor (CAR) and endothelial cell-selective adhesion molecule (ESAM). JAM molecules contain two immunoglobulin-like extracellular domains, a transmembrane domain and a short cytoplasmic tail with a PDZ domain binding motif (post-synaptic density protein 95), Drosophila disc large tumor suppressor (Dlg1) and zona occludens protein (Z0-1) binding motif (3). The crystal structure of human JAM-A contains a V-type and I-type domain whereas the murine crystal structure of JAM-A contains two V-type domains. However there is no clear consensus regarding the

sub-types of Ig-like domains in JAM-A. JAM-B, JAM-C and JAM-L have been reported to contain V- and C2-type domains while JAM4 has been reported to contain a pair of V-type domains (4-8). With the exception of JAM-L (which lacks a PDZ domain), the JAM family members have been shown to interact with TJ-associated scaffold proteins such as ZO-1, AF6, CASK, MUPP1, PAR3, LNX-1, -2 and MAGI-1 (3, 4). Sequence and structural homologies between the JAM family members are summarised in **Table 1**, and readers interested in their nomenclature and tissue expression patterns are directed to the following recent review (3).

JAM family members are abundantly expressed on epithelial and endothelial cells but also feature on hematopoietic cells including platelets, leukocytes and hematopoietic stem cells (9). They form homophilic and heterophilic interactions in *cis* and *trans* configurations and are known to regulate several biological functions. Accordingly, deregulation of JAM expression has recently been linked with several pathological conditions. Specifically, JAM-A knockout mice have been described to exhibit structural and functional features consistent with intestinal inflammation (10), while JAM-A-deficient platelets are reportedly pro-thrombotic (11, 12) and pro-atherosclerotic in hyper-lipidemic mice (13).

Within its family, the role of JAM-A has been most highly studied in cancer. Overexpression of JAM-A has been linked with increased risk of metastasis in several independent cohorts of breast cancer patients (14-16). Mechanistically, growth impairment of JAM-A-deficient tumors has been demonstrated in a mammary gland-specific polyoma virus middle T-antigen (MMTV-PyVmT) mouse model of breast cancer, while abrogation of JAM-A expression in breast cancer cells has been linked with the induction of apoptosis and with reduced breast cancer progression (16). Further evidence implicating JAM-A in breast cancer cell survival

signaling has accrued from a study in which it was described as a novel regulator of HER2 protein degradation and signaling (15). In terms of the therapeutic potential of targeting JAM-A, its pharmacological inhibition using a monoclonal antibody has been demonstrated to significantly inhibit tumor growth in murine xenograft models of human tumors (17). Interestingly, inactivation of JAM-A has also been shown to enhance the anti-tumoral immune response by promoting dendritic cell and T lymphocyte infiltration (18). Accordingly, in our own laboratory, we have observed significant anti-tumor efficacy (without systemic toxicity) of a novel JAM-A antagonist in an immunocompetent murine model of cancer (Brennan et al, unpublished).

Aside from breast cancer, alterations in JAM-A expression or function have also been implicated in other cancers. In nasopharyngeal cancer cells (NPC), up-regulation of JAM-A has been shown to induce epithelial to mesenchymal transition (EMT) via activation of the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathway, and correlations with high JAM-A expression in NPC patients suggest that high JAM-A expression positively correlates with poor prognosis (19). In non-small cell lung cancer (NSCLC) patients, JAM-A was found to be expressed in 37% of lung tumor specimens compared to corresponding normal tissues, with high expression significantly correlating with TNM stage, lymph node metastasis and decreased overall survival (20). Moreover, gene silencing of JAM-A inhibited tumor cell proliferation and induced cell cycle arrest in lung cancer cells (20). JAM-A has also been shown to promote proliferation and to inhibit apoptosis in gastric cancer cells (21). In addition, recent data suggest a role for JAM-A in maintenance of the cancer stem cell phenotype in brain and breast cancer cells (22, 23). JAM-A overexpression has also been reported in primary cells derived from multiple myeloma patients, in several multiple

myeloma cell lines and, notably, elevations in JAM-A expression have been recorded at relapse compared to diagnosis in multiple myeloma patients (24).

Though abundant evidence suggests an association between high JAM-A expression and disease progression in many cancers, it must be acknowledged that spatial and temporal differences in the regulation of physiologically-important molecules like JAM-A might in turn influence their participation in malignant conditions. In breast cancer cells it was originally thought that attenuation of JAM-A either directly (25) or by upstream regulation of transforming growth factor-β1 expression (26) contributed to invasive potential. In pancreatic cancer low expression of JAM-A has been associated with metastasis and poor patient survival (27), while downregulation of JAM-A has also been linked with clear renal cell carcinoma (28). It will be interesting to determine in future studies whether JAM-A represents merely a biomarker in some cancers versus a viable therapeutic target in others.

CAR

The Coxsackievirus-Adenovirus receptor (CAR) is a member of the IgSF which functions as an adhesion molecule at epithelial tight junctions (29) and has been found to regulate the Notch signaling pathway by binding with protein ligand-of-Numb protein X (LNX), a binding partner of Numb in mammalian cells (30). Pathophysiologically, CAR has been mostly studied in terms of its confirmed role as a mammalian receptor for coxsackie and adenovirus pathogens (31, 32). However emerging literature suggests intriguing new roles for CAR in the control of cancer initiation and/or progression.

The expression levels of CAR across different tumor tissues and subtypes have been typically examined by correlating CAR immunopositivity with tumor grade, growth or nodal status (33). Interestingly, CAR expression levels vary greatly across tissues, suggesting that CAR may be regulated differentially based on organ origin (33, 34). Furthermore, the potential for hormonal regulation of CAR has been highlighted by reports of receptor upregulation in cancers associated with the female reproductive organs (including the endometrium, cervix and ovary) in contrast to its downregulation in both prostate and testicular neoplasms (33).

Despite the lack of a single paradigm explaining the role of CAR in cancer, it remains an area of significant interest due to the possibility of utilising it as a targeting molecule for virally-based gene therapies (35). Several studies have established that CAR expression levels are upregulated in cancer tissues. Some lung cancer subtypes have been found to overexpress CAR, and it has been demonstrated that its inhibition decreased tumor formation and prevented tumor xenograft growth in *scid* mice (36, 37). Furthermore, inhibiting CAR in lung cancer cells has been shown to reduce cell adhesion, invasion and colony formation; conversely, overexpression of CAR has been linked with increased cell adhesion, invasion and colony formation (38). Similarly, the silencing of CAR in breast cancer cells resulted in decreased proliferation (39), whilst increased CAR expression was noted in higher grade breast tumors and associated with poor patient prognosis (40). Other evidence has suggested that CAR may be regulated by estrogen in breast cancers, having established the ability of estradiol to induce both RNA and protein expression of CAR (39).

In contrast, others have labeled CAR as a potential tumor suppressor, in studies where its downregulation has been associated with increased proliferation (41-43), invasive phenotypes (44, 45) and hypoxic conditions; which themselves downregulate the transcription of CAR (46). In gastric cancer cells CAR gene silencing has been shown to induce increased

proliferation as well as migration and invasion, with the reverse occurring under CAR over-expression conditions (43). Similarly, bladder carcinomas and those of the head and neck have displayed stepwise reductions in CAR expression in parallel with increasing tumor grade (41, 44, 45). Additionally, this phenomenon was also seen in cancer cells originating from the prostate, but interestingly CAR expression re-appeared in metastatic lesions (42). Further studies have noted that CAR expression levels vary throughout cancer progression, suggesting that CAR may play both tumor suppressive and tumor promoting roles dependent upon the stage of cancer (47).

Mechanistically, CAR down- or upregulation in cancer is poorly understood, and, due to its differential expression across tissues, the main interest of targeting CAR in cancer may remain with its role in adenoviral gene therapies (48). However, the variable expression of CAR across cancer types and subtypes makes it important to establish individual patient expression levels before treatment, in order to determine those who will be most responsive to adenoviral gene therapies (41, 42). Interestingly, the discovery that CAR expression may be influenced transcriptionally by hypoxic environments means that alternative adenoviruses may be required in cases where CAR expression is low; in order to select those capable of cell attachment via attachment/invasion co-receptors independent of CAR (46).

Nectins

Nectins are immunoglobulin-like cell adhesion molecules containing one extracellular region with three Ig-like loops, a transmembrane segment and a cytoplasmic region containing an afadin-binding motif (49). The distal IgV extracellular loop participates in a complex network of protein-protein interactions, among them homophilic *trans* interactions contributing to cell-cell adhesion and heterophilic *trans*-interactions contributing to cellular functions such as

the formation of adherens junctions, TJs and apical-basal polarity (49). Four subtypes of nectins have been reported: nectins-1-3 are expressed on a variety of cells like epithelial, endothelial, haematopoietic, neural and fibroblastic whereas nectin-4 expression is mainly restricted to the placenta in humans (50, 51). With the exception of nectin-4, the other nectins have known splice variants including nectin-1α, nectin-1β, nectin-1γ, nectin-2α, nectin-2δ, nectin-3α, nectin-3β and nectin-3γ (51). The three splice variants nectins-1γ, -1β and -3γ in addition to nectin-4 lack a binding motif for the adaptor molecule afadin in their cytoplasmic regions (50), whereas the afadin-binding motif of all other nectins is a conserved sequence (Glu/Ala-XTyr-Val) that binds to the PDZ domain of afadin (50). Despite lacking this conserved motif, nectin-4 can bind afadin through its C-terminal Gly-His-Leu-Val motif (52). Five additional IgSF members, known as nectin-like molecules (necl-1 to necl-5) also share the nectin ectodomain architecture and play roles in cell–cell adhesion despite lacking afadin-binding motifs (49). Afadin is an F-actin binding protein that is expressed in epithelia, neurons, fibroblasts and endothelial cells. Afadin has two splice variants: l-afadin and s-afadin. l-Afadin, the larger splice variant, is a nectin- and F-actin-binding protein with two Ras association (RA) domains, a forkhead-associated (FHA) domain, a DIL domain, a PDZ domain, three proline-rich (PR) domains and an F-actin-binding domain. s-Afadin, the smaller splice variant, lacks the F-actin-binding domain and the third proline-rich domain and its expression is restricted to neuronal tissues (53). Afadin has been reported to have tumor suppressive functions, and accordingly its loss has been associated with progression of cancers including breast, colorectal, endometrial and pancreatic (54-57).

Nectins and Necls are involved in a variety of developmental processes, and their dysregulation has been implicated in various diseases including cancer. However there is currently no single paradigm for the contribution of nectins and Necls to cancer, with both

cancer-promoting and cancer-suppressive functions having been ascribed to them. In breast cells nectin-3 has been reported as a key component in the formation of intercellular junctions, and therefore a putative suppressor of breast cancer cell invasion (58). However expression of nectin-3 has been *positively* associated with poor prognosis in a pancreatic adenocarcinoma patient population (50). Increased expression of nectin-1/-2 has also been linked with metastatic disease in breast cancer patients (58), and high levels of nectin-2 have been detected in serum and in primary tumors of breast, ovarian and colorectal cancer patients (59, 60). Furthermore, nectin-2 has been proposed as a biomarker for metastasis and poor prognosis in certain gallbladder carcinomas (61). Nectin-4 is mainly expressed during embryogenesis and is not typically detected in normal adult tissues or serum. However in ovarian, breast and lung cancer, nectin-4 has been detected in primary tumor tissue and in patient serum (62-65). In colorectal cancer nectin-4 expression has been associated with chemotherapeutic resistance to 5-fluorouracil by inducing the PI3K–AKT cascade (66), and its expression has been proposed as a risk factor for distant relapse of T1-T2, node-negative luminal A early breast cancer (67).

Thus an emerging consensus seems to suggest that the tumor-promoting properties of nectins supersede their tumor-suppressive capabilities. However spatial and temporal context may play important roles influencing their involvement in cancer, in addition to the balance between expression of different nectins or even Necls. While comparatively less is known about the role of the Necl family in cancer, forced expression of Necl-1,-4 in colon cancer cells has been described to suppress growth and tumorigenic ability, in conjunction with enhanced rates of apoptosis (68). It will be intriguing to follow how the interplay between nectins and Necls is found to influence cancer progression, if at all.

Occludin

Occludin is a member of the tight junction-associated Marvel protein family containing four transmembrane domains and two extracellular loops; with its extracellular domain containing a high ratio of Gly/Tyr rich residues and its C-terminal binding ZO-1 (49-51). Occludin has four known splice variants (52), and knockout studies of the parent molecule have indicated its importance in tight junction stability and maintenance in epithelial cells (53). Additionally, occludin has been established as a regulator of epithelial cell migration, with phosphorylation at the Y473 residue resulting in PI3K activation at the leading edge of migratory cells (54).

Occludin is widely expressed in both endothelial and epithelial cells (55), and has been identified in multiple tissues including kidney, skin, blood vessels, liver and bladder (56). Reports of discordance between the gene and protein expression levels of occludin (56) support the theory that post-translational modifications of occludin may alter its expression in different tissues, the modifications of which have previously been comprehensively reviewed (53).

Evidence for a role of occludin in cancer formation and progression is steadily growing. In order to locally invade as an early pre-requisite for metastasis to distant sites, one paradigm suggests that primary tumor cells must develop the ability to loosen TJs, and accordingly decreased expression of occludin has been associated with some cancers (56). Loss of occludin expression has been associated with altered TJ function and increased invasiveness in breast cancer cell lines, and expressional downregulation of occludin protein (but not mRNA) has been noted in conjunction with increasing stage in breast cancer tissue (55). Additionally, 10-year follow up data has correlated decreased occludin expression with poor patient prognosis (55).

Other studies have also associated occludin downregulation with metastatic phenotypes and glandular de-differentiation in endometrial carcinomas (57). Supportive research has highlighted how, when overexpressed, occludin can decrease tumorgenic phenotypes both *in vitro* and *in vivo*, and that epigenetic regulation of the OCLN promoter is sufficient to influence metastatic potential via direct regulation of apoptotic pathways (58). Furthermore, occludin expression has been correlated with oxidative stress-induced premature senescence in breast cancer cells, by upregulating negative cell cycle regulators such as p16, p21 and p27 but not p53 (59). In the same study, endogenous re-expression of occludin (via demethylation) induced apoptotic signaling (59), by a mechanism likely involving occludin mislocalization at TJs with consequent induction of the extrinsic apoptotic pathway (60). Further evidence of occludin-mediated regulation of apoptosis has come from a study in which its downregulation in keratinocytes reduced sensitivity to TRAIL, an apoptotic ligand involved in the extrinsic pathway (61). Interestingly, natural expressional regulation of occludin may be achievable via a number of pathways, as typified by the demonstration of occludin transcriptional activation by thyroid transcription factor 1 (TTF-1). Significantly, when silenced, TTF-1 reduced occludin expression in conjunction with the induction of invasive/ metastatic behavior in lung carcinoma cells (62). Furthermore, recent work has highlighted the potential role miRNAs may play in regulating occludin expression (63), particularly miRNAs that bind in the non-coding region of occludin and induce degradation of its mRNA (64).

With accumulating data indicating occludin as an essential player in apoptotic signaling following TJ disruption and its downregulation predisposing to metastatic behaviors *in vitro* and *in vivo*, it is exciting to speculate that re-expressing occludin in cancer may act as a potential therapeutic target. In support, a previous *in vitro* study demonstrated how re-expression of occludin (in occludin-absent oncogene-transformed cells) was sufficient to

restore the appearance of a normal epithelial phenotype (65). Though no clinical trials are currently underway, emerging research might potentially focus upon the epigenetic regulation of occludin via TTF-1 and other activators as a promising strategy to correct the deficits associated with occludin loss in cancer.

Tricellulin (MarvelD2)

Tricellulin (marvel D2) is a 64 kDa protein located primarily at tricellular but also bicellular TJs, which plays an important role in maintaining the epithelial barrier (69-71). In addition to occludin and marvelD3, tricellulin is a member of the marvel protein family, all of whose members contain four transmembrane domains (69, 72, 73). Several isoforms of the gene encoding tricellulin, *TRIC*, have been identified including *TRIC-a, TRIC-a1, TRIC-b* and *TRIC-c*; with *TRIC-a* and *TRIC-a1* having 32% structural homology with the C-terminus of occludin (70). Gene silencing of tricellulin in epithelial cells has been shown to induce irregularities in cellular shape and disorganisation of F-actin fibers, owing to the inactivation of Cdc42 by tricellulin and accordingly impairment of junctional tension (74). Furthermore, in normal human pancreatic duct epithelial cells, the c-Jun N-terminal kinase (JNK) pathway has been found to regulate tricellulin expression at tricellular junctions in addition to epithelial barrier function (75). Interestingly, occludin silencing has been linked with tricellulin relocalisation to bicellular tight junctions, highlighting an important role for occludin in tricellular tight junction maintenance (71) despite the fact that occludin and tricellulin are not known to physically interact with each other (73). Tricellulin does however directly bind other tight junction proteins including ZO-1 (70) and marvelD3 (73).

The specific role and downstream effects of tricellulin expression in carcinomas is still in the early stages of investigation, so it is as yet unclear whether it represents a biomarker or a potential therapeutic target. High expression of tricellulin has been noted in human pancreatic cancer cell lines (including HPAC and PANC-1 (76)) and in highly-differentiated, low grade pancreatic ductal adenocarcinomas (77). In hepatocellular carcinoma, high tricellulin expression was shown to correlate with decreased overall survival, however, in another type of primary liver carcinoma (intrahepatic cholangiocarcinoma), tricellulin expression was associated with *increased* overall survival (78). In support of a potential link between high tricellulin expression and good patient prognosis, low tricellulin expression has been described in poorly-differentiated regions of fibrolamellar hepatocellular carcinoma relative to normal liver tissue or areas of well-differentiated tumor (79). Tricellulin expression has also been observed in gastric cancer, with the transcription factor snail being implicated in repressing tricellulin expression during EMT induction (80). Accordingly, expression of tricellulin was particularly associated with well-differentiated and EMT-negative tumors while it was lost in undifferentiated or EMT-positive tumors (80). An additional study investigating the expression of tricellulin in tonsillar squamous cell carcinoma found a loss of tricellulin expression (regardless of tumor stage) in comparison to normal tissue (81).

The specific role of tricellulin at TJs remains elusive but nonetheless its importance in epithelial barrier function, cellular organisation and signaling is becoming apparent. Accordingly, the disruption of tricellulin expression and function may represent a diagnostic or prognostic biomarker associated with advancing tumor stage, aggressiveness and overall patient outcome.

MarvelD3

MarvelD3 represents one of the newer and lesser known TJ proteins. Similar to occludin and tricellulin, MarvelD3 is a member of the tight junction-associated marvel protein family (82). Predictive studies have identified two splice variants of MarvelD3 with a shared cytoplasmic N-terminal domain but different transmembrane domain-containing C-terminal domains (82). Expression of MarvelD3 has been noted in endothelial cells of the brain, cornea, umbilical vein and in murine/human epithelial cells of the cornea, prostate, small intestine, colon, stomach, liver, kidney, spleen and pancreas (73, 76, 82). Gene silencing experiments have revealed that MarvelD3 is not required for epithelial tight junction formation, but that it may influence paracellular permeability of established epithelial barriers (82). Accordingly, MarvelD3 has been shown to interact with other TJ proteins that regulate barrier assembly and function, including occludin, tricellulin, claudin-1 and claudin-3 (83).

Some interesting potential contributions of MarvelD3 to cancer have been emerging of late. Specifically, its expression has been observed in colon, pancreatic, lung, colorectal and breast cancer tissues (73, 76, 82, 84, 85), with mounting evidence pointing towards its regulatory functions more than its structural role. In a trend similar to that seen with tricellulin in gastric cancer (80), MarvelD3 has been described as highly expressed in well-differentiated pancreatic cancer cells but less well expressed in poorly-differentiated pancreatic cancer cells co-expressing the transcription factor snail (76). Accordingly the induction of EMT by snail downregulated MarvelD3 in highly differentiated pancreatic cancer cells (76), and, in a potential feedback loop, MarvelD3 gene silencing has been shown to increase snail2 expression in lung cancer cells and to reduce anchorage-independent growth (84). This raises the intriguing possibility of reciprocal crosstalk between MarvelD3 and snail transcription factors.

Studies examining the functions of MarvelD3 in cancer are providing increased insight into its upstream regulators and potential mechanisms of action. For example, increased methylation of the MARVELD3 gene has been reported in lung tumors from non-smokers compared to current or former smokers, suggesting MarvelD3 as a potential biomarker in lung cancer (84). Downregulation of MarvelD3 in differentiating colorectal cancer cells has been shown to increase cell migration and proliferation, with re-introduction of MarvelD3 inhibiting migration, proliferation and tumor growth (85). Furthermore MarvelD3 has been revealed to regulate the MEKK1-JNK signaling pathway, via recruitment of MEKK1 to cell junctions and subsequent downregulation/functional inhibition of JNK (85). Such findings highlight the emerging potential importance of molecules like MarvelD3 in cancer initiation, growth and progression.

Conclusion

A wealth of information continues to emerge implicating various non-claudin transmembrane TJ proteins in tumorigenesis. Whether some of the reported expressional changes are capable of driving disease processes versus passively reporting their presence is not unequivocally clear, but careful consideration of spatial, temporal and translational contexts should inform future discussions around the significance of each finding. What *is* becoming increasingly clear is that TJ proteins are not restricted to adhesion-dependent functions, but may also participate in (or even initiate) adhesion-independent signaling mechanisms that can drive tumorigenic behaviors. In the area of greatest interest to our own laboratory, our published and unpublished evidence is consistent with a model whereby overexpression of JAM-A on breast tumor cells facilitates a switch from principally regulating cell-cell adhesion to regulating signaling pathways required for cell survival, cell migration and downstream control of certain oncogenes. The possibility of uncovering mechanistic differences between

the regulation of adhesion-dependent physiological signaling versus adhesion-independent pathophysiological signaling offers hope that easily-accessible cell surface targets like the transmembrane TJ proteins could be developed as future druggable targets for certain subsets of cancer patients.

Compliance with Ethics Guidelines

Conflict of Interest

Sri HariKrishna Vellanki, Cathy E Richards, Yvonne E Smith, and Ann M Hopkins declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

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