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Review

Tight junctions and their role in cancer metastasis

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Summary. Tight Junctions govern the permeability of endothelial and epithelial cells and are the most topical structures of these cell types. Tight junctions create an intercellular barrier and intramembrane diffusion fence. An important step in the formation of cancer metastases is interaction and penetration of the vascular endothelium by dissociated cancer cells.

Early studies demonstrated a correlation between the reduction of tight junctions and tumour differentiation and experimental evidence has emerged to place tight junctions in the frontline as the structure that cancer cells must overcome in order to metastasise. Changes in tight junction function are thus an early and key aspect in cancer metastasis.

Further work is required to fully realise the potential that this structure has in cancer invasion and metastasis in order to develop new and novel therapies in the prevention of tumour metastasis.

Key words: Tight junctions, Cancer metastasis

Introduction

Tight Junctions are the apical most structure in epithelial and endothelial cells (Jiang et al., 2000) (Fig. 1). The tight junction structure creates a physiological barrier and intramembrane fence, which helps to maintain distinct tissue spaces and to separate the apical space from the lateral plasma membrane (Tsukita et al., 1996). This membrane domain creates a primary barrier preventing the paracellular transport of solutes, and also creates and controls the lateral diffusion fence (Morita et al., 1999). Endothelial cells form a continuous monolayer *in vivo* with functions as a selective barrier to the passage of proteins and extravasation of inflammatory cells in blood vessels (Satoh et al., 1996). This barrier function is carried out by tight junctions. The regulation of vascular permeability is one of the most important functions of endothelial cells, where the degree of permeability differs at different sites in the body (Utoguchi et al., 1995).

Polarised epithelial cells possess two functionally and biochemically distinct plasma membrane domains that are separated by tight junctions (Matter and Balda, 1998).

The tight junction can be regulated in response to physiological and tissue-specific requirements (Wong and Gumbiner, 1997). In response to stimuli, tight junctions are able to rapidly change their permeability and functional properties, which permits dynamic fluxes of ions and solutes in addition to the passage of whole cells (Chen et al., 1997).

Over the last few years there has been an explosion in the knowledge of the structure of tight junctions at the molecular level, with an ever expanding panel of molecules identified as being either part of, or associated with the tight junction. Evidence is also accumulating as to how tight junction function is regulated, and how this plays a role in cellular responses to external stimuli. In addition, evidence suggests that, as we shall discuss here, tight junctions are involved in the development and penetration of the endothelium and mesothelium by cancer cells (Jiang et al., 2000).

Tight junction structure

Tight junctions appear as discrete sites of fusion between the outer plasma membrane of adjacent cells when visualised in ultra-thin section electron microscopy. When visualised using freeze-fracture, they appear as continuous intramembrane particle strands in the protoplasmic face with complementary grooves in the extracellular face (Furuse et al., 1998), which completely circumscribe the apices of the cells as a network of intramembrane fibrils (Wong and Gumbiner, 1997). This identifiable ultrastructure represents a conglomeration of molecules that are thought to either constitute, associate with or regulate tight junctions (Fig. 2). Although a number of these molecules have been known since the mid eighties, there is an expanding list of additional molecules now shown to be involved also (Table 1).

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From extensive investigations (Fanning et al., 1999; Jiang et al., 2000) into the molecular components of the tight junction structure, it has become apparent that the junctions are composed of three regions: (i) the integral membrane proteins – occludin, claudins, junctional adhesion molecules; (ii) the peripheral proteins – zonula occluden (ZO)-1, -2, -3; and (iii) proteins associated with tight junctions (see below).

The integral proteins are essential for the correct assembly of the structure and controlling tight junction function by homotypic or heterotypic interactions. For successful assembly however, the integral proteins must be anchored to the relevant peripheral proteins such as ZO-1, which binds to the actin system of the cell. This is in conjunction with the other associated proteins that are involved in the regulation of tight junction function and cell signalling (Table 1).

Tight junction molecules

Zonular occludens (ZO-1)

ZO-1 was the first molecule to be identified in tight junctions, as a phosphoprotein of 210-225 kDa in size (Stevenson et al., 1986; Anderson et al., 1988) localised in the immediate vicinity of the plasma membrane of both endothelial and epithelial cells (Furuse et al., 1998). ZO-1 is concentrated at the tight junctions themselves although it can also be found in adherens junctions, the nucleus and in cells that do not have a distinct tight junction structure. ZO-1 is phosphorylated on serine residues under normal conditions, but when stimulated becomes phosphorylated on tyrosine residues. It is a member of the MAGUK protein family (membrane associated and having the presence of a guanylate kinase

Table 1. Proteins involved in tight junction structure and function.

INTEGRAL MEMBRANE PROTEINS	PERIPHERAL PROTEINS	ASSOCIATED PROTEINS
Occludin	Zonula occludens-1 (ZO-1)	Cingulin, 7H6
Claudins 1-15	ZO-2	Symplekin, ZONAB
Junctional adhesion protein (JAM)	ZO-3	Rab-13, 19B1
Paracellin-1		AF-6, Rab 3B, PKC
Senescence-associated epithelial membrane protein 1 (SEMP-1)		c-src, Gαi-2

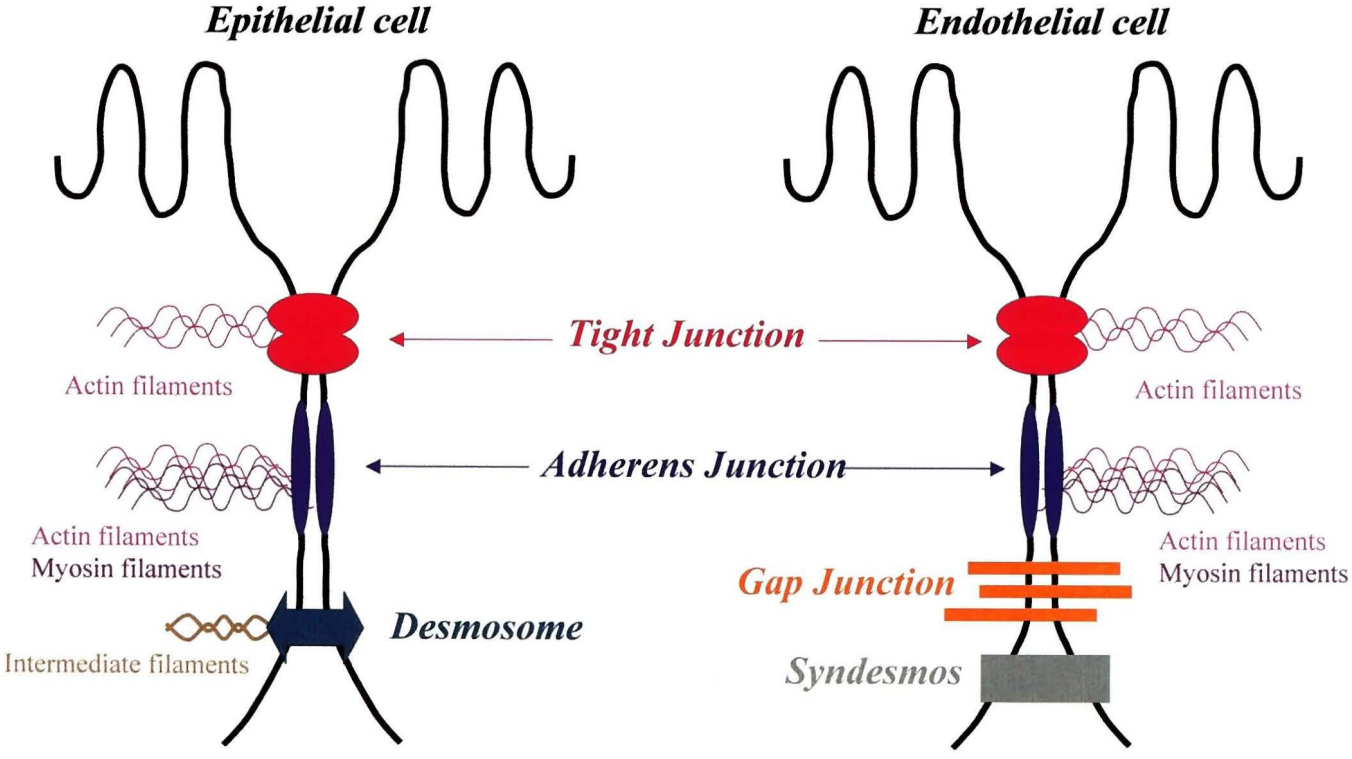

Fig. 1. The main structures providing cell-cell interactions in epithelial and endothelial cells. Tight junctions are located at the most apical location, and as such are the first barrier to penetration.

or GUK domain) of which the members share the common domains of SH3, homologous to GUK and have one or more PDZ domains (Itoh et al., 1999; Balda and Matter, 2000). The PDZ domains are thought to mediate a reversible and regulated protein to protein interaction through contact with other PDZ domains, or by recognition of sequence motifs at the C termini of integral proteins (Itoh et al., 1999). The SH3 domain act as protein to protein interaction molecules binding to the PXXP motif. In addition, the UGK domain is also involved in protein to protein interactions.

ZO-2 and ZO-3 are also members of this MAGUK family, of 160 kDa and 130 kDa respectively. ZO-2 is a phosphoprotein that co-precipitates with ZO-1 and is located only in tight junctions. ZO-3 interacts with both ZO-1 and ZO-2, sharing a high sequence homology with both (Itoh et al., 1999).

Sequence analysis of ZO-1 and ZO-2 shows them to be homologous to members of the lethal discs large-1 (Dlg), PSD-95/SAP90 and p55 protein family suggesting a role in signal transduction (Itoh et al., 1997; Furuse et al., 1998). Mutations in the Dlg gene cause neoplastic overgrowth of the imaginal discs in Drosophila larvae, suggesting a tumour suppressor role (Chlenski et al., 1999). Such evidence suggests that this may be a role of ZO-1 in mammals.

Occludin

The first transmembrane tight junction protein identified was occludin, with a molecular weight of 62-82 kDa (Furuse et al., 1993; Itoh et al., 1993). Occludin consists of 4 membrane-spanning elements in its NH2- and COOH-terminii located in the cytoplasm which form two extracellular loops. The discrepancy in the size of the occludin protein is a result of serine and threonine phosphorylation. Occludin binds directly with ZO-1 at its COOH-terminal and the cytoplasmic domain also (domain E) interacts with both ZO-1 and ZO-1 (Itoh et al., 1997; Furuse et al., 1998). Occludin is a functional component of tight junctions (Wong and Gumbiner 1997; Matter and Balda 1999) and is widely expressed in both endothelial and epithelial cells, but does not exist in cells and tissues without tight junctions (Jiang et al., 2000). Although evidence shows that occludin is an important component of the tight junction (permeability function of tight junctions can be damaged by peptides inhibitory to occludin), in other cells types, such as stem cells, tight junctions are still formed even after removal of occludin (Balda et al., 1996; Wong 1997; Furuse et al., 1998) suggesting the existence of other tight junction integral membrane proteins.

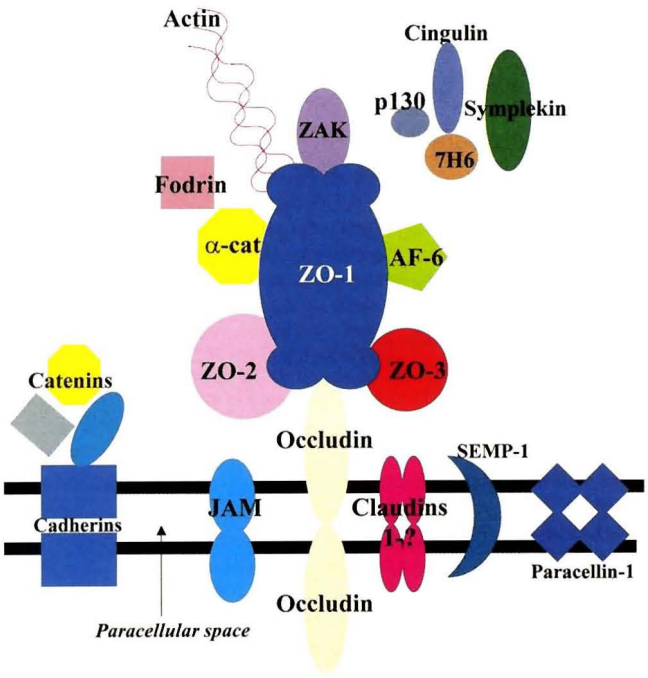

Fig. 2. Schematic representation of the interactions suggested for proteins involved in tight junction structure and function (see text).

The two extracellular loops of occludin project into the paracellular space and are thought to interact with loops originating from occludin or other as yet unidentified molecules in the neighbouring cells, so promoting interaction and "sealing" of the paracellular space (Fig. 1) (Denker and Nigam, 1998). It is the larger phosphorylated form of occludin that is found exclusively localised in the tight junction; smaller less phosphorylated forms are found in the basolateral membrane and cytosol also (Sakakibara et al., 1997; Denker and Nigam, 1998). Therefore, the phosphorylation of the occludin molecule is directly involved in it's function. An alternatively spliced form of occludin, occludin 1B has been identified in MDCK cells and cultured T84 human colon cancer cells (Muresan et al., 2000).

Claudins

Claudins are a family of integral transmembrane proteins found in tight junctions (Furuse et al., 1998). Claudin-1 and claudin-2 are structurally related, are 23 kDa in size and have four membrane spanning regions, although share no homology with occludin. Both are found in cells with and without tight junctions, but exhibit a high level in the tight junctions of those that do. New members of the claudin family have been found, with there being at least 15 members (Morita et al., 1999). When occludin is absent, claudins are the candidates thought to be responsible for the primary seal-forming elements of the extracellular space (Fanning et al., 1999). There is some evidence that claudin can bind to ZO-1 in the absence of occludin, either directly, or indirectly through the mediation of ZO-2 and ZO-3 (Morita et al., 1999). The claudin family exhibits a tissue specific distribution, for example, claudin-5 is the main form in endothelial cells (Fanning et al., 1999; Liebner et al., 2000), which suggests that

they may contribute to functional differences, where the type of claudin may be a determining factor in the tightness of the tight junctions (Fanning et al., 1999; Tsukita and Furuse 1999). Claudins possess Ca^{2+}-independent cell adhesion activity which is much stronger than that of occludin (Kubota et al., 1999).

Junctional Adhesion Molecule (JAM)

Junctional adhesion molecule (JAM) is a 32 kDa type 1 transmembrane protein (Fanning et al., 1999). It has the structural and sequence conservation features of an IgSF molecule with two extra-cellular Ig-like domains and two sites for N-glycosylation and is expressed at cell-cell contacts in endothelial and epithelial cell monolayers (Williams et al., 1999). It is also expressed in high levels by circulating immune cells. The distribution of JAM is similar to that of ZO-1. The ligand for JAM is still unknown, but it has been suggested that weak JAM-JAM binding may play a role in the induction of transmembrane signalling during the normal passage of migrating cells and inherent junctional disruption (Fanning et al., 1999; Williams et al., 1999).

Paracellin-1

Paracellin-1 (PCLN-1) is a more recently described tight junction molecule with a structure of four transmembrane domains and intracellular NH2- and COOH-terminii (Simon et al., 1999). The sequence and structure shows a similarity to the claudin family. PCLN-1 is uniquely expressed in kidney tissues where it plays a key role in the magnesium and calcium diffusion through tight junctions and is thus involved in the formation of paracellular channels.

Senescence-Associated Epithelial Membrane Protein 1 (SEMP1)

SEMP-1 is an epithelial membrane protein that is homologous to the mouse claudin-1 and is co-localised with tight junctions. It has 3 transmembrane domains with a COOH-terminal site (Swisshelm et al., 1999). SEMP-1 has a suggested role in cell specific functions such as maintenance of differentiated cell shape, permeability, polarity and/or signal transduction (as compared with claudins). It is expressed in a number of tissue types (both adult and foetal), but is expressed at low or undetectable levels in human breast cancer cell lines. Moreover, SEMP-1 has enhanced expression in senescent human mammary epithelial cells, indicating that it may act as a tumour suppressor gene in such tissues (Swisshelm et al., 1999).

Tight junction - associated molecules

Cingulin

Cingulin is a 140 kDa protein found in the tight junctions of both endothelial and epithelial cells (Citi et al., 1988). The molecule appears as two peptides entwined as a "coiled coil" and is located in close proximity to the vinculin-rich cytoskeletal belt (Stevenson et al., 1989, Denker and Nigam, 1998).

7H6

7H6 is a 155 kDa protein within the tight junctions of hepatocytes, epithelial cells and endothelial cells. It is believed to function in the regulation of paracellular permeability and barrier function of both endothelial and epithelial cells *in vitro* (Zhong et al., 1994; Tobioka et al., 1996; Denker and Nigam, 1998). 7H6 disappears rapidly (and reversibly) from tight junctions of MDCK cells when the paracellular barrier function is perturbed experimentally (Satoh et al., 1996).

Symplekin

Symplekin is a 127 kDa protein found in the tight junctions of a range of cell types, but not in endothelial cells (Denker and Nigam, 1998).

Others molecules

A number of other proteins are also associated with tight junctions, including rab-13, 19B1, AF-6, rab 3B, protein kinase C, c-src and Gαi-2 (Keon et al., 1996; Merzdorf et al., 1997; Yamamoto et al., 1997; Denker and Nigam, 1998; Weber et al., 2000). However, their precise role is not yet fully understood. ZO-1-associated-nucleic-acid-binding-protein (ZONAB) is a recently described molecule that associates directly with ZO-1 in a regulatory capacity (Balda and Matter, 2000).

Tight Junction Functions

Tight junctions function as: (i) barriers to cell migration, (ii) regulators of cellular permeability, (iii) intermediates/transducers in cell signalling; and (iv) cell-cell adhesion molecules. Tight junctions form selective paracellular diffusion barriers that regulate the diffusion of solutes across epithelia and constitute intramembrane diffusion barriers that prevent the intermixing of apical and basolateral lipids in the extracytoplasmic leaflet of the plasma membrane (Balda et al., 2000). In MDCK cells, occludin has been shown to be critically involved in both of these tight junction functions and that its COOH-terminal cytoplasmic domain is of functional importance. Mutant occludin studies have also shown that the extra-cytoplasmic domains of occludin and at least one of the transmembrane domains are also involved in selective paracellular permeability, probably in a regulatory capacity (Balda et al., 2000).

A recently discovered ZO-1-associated molecule ZONAB, is thought to directly participate in the control of gene expression, in the regulation of epithelial/endothelial cell differentiation and in cell growth and differentiation (Balda and Matter 2000).

ZO-1 may directly participate in the regulation of cellular differentiation as localisations of both ZO-1 and ZO-2 were disrupted when C-terminally truncated mutants and a deletion mutant of ZO-1 were stably expressed in corneal epithelial cell lines (Reyom et al., 2000). Mutants showed distinctly different cell morphologies suggesting a partial transformation to mesenchymal cell types, and a corresponding down-regulation in occludin expression. ZO-1 is also thought to be involved in modulation of ß-catenin signalling in MDCK cells (Reichert et al., 2000). The ZO-1 PDZ domain, when no longer localised at the plasma membrane, induces a dramatic epithelial to mesenchymal transition with repression of epithelial marker genes and significantly induced tumourigenicity. This is in conjunction with an activated ß-catenin signalling pathway.

The blood brain barrier separates brain tissues from the blood circulation and prevents hydrophilic and toxic substances from entering the brain. Whilst astrocytes and endothelial cells are important in the blood-brain barrier, endothelial cells are of fundamental importance in barrier function. These endothelial cells have strong tight junctions (with high levels of occludin) and low endocytic vesicles and specific transport mechanisms for molecules such as L-DOPA, but reduced entry for almost all other molecules form blood to brain (Hirase et al., 1997). A discussion of the role of tight junction function in the blood-brain barrier can be found in Kniesel and Wolburg (2000).

In mammalian cells, the tight junction functions as a barrier with controlled permeability, in that compartments with different solute composition are separated, but are not absolutely unconnected (Lapierre, 2000). The permeability of this paracellular zone needs to be controlled by both internal and external factors allowing for modulation of permeability under certain circumstances. Occludin has been shown to influence ion and solute permeability; claudins are though to co-ordinate with occludin and also provide cell-cell adhesional strength; JAM influences paracellular transmigration of immune cells; and the plaque of cytoplasmic proteins under the junction may responsible for scaffolding the transmembrane proteins, creating a link to the perijunctional actin cytoskeleton and transducing regulatory signals that control the paracellular link (Fanning et al., 1999).

Tight junctions can also be involved in cell-cell adhesion, although in a weak interaction. Occludin expression induces adhesion in the absence of Ca^{2+} (so independent of cadherin-cadherin contacts) in NRK and Rat-1 fibroblasts, and occludin-induced cell adhesion has been observed in fibroblasts (L-cell) that do not normally express occludin (VanItallie and Anderson, 1997). Results from antibody studies suggest that the extracellular surface is directly involved in cell-cell adhesion and that the ability to confer adhesiveness correlates with the ability to colocalise with ZO-1 (VanItalle and Anderson, 1997). It remains to be seen whether occludin is a homotypic cell adhesion molecule, or if it has an as yet unidentified receptor (Fanning et al., 1999). Occludin is also capable of organisation laterally through side-to-side associations, perhaps within the membrane bilayer (Chen et al., 1997). Occludin may then create a paracellular barrier by polymerising laterally in the membrane, creating a continuous line of adhesion between cells (Fanning et al., 1999).

Mouse L-fibroblasts transfected with claudin-1, -2 or 3 also exhibit Ca^{2+}-independent cell-adhesion (Kubota et al., 1999). Furthermore, the adhesive qualities of occludin are negligible when compared to those of the claudins. There are indications that JAM may also be involved in cell adhesion, as it has been shown to accumulate at sites of cell-cell contact, inducing cell-cell adhesion in cells that do not have tight junctions, such as COS cells (Fanning et al., 1999). Experimental evidence suggests that JAM can mediate both homotypic adhesion and influence monocyte migration, as it provides an adhesive contact for monocytes to find the intercellular pathway (Martin-Padura et al., 1998; Fanning et al., 1999).

Structure and function

The red cell protein 4.1R expressed in non-erythroid cells interact with ZO-2 in *in vitro* binding studies (Mattagajasingh et al., 2000). Furthermore, 4.1R is co-localised with both ZO-2 and occludin on MDCK cell tight junctions and suggest that 4.1R might play a role in the organisation and function of the tight junction by establishing a link between the tight junction itself and the actin cytoskeleton.

ZO-1, -2, -3, in addition to the MAGUK characteristics (PDZ, SH3, GK) have distinctive a carboxyl terminal with splicing domains, acidic- and proline-rich regions. The modular organisation of these proteins allows them to function as scaffolds, which associate with transmembrane tight junction proteins, the cytoskeleton and signal transduction molecules. They are able to shuttle between the tight junction and the nucleus, where it is suggested they regulate gene expression (Gonzalez-Mariscal et al., 2000).

Mature epithelial cells have a complex chain of reactions involving α-, ß- and γ-catenins, G proteins, phospholipase C, PKC, calmodulin, mitogen-activated protein kinase, and cytoskeletal proteins which keeps the tight junction sensitive to physiological requirements and local conditions throughout the life of the cell (Cereijido et al., 2000).

Cingulin has been suggested as a functionally important component of the tight junction by linking the sub-membrane plaque domain of the tight junction to the actomyosin cytoskeleton (Cordenonsi et al., 1999).

Tight junction regulation

Evidence for the regulation of tight junction function has been accumulating in the last few years. A number

of factors are believed to be involved (Table 2). Numerous factors are involved in the regulation of tight junction function in the blood brain barrier, amongst them: G-proteins, serine, threonine, and tyrosine kinases, extra- and intracellular calcium levels, cAMP levels, proteases and TNF-α (Kniesel and Wolberg, 2000).

Lipids

Polyunsaturated fatty acid gamma linoleic acid (GLA), an anti-cancer essential fatty acid has previously been shown by the authors to increase transendothelial resistance and decrease paracellular permeability in vascular endothelial cells (Jiang et al., 1998a,b). Linoleic acid and eicosatrienoic acid however, decrease transendothelial resistance and increase permeability via changes in expression of occludin. Both these acids are involved in the development of certain tumours, such as breast cancer.

Protein Kinase C

During the assembly of tight junctions, activity of Protein kinase C (PKC) markedly increases; RhoA and Rad GTPases regulate tight junction structure and function (Jou et al., 1998). PKC is thought to play a role in tight junction opening in response to calcium withdrawal (Lacaz-Vieira, 2000). In LLC-PK1 renal epithelial sheets, activation of PKC results in rapid increase in paracellular permeability and decrease in TER (Clarke et al., 2000a). No change was seen in tight junction structure of the cells, but the membrane distribution of ZO-1 changed significantly. PKC activation did however, result in a time-dependant decrease in threonine phosphorylation of occludin indicating that PKC is apparently further upstream in the signalling pathway regulating epithelial barrier function, with a downstream serine/threonine phosphatase acting upon occludin. Clarke et al. (2000b) discusses the features of PKC proteins in tight junction regulation by their effect on the phosphorylation states of tight junctional proteins, suggesting that downstream kinases and/or phosphatases mediate PKC's effect on tight junction permeability. This also suggests that the tight junctional leakiness associated with PKC activation suggests a potentially useful role for tight junction leakiness as a marker for early cancer diagnosis.

Cytokines, growth factors and hormones

Regulation of tight junctions by cytokines, indicates that disruption of tight junction function and accompanying alterations in paracellular permeability is a hallmark of many pathological states (Walsh et al., 2000). Proinflammatory cytokines such as TNF-α and IFN-γ can downregulate the expression of occludin, paralleling decreased TER (Mankertz et al., 2000). Both cytokines appear to diminish occludin promotor activity both alone and synergistically, suggesting genomic regulation of alterations in paracellular barrier function. TNF-α also causes decreases in transendothelial resistance and fragmentation of the basal continuous interendothelial cell ZO-1 distribution (Schimtiz et al., 1999). Spontaneous and induced (by TNF-α) apoptosis of intestinal epithelial cells is accompanied by increased paracellular permeability that may facilitate loss of solutes and uptake of noxious agents in the gut (Gitter et al., 2000).

Interferon gamma (IFN-γ) induces loss of ZO-1, ZO-2 and occludin from T84 cells, together with diffused localisation of these molecules in the cells (Youakim and Ahdieh, 1999).

VEGf increases BMEC permeability and reduces TER with loss of occludin and ZO-1 from tight junctions, with the level of occludin protein decreased from westerns suggesting VEGf increases permeability by reducing occludin expression and so disrupting ZO-1 and occludin organisation (downstream effects of the VEGf signalling pathway) (Wang et al., 2001). Cerebral endothelial cells (CECs) show loss of occludin in the presence of growth factors such as endothelial cell growth factor (ECGf) with has an important bearing on the formation of the blood-brain barrier formation (Krizbai et al., 2000). The loss of junctional proteins was accompanied by an increase in migratory behaviour and altered protease activity. Epidermal growth factor (EGF) induces tyrosine phosphorylation of ZO-1 and increases its affinity for cytoskeleton interaction (VanItallie and

Table 2. Factors that disrupt tight junction function.

DISRUPTIVE FACTOR	TIGHT JUNCTION PROTEIN AFFECTED	REFERENCE
Linoleic acid, eicosantrienoic acid	Occludin	Jiang et al., 1998a,b
Protein Kinase C (PKC)	ZO-1, occludin	Clarke et al., 2000a,b
TNF-α	Occludin, ZO-1	Manketz et al., 2000
IFN-γ	Occludin, ZO-1, ZO-2	Youakim and Ahdieh, 1999; Manketz et al., 2000
VEGf	Occludin, ZO-1	Wang et al., 2001
ECGf	Junctional proteins	Krizbai et al., 2000
EGF	ZO-1	VanItallie and Anderson, 1999
TGFß	ZO-1	Buse et al., 1995
HGF/SF	ZO-1, occludin	Jiang et al., 1999b
Protease	Occludin	Tilling et al., 1998
Phenylarsine oxide	Occludin	Wachtel et al., 1999

Anderson, 1999).

TGFß has been shown to reduce the function of tight junctions by inducing reorganisation of apical F-actin and the development of stress fibres, with a loss of normal cell border-associated ZO-1 distribution in endothelial cells (Petroll et al., 1996; Woo et al., 1996). TGF-α modifies the location of ZO-1 from the cell-cell border to the cytoplasm, with a corresponding alteration of the growth characteristics of these cells (Buse et al., 1995).

Hepatocyte growth factor/scatter factor (HGF/SF) is a cytokine that regulates a number of cellular functions, including cell adhesion and communication (Jiang et al., 1999a). HGF/SF has been reported to regulate paracellular permeability and phosphorylation of ZO-1 in epithelial cells (Nusrat et al., 1994), probably by the phosphorylation of ZO-1 (Grisendi et al., 1998). In the human cell line ATCCC1998, HGF/SF has been shown to decrease transepithelial resistance and to increase paracellular permeability, mainly by the alteration or phosphorylation status of occludin and its subsequent relocation from the cell-cell border to the cytoplasm (Jiang et al., 1999b). Prolonged treatment with HGF/SF may reduce the expression of occludin and ZO-1 via mechanisms yet to be identified (Jiang et al., 1999b). Human vascular endothelial cells are also effected by treatment with HGF/SF, in that transendothelial resistance and paracellular permeability are decreased and increased respectively (Martin et al., 2000).

Prostaglandins 12 and E2 exert a synergistic effect on restoration of ischemia reduced transepithelial resistance, probably due to their induction on intracellular cAMP (Noske and Hirsch, 1986; Nlikslanger et al., 1997).

Proteases

Proteinases are also thought to be involved in the regulation of tight junction assembly in infections such as *Vibrio cholerae*, a bacterium that secretes the haemagglutinin/protease toxin (Wu et al., 2000). This toxin has been shown to enzymatically cleave occludin, thus preventing tight junction assembly, leading to microbial pathogenesis. It appears that this is controlled by the direct effect of the protease degrading occludin, the breakdown of which may send signals to ZO-1 (to which it is normally associated) that causes redistribution of ZO-1 resulting in reorganisation of the F-actin cytoskeleton.

Extracellular matrix proteins such as type IV collagen, fibronectin and laminin have been shown to increase transendothelial resistance of primary microvascular endothelial cells (Tilling et al., 1998).

Protein tyrosine phosphatase (PTP) inhibitor phenylarsine oxide (PAO) induces proteolysis of occludin which was inhibited by the metalloproteinase inhibitor 10-phenathroline (PHEN) (Wachtel et al., 1999). The PAO treatment under conditions for occludin proteolysis caused a corresponding increase in endothelial paracellular permeability, suggesting a method of control for paracellular permeability in endothelial cells.

Steroids

Dexamethasone, a glucocorticoid, induces the reorganisation of tight junctions and stimulated TER of epithelial monolayers in Con8 mammary tumour cells (Woo et al., 2000). Increased TER was caused by dexamethasone stimulation of Id-1 protein, a helix-loop-helix transcriptional factor that appears to act as a critical regulator of mammary epithelial cell-cell interactions at an early step in the glucocorticoid-dependant signalling pathway controlling tight junction integrity. Dexamethasone also regulates matrix-metalloproteinase (MMP) expression in CNS vascular endothelium particularly alteration in expression of ZO-1 by MMP-9 (Harkness et al., 2000). Whereas TNF-α and Il-1ß upregulated MMP-9 activity, so causing decreased ZO-1 expression, dexamethasone prevented this effect. This is of import as such changes in MMP activity by the CNS vascular endothelium may play a role in the pathogenesis of blood-brain and blood-retinal barrier breakdown *in vivo*. Hormones such as prolactin and dexamethasone stimulate tight junction formation, with additive effects when both used together on mouse mammary cell lines (HC11 and Comma-1D) which exert their effect on tight junctions via regulation of occludin (Stelwagen et al., 1999).

Other factors

The function and assembly of tight junctions has been shown to be enhanced by endogenous and exogenous cAMP. CB-cAMP added to cells results in increased TER, probably via a direct effect on tight junctions and an indirect effect via cell adhesion mechanisms (Duffey et al., 1981; Wolburg et al., 1994).

Tight junction assembly occurs in the presence of extracellular calcium, but is disrupted in the presence of calcium chelators (Conteras et al., 1992). This calcium dependency may be a secondary effect of calcium on other structures, such as cadherin-mediated cell-cell adhesion.

Nusrat et al. (2000) reviewed factors involved in the regulation of tight junctions across the epithelial lining of the gastrointestinal tract. Luminal glucose can increase paracellular permeability to small molecules and cytokines and leukocytes are also able to regulate tight junction structure and paracellular permeability via changes in the tight junction complex or association with the actin cytoskeleton.

Sheth et al. (2000) have looked at the different occludin forms expressed during cleavage in mouse blastocytes, and propose that the phosphorylation of only one of the forms, which leads to its exclusive conversion from a Triton-X-100-soluble to -insoluble may regulate occludin association with ZO-1 and membrane

assembly, thus acting as a control on completion of tight junction biogenesis.

Cancer invasion, angiogenesis and metastasis

Metastasis of cancer cells proceeds via a number of steps (Fig. 3): tumour cells must first invade the surrounding stroma, initialise angiogenesis, the tumour must then develop which requires transport of nutrients to and removal of waste products from the tumour site (Brooks, 1996). Local diffusion will suffice for tumours up to 2 mm in diameter, but for tumours to continue to grow, a connection must be made to the blood supply. The blood vessels within the tumour can then provide a route for detached tumour cells to enter the circulatory system and metastasize to distant sites (Folkman and Shing, 1992; Folkman, 1996). As most tumour cells are surrounded by stroma, interaction between the stroma and the malignant cells are extremely important in the development of tumour angiogenesis (Ono et al., 1999). To metastasise, the detached tumour cells must enter the blood circulation, survive the immune system and arrive at a distant site. Here they interact with the endothelial cells by undergoing biochemical interactions, (mediated by carbohydrate-carbohydrate locking reactions, which occur weakly but quickly) develop adhesion to the endothelial cell to form stronger bonds, thus penetrating the endothelium and the basement membrane. The new tumour can them proliferate.

As tight junctions exist between the cells of the stroma, the endothelium and the tumour itself, tight junctions are the first structure blocking the way for cancer cells to metastasise. For the cancer cell to proceed, the tight junction structure must be disturbed and broken to allow penetration of the cancer cell.

Metastasis and tight junctions

The interaction and penetration of the endothelium by the cancer cell is thus a key step in the formation of metastases (Hart et al., 1989; Jiang et al., 1994). As the molecular structure and mechanism of how tight

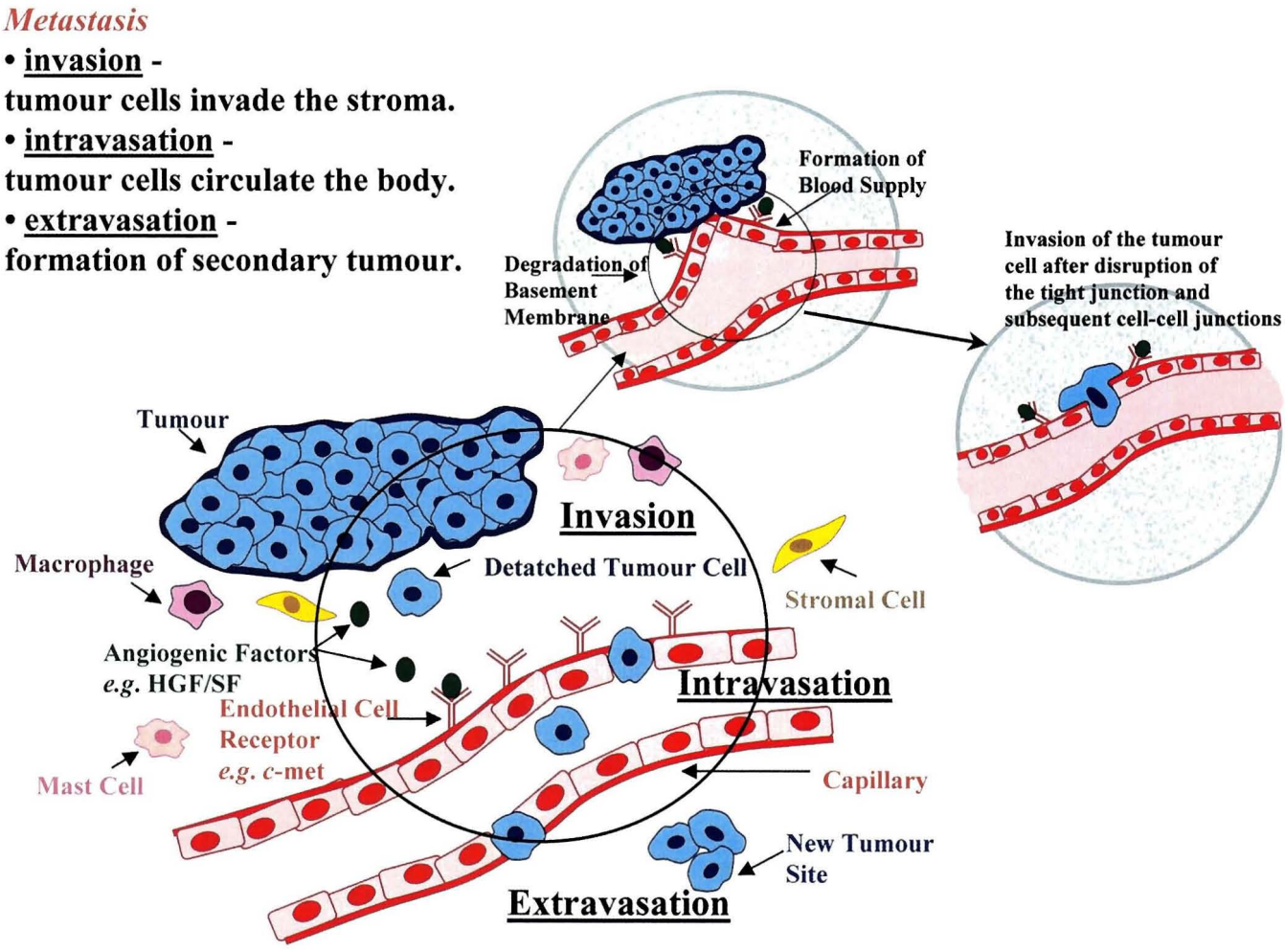

Fig. 3. Schematic showing the process of metastasis.

junction function is a relatively new area, it is only now that tight junctions are viewed as a potentially important target for anti-cancer research and evidence is slowly accumulating to support this idea.

Penetration of the mesothelium

Soler et al. (1999) investigated the association between tight junction permeability of the epithelium in normal human and rat colon epithelia and in colon tumours. They looked at TER and the paracellular influx rate, showing that the tight junctions of colon tumours (both natural and induced) were 'leakier' than those of normal colon, suggesting that the increased permeability of colon epithelium and decrease in epithelium barrier function precedes the development of colon tumours. Tobioka et al. (1996) have shown that enhancement of the function of tight junctions also reduced the penetration of tumour cells through mesothelial cells.

Penetration of the endothelium

Cancer cells have been shown to release factors that assist in their transmigration through the endothelium by treating endothelial cells with conditioned medium from a highly invasive/metastatic melanoma cell line (Utoguchi et al., 1995). Tight junction function was damaged (as indicated by changes in TER) which was irreversible.

Malotilate (MT) has been found to inhibit the invasion and metastasis of rat mammary carcinoma cells through the modification of host endothelial cells. However, MT does not affect the growth of the human oral squamous cell carcinoma cells (SAS, Ca9-22, HSC-2, 3, 4) through a rat lung endothelial invasion model. However, if the rat endothelial cells were treated with MT, invasion was significantly inhibited (in cell lines SAS, Ca9-22 and HSC-4), and with tendency towards inhibition in the remaining two cell lines (Shibata et al., 2000). Protein levels of ZO-1 were elevated dose dependently, with enhanced tight junction function.

Tight junction molecules in cancer cells

The oncogenes of small DNA tumour viruses are believed to promote tumourigenesis by complexing with cellular factors intimately involved with cell proliferation (Glaunsinger et al., 2000). The E4-ORF1 and E6 proteins are the oncogenic determinants for the human adenovirus and papillopmaviruses, and depend on the carboxy-terminal PDZ-domain binding motifs of cell membrane proteins to bind to, also binding to MAGI-1, a dlg-related PDZ protein. The tumourigenic potential of such viruses is dependant on their ability to inhibit the function of such MAGUK proteins in human cells.

Tight junctions are markedly reduced in a number of oncocytic malignant tumours of the thyroid (CochandPriollet et al., 1998).

Claudin-1 is expressed in human mammary epithelial cells, but is only expressed in low or undetectable levels in breast tumours and breast cancer cell lines (Kramer et al., 2000). There appears to be no evidence to propose the involvement of an aberrant form of claudin-1 in breast tumourigenesis, as no genetic alterations in promotor or coding sequences for claudin-1 have been found in breast cancer cell lines or in breast cancer patients (either somatic or germline mutations). It appears that other regulatory or epigenetic factors may be involved in the down-regulation of the claudin-1 gene during breast cancer development. Claudin-1 expression was also lost in the majority of interendothelial junctions in tumour microvessels of cases of human glioblastoma multiforme with associated changes in the morphology of the tight junctions visualised by freeze-fracture (Liebner et al., 2000). Claudin-5 and occludin was only down-regulated in hyperplastic vessels. Such results suggest a relationship between claudin-1 suppression and the alteration of tight junction morphology, which is likely to correlate with the increase of endothelial permeability.

In breast tissues, ZO-1 was found be at high levels, immunohistochemically in normal tissues; this level was reduced or lost in 69% of breast cancer tissues (Hoover et al., 1998). In infiltrating ductal carcinomas, a reduction in ZO-1 staining in 42% of well differentiated, in 83% of moderately differentiated and in 93% of poorly differentiated tumours was seen. The ZO-1 staining correlated well with tumour differentiation and a reduced staining of the epithelial cell adhesion molecule E-cadherin.

ZO-1 was also expressed in gastric and intestinal tissues, together with occludin (Kimura et al., 1997). There was however, a reduction in both these tight junction molecules was observed in associated tumours which was inversely correlated with the histological grade of the tumours.

ZO-2 exists in different isoforms, ZO-2A and ZO-2C, with ZO-2C being a truncated form of ZO-2A (a deletion of 23 amino acids at the N-terminus of ZO-2). The ZO-2A isoform has been found to be expressed in normal epithelial cells and cell lines, whilst ZO-2C is only expressed in the majority of pancreatic cancer cell lines (Chilenski et al., 1999).

Prevention of metastasis

If the tight junction is the first obstacle that cancer cells must overcome in order to extravasate and to metastasis successfully, agents that can strengthen tight junction functions would be essential in the fight against cancer. As discussed earlier, dexamethasone, a synthetic glucocorticoid is able to increase tight junction function, as does GLA.

Satoh et al. (1996) investigated the impact of the tight junction in the penetration of the endothelium. cAMP and trans-retinoic acid (RA) have been shown to regulate tight junction function in endothelial cells by reducing tight junction mediated paracellular influx and increasing transendothelial resistance (TER), indicating

an enhanced tight junction function. This improvement was demonstrated to be the result of an increased level of 7H6, and although there was no change in adhesion of breast cancer cells to the endothelium, there was a decrease in the number of cancer cells penetrating. Tobioka et al. (1996) investigated the effect of enhanced tight junction function of mesothelial cells reducing their penetration by tumour cells. Again, as Satoh et al. (1996), they demonstrated that enhancement could be achieved by treatment with retinoic acid, and that this caused an increase in levels of 7H6 at the cell-cell borders of the mesothelial cells, associated with increased TER. Again, the number of penetrating tumour cells was reduced.

Tumour cell transmigration is very different to that seen in neutrophil passage through the endothelium (Burns et al., 1997), where transendothelial migration is independent of destruction of tight junctions, but occurs instead at the tricellular regions in the endothelial cells. Such a different transmigratory mechanism may provide further clues to the prevention of metastasis.

Agents that inhibit the effects of cytokines and growth factors such as TNF-α, TGF-ß, VEGf and HGF/SF, all of which are able to decrease transepithelial/endothelial resistance and increase paracellular permeability, could be a useful tool in the fight against cancer metastasis. The HGF/SF variant, NK4 is able to successfully inhibit HGF/SF induced decrease in human vascular endothelial cells transendothelial resistance and increased paracellular permeability (Martin et al., 2000).

Conclusions

Tight junctions were found to be altered in tumour cells and tissues by reduction in number over twenty years ago (Pauli et al., 1977; Kerjaschki et al., 1979; Polak-Charcon et al., 1980). Early studies such as these demonstrated a correlation between the reduction of tight junctions and tumour differentiation: lower levels of tight junction number correlated with poorer differentiation of tumours (Jiang et al., 2000). Since then, evidence has slowly accumulated to show that tight junctions may have a vital role to play in the prevention of cancer metastasis. Experimental evidence has emerged to place tight junctions in the frontline as the structure that cancer cells must overcome in order to metastasise. Further work is required to fully realise the potential that this structure has in cancer invasion and metastasis in order to develop new and novel therapies in the prevention of tumour metastasis.

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