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Biophysical approaches for studying the integrity and function of tight junctions

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Abstract: Cell-cell adhesion is an extremely important phenomenon as it influences several biologically important processes such as inflammation, cell migration, proliferation, differentiation and even cancer metastasis. Furthermore, proteins involved in cell-cell adhesion are also important from the perspective of facilitating better drug delivery across epithelia. The adhesion forces imparted by proteins involved in cell-cell adhesion have been the focus of research for sometime. However, with the advent of nanotechnological techniques such as the atomic force microscopy (AFM), we can now quantitatively probe these adhesion forces not only at the cellular but also molecular level. Here, we review the structure and function of tight junction proteins, highlighting some mechanistic studies performed to quantify the adhesion occurring between these proteins and where possible their association with human diseases. In particular, we will highlight two important experimental techniques, namely the micropipette step pressure technique and the AFM that allow us to quantify these adhesion forces at both the cellular and molecular levels, respectively.

keyword: Tight junctions, Cell-cell adhesion, Atomic force microscopy, Micropipette step pressure technique.

1 Introduction

The epithelial cell-cell adhesion complex consists of several components that include the adherens junctions, tight junctions**,** gap junctions and desmosomes. In particular, cell-cell adhesion proteins such as tight junction (TJ) proteins and adherens junction (AJ) integral membrane proteins play an important role in maintaining homeostasis in multicellular organisms. These proteins not only act as barriers guarding the paracellular route for solute transport across epithelia, but also play an important role in regulating cell proliferation and differentiation (Gumbiner 1993; Balda and Matter 2003; Schneeberger and Lynch 2004). Changes in these proteins have also been implicated in the causation and more importantly, pathogenesis of several diseases. Finally, study of these proteins is important for testing and improving drug delivery across cell layers. (Gonzalez-Mariscal and Nava 2005).

While the role of AJ integral membrane proteins in cell-cell adhesion is well established and has been characterized in some detail, little is known about the strength of adhesion forces mediated by TJ integral membrane proteins. This is important if one ultimately wants to build quantitative models accurate at the molecular level for cancer metastasis. Furthermore, the role of forces in the regulation and control of cellular processes is increasingly being recognized throughout biology. In this review, we will present the composition, structure and functions of TJ proteins, their association with various diseases and the assays used to monitor their functions and quantify the adhesion forces they generate. We will also focus on the use of micropipette step-pressure technique and the atomic force microscope for resolving these adhesion forces.

2 Architecture of tight junctions

Tight junctions constitute the most apical component of the intercellular junctions. They form a continuous belt around the apical membrane of the cells resulting in close contact with each other. Several recent reviews have dealt in detail with the structural composition and organization of tight junctions (Goodenough 1999; Tsukita, Furuse and Itoh 2001; Balda and Matter 2003; Harhaj and Antonetti 2004). Proteins that make up tight junctions can be divided into two groups (Fig. 1):

1. transmembrane proteins such as occludin, claudins and junctional adhesion molecules (JAMs), and

2. cytoplasmic plaque components including the ZO family of proteins and others.

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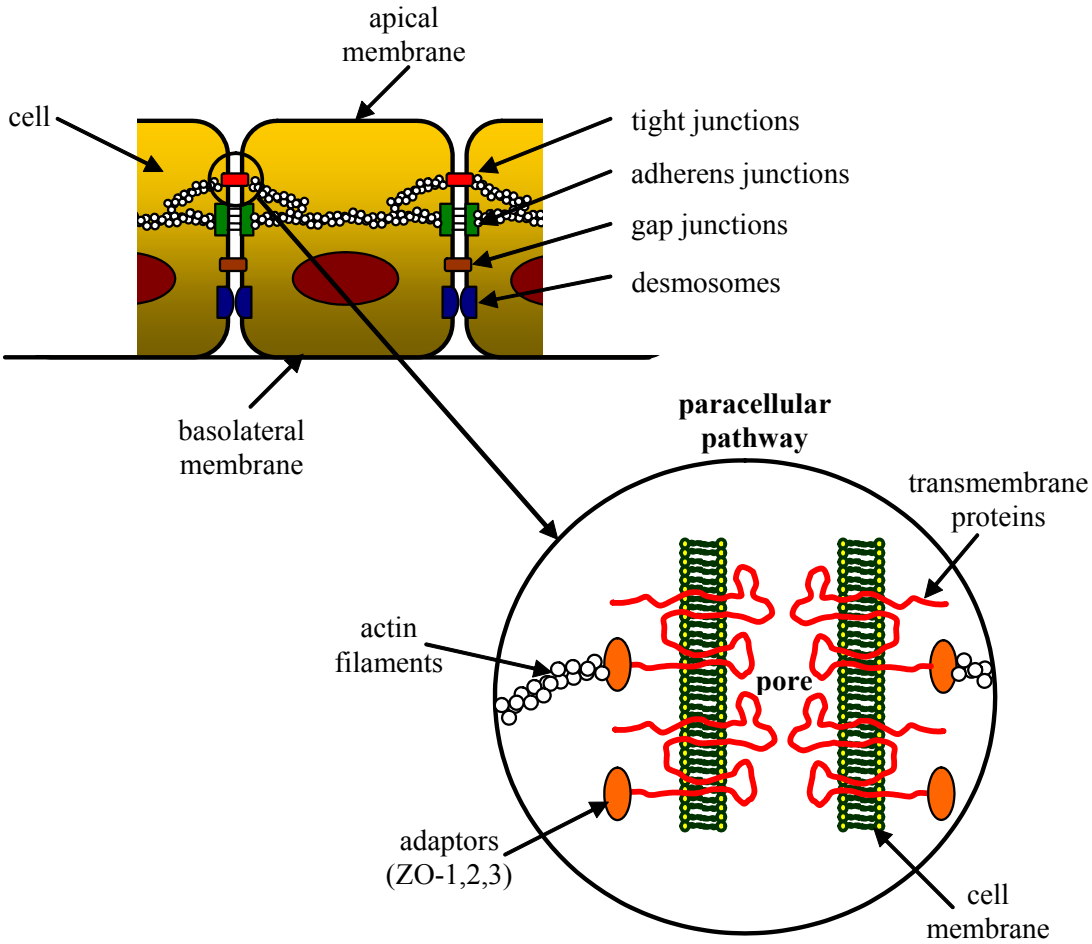

Figure 1 : Schematic diagram of the tight junction organization. The transmembrane proteins of adjacent cells come in close contact with each other forming adhesions. These proteins are linked to the cytoskeleton and other regulatory proteins via adaptor molecules.

Occludin and claudins are proteins that traverse the cell membrane four times forming two extra-cellular loops (Fig. 1). The extra-cellular loops of these transmembrane proteins on adjacent cells come into contact with each other forming the actual paracellular diffusion barrier. On the other hand, the cytoplasmic plaque contains the adaptor, regulatory and transcription factor proteins that link the transmembrane proteins to the actin cytoskeleton and are important in the bidirectional signal transduction between the tight junctions and the cell interior.

Occludin was the first discovered transmembrane TJ protein and was isolated from chick livers (Furuse, Hirase, Itoh, Nagafuchi, Yonemura and Tsukita 1993). As it could only be extracted using detergent, this suggested that it was an integral membrane protein. Cloning and sequencing studies revealed that the protein was 504 amino acids long with molecular weight of 55.9kD. The first and second extra-cellular loop and its C-terminal are considered to be extremely important for occludin mediated cell adhesion and localization, respectively. (Balda, Whitney, Flores, Gonzalez, Cereijido and Matter 1996; Van Itallie and Anderson 1997; Medina, Rahner, Mitic, Anderson and Van Itallie 2000). The C-terminal is linked to the actin cytoskeleton by the ZO family of proteins. Occludin has quantitatively been well associated with barrier properties of tissues. Tissues that are less permeable have been shown to have higher content of occludin as compared to more permeable tissues (Hirase, Staddon, Saitou, Ando-Akatsuka, Itoh, Furuse, Fujimoto, Tsukita and Rubin 1997; Kevil, Okayama, Trocha, Kalogeris, Coe, Specian, Davis and Alexander 1998). In spite of

this correlation, occludin expression is neither necessary nor sufficient for the formation of intact TJ strands. (Furuse, Sasaki, Fujimoto and Tsukita 1998; Saitou, Fujimoto, Doi, Itoh, Fujimoto, Furuse, Takano, Noda and Tsukita 1998) or paracellular barrier function. Formation of TJ strands in occludin-deficient mice has led to the discovery of claudins (Furuse, Fujita, Hiiragi, Fujimoto and Tsukita 1998). Also first discovered in chick livers, there are now about 24 types of claudins constituting the claudin gene family in mammals (Morita, Furuse, Fujimoto and Tsukita 1999). In spite of having four transmembrane domains like occludin, they share little sequence similarity. These proteins have a molecular weight in the range of ∼25kD. All claudins have been shown to have a C-terminal YV (except claudin-16, which has a type I TRV) PDZ-binding motif that is important for the interaction with the PDZ domain of the "cytoplasmic plaque" proteins like ZO group of proteins (Itoh, Furuse, Morita, Kubota, Saitou and Tsukita 1999; Hamazaki, Itoh, Sasaki, Furuse and Tsukita 2002; Muller, Kausalya, Claverie-Martin, Meij, Eggert, Garcia-Nieto and Hunziker 2003). Claudins associate laterally with each other to form the strands even in the absence of Ca^{2+} (Sasaki, Matsui, Furuse, Mimori-Kiyosue, Furuse and Tsukita 2003). These associations can be homo- or heterogenic, but in the case of heterogenic interactions, only certain combinations are possible (Furuse, Sasaki and Tsukita 1999).

JAMs belong to the immunoglobulin superfamily and in contrast to claudins and occludin, only span the membrane once. JAMs were found to be important for leukocyte migration across epithelia (Martin-Padura, Lostaglio, Schneemann, Williams, Romano, Fruscella, Panzeri, Stoppacciaro, Ruco, Villa, Simmons and Dejana 1998; Williams, Martin-Padura, Dejana, Hogg and Simmons 1999).

ZO (ZO-1, ZO-2, and ZO-3) proteins belong to the group of membrane associated guanylate kinase family of proteins (MAGuK's). They contain three PDZ domains, an SH3 domain and a GUK domain that lacks kinase activity. The PDZ domains interact with the cytoplasmic tail of claudins and JAMs while the C-terminal proline rich tail binds to the actin filaments. Binding to occludin occurs via the GUK domain. A wide variety of cellular proteins have now been identified that are associated either directly or via adaptors to the TJs (e.g. cingulin and symplekin) (Harhaj and Antonetti 2004).

3 Functions of tight junctions

The tight junctions form a continuous belt around the apical membrane of the cell and prevent the apical proteins and outer leaflet lipids from diffusing to the basolateral membrane and vice versa (Dragsten, Blumenthal and Handler 1981; van Meer and Simons 1986). This is referred to as the "fence function" of the tight junctions, which is critical for maintaining polarity ((the asymmetric distribution of the integral membrane proteins and lipids in the apical and basolateral plasma membrane) of the epithelial cells.

There is a bidirectional signaling between the cell and TJs. As described above, the tight junction proteins of adjacent cells come into contact with each other to form the paracellular barrier, a second well characterized function of TJs. However, the barrier formed is not passive but dynamic in nature. Its permeability is actively regulated by signals that come from within the cell. This is referred to as the "gate function" of the tight junctions. As described above, the tight junctions are intimately associated with the actin cytoskeleton and small GTPases (e.g. Rho GTPase). It is thought that these small GTPases regulate the paracellular permeability by inducing changes in the actin cytoskeleton through Rho effector proteins (e.g. ROCK) (Fujita, Katoh, Hasegawa, Yasui, Aoki, Yamaguchi and Negishi 2000). It is also possible that the change in the permeability might be due to the direct action of kinases on the tight junction proteins (e.g. phosphorylation of occludin) (Hirase, Kawashima, Wong, Ueyama, Rikitake, Tsukita, Yokoyama and Staddon 2001). The Rho GTPases can also possibly activate myosin leading to contraction of actin filaments and opening up of the paracellular barrier (Hecht, Pestic, Nikcevic, Koutsouris, Tripuraneni, Lorimer, Nowak, Guerriero, Elson and Lanerolle 1996; Turner, Rill, Carlson, Carnes, Kerner, Mrsny and Madara 1997). The activation of these small GTPases in turn could involve several signaling molecules and is still not well understood.

Furthermore, the barrier is ion selective meaning that its permeability is different for the diffusion of different ions. This selectivity is determined by the type of claudins present. For example expression of claudin-2 in MDCK I cells caused an increased permeability to cations (Furuse, Furuse, Sasaki and Tsukita 2001). On the other hand, expression of claudin-8 in MDCK II cells lead to decreased permeability of mono and divalent

Table 1 : Diseases associated with changes in tight junction structure, function or localization in various organ systems

Organ system	Associated diseases
Central Nervous System	Multiple sclerosis (Plumb, McQuaid, Mirakhur and Kirk 2002; Kirk, Plumb, Mirakhur and McQuaid 2003; Minagar, Ostanin, Long, Jennings, Kelley, Sasaki and Alexander 2003) Alzheimer's disease (Fiala, Liu, Sayre, Pop, Brahmandam, Graves and Vinters 2002) HIV encephalitis and dementia (Dallasta, Pisarov, Esplen, Werley, Moses, Nelson and Achim 1999; Boven, Middel, Verhoef, De Groot and Nottet 2000; Andras, Pu, Deli, Nath, Hennig and Toborek 2003; Annunziata 2003; Nitta, Hata, Gotoh, Seo, Sasaki, Hashimoto, Furuse and Tsukita 2003) Auto immune encephalitis, astrocytomas and glioblastoma multiforme (Liebner, Fischmann, Rascher, Duffner, Grote, Kalbacher and Wolburg 2000; Papadopoulos, Saadoun, Woodrow, Davies, Costa-Martins, Moss, Krishna and Bell 2001; Wolburg, Wolburg-Buchholz, Kraus, Rascher-Eggstein, Liebner, Hamm, Duffner, Grote, Risau and Engelhardt 2003; Schneider, Ludwig, Tatenhorst, Braune, Oberleithner, Senner and Paulus 2004) Hyperthermia (Chen, Xu, Huang, Xu, Jiang and Cai 2003) Duchene's muscular dystrophy (Nico, Frigeri, Nicchia, Corsi, Ribatti, Quondamatteo, Herken, Girolamo, Marzullo, Svelto and Roncali 2003) Hypoxia (Fischer, Wobben, Marti, Renz and Schaper 2002; Mark and Davis 2002)
Gastro- Intestinal System	Diarrhea induced by : various bacterial toxins (Gerhard, Schmidt, Hofmann and Aktories 1998; Sonoda, Furuse, Sasaki, Yonemura, Katahira, Horiguchi and Tsukita 1999; Nusrat, von Eichel-Streiber, Turner, Verkade, Madara and Parkos 2001; DeMeo, Mutlu, Keshavarzian and Tobin 2002; Fasano and Nataro 2004; Viswanathan, Sharma and Hecht 2004) Giardia lamblia duodenitis (Buret, Mitchell, Muench and Scott 2002) Helicobacter pylori (Papini, Satin, Norais, de Bernard, Telford, Rappuoli and Montecucco 1998; Terres, Pajares, Hopkins, Murphy, Moran, Baird and Kelleher 1998; Amieva, Vogelmann, Covacci, Tompkins, Nelson and Falkow 2003), Rota virus. (Nava, Lopez, Arias, Islas and Gonzalez-Mariscal 2004) Inflammatory bowel diseases (Marin, Greenstein, Geller, Gordon and Aufses 1983; Schurmann, Bruwer, Klotz, Schmid, Senninger and Zimmer 1999; Kucharzik, Walsh, Chen, Parkos and Nusrat 2001; Bruewer, Luegering, Kucharzik, Parkos, Madara, Hopkins and Nusrat 2003; Poritz, Garver, Tilberg and Koltun 2004) Ccolitis (Lora, Mazzon, Martines, Fries, Muraca, Martin, d'Odorico, Naccarato and Citi 1997; Mazzon and Cuzzocrea 2003) Celiac disease (Kohl, Ashkenazi, Ben-Shaul and Bacher 1987; Schulzke, Bentzel, Schulzke, Riecken and Fromm 1998; Fasano, Not, Wang, Uzzau, Berti, Tommasini and Goldblum 2000; Pizzuti, Bortolami, Mazzon, Buda, Guariso, D'Odorico, Chiarelli, D'Inca, De Lazzari and Martines 2004) Gastro-esophageal reflux disease (GERD) (Miwa, Asaoka, Hojo, Iijima and Sato 2004) Menetrier's disease (Kelly, Miller, Malagelada, Huizenga and Markowitz 1982)

Hepato-biliary-pancreatic system	Cholestasis associated with common bile duct ligation (Fallon, Mennone and Anderson 1993; Landmann 1995) Primary biliary cirrhosis and primary sclerosing cholangitis (Sakisaka, Kawaguchi, Taniguchi, Hanada, Sasatomi, Koga, Harada, Kimura, Sata, Sawada, Mori, Todo and Kurohiji 2001) Cholelithiasis (Snigerevskaia and Veselov 1986) Acute pancreatitis (Fallon, Gorelick, Anderson, Mennone, Saluja and Steer 1995; Schmitt, Klonowski-Stumpe, Eckert, Luthen and Haussinger 2004)
Respiratory system	Asthma (Ranga, Powers, Padilla, Strope, Fowler and Kleinerman 1983; Elia, Bucca, Rolla, Scappaticci and Cantino 1988; Winton, Wan, Cannell, Gruenert, Thompson, Garrod, Stewart and Robinson 1998; Wan, Winton, Soeller, Tovey, Gruenert, Thompson, Stewart, Taylor, Garrod, Cannell and Robinson 1999; Wan, Winton, Soeller, Gruenert, Thompson, Cannell, Stewart, Garrod and Robinson 2000; Hogman, Mork and Roomans 2002; Shahana, Kampf and Roomans 2002) Shock lungs (Barrios, Inoue and Hogg 1978) Interstitial lung disease (Gardner, Brody, Mangum and Everitt 1997) Ventilator induced lung injury (Cavanaugh, Oswari and Margulies 2001; Cavanaugh and Margulies 2002)
Renal System	Familial hypomagnesaemia and hypercalciuria (Simon, Lu, Choate, Velazquez, Al-Sabban, Praga, Casari, Bettinelli, Colussi, Rodriguez-Soriano, McCredie, Milford, Sanjad and Lifton 1999; Blanchard, Jeunemaitre, Coudol, Dechaux, Froissart, May, Demontis, Fournier, Paillard and Houillier 2001; Muller, Kausalya et al. 2003) Pseudo-hypo aldosteronism (Yamauchi, Rai, Kobayashi, Sohara, Suzuki, Itoh, Suda, Hayama, Sasaki and Uchida 2004)
Carcinomas	Hepatocellular carcinoma. (Schmitt, Horbach, Kubitz, Frilling and Haussinger 2004) Endometrial carcinoma (Tobioka, Isomura, Kokai, Tokunaga, Yamaguchi and Sawada 2004) GI tract carcinomas (Kimura, Shiozaki, Hirao, Maeno, Doki, Inoue, Monden, Ando-Akatsuka, Furuse, Tsukita and Monden 1997) Pancreatic carcinoma (Michl, Barth, Buchholz, Lerch, Rolke, Holzmann, Menke, Fensterer, Giehl, Lohr, Leder, Iwamura, Adler and Gress 2003), oral carcinomas (Palekar and Sirsat 1975) Breast carcinoma (Kominsky, Argani, Korz, Evron, Raman, Garrett, Rein, Sauter, Kallioniemi and Sukumar 2003)
Other diseases	Autosomal recessive hearing loss (Wilcox, Burton, Naz, Riazuddin, Smith, Ploplis, Belyantseva, Ben-Yosef, Liburd, Morell, Kachar, Wu, Griffith and Friedman 2001; Ben-Yosef, Belyantseva, Saunders, Hughes, Kawamoto, Van Itallie, Beyer, Halsey, Gardner, Wilcox, Rasmussen, Anderson, Dolan, Forge, Raphael, Camper and Friedman 2003) Diabetic retinopathy (Antonetti, Barber, Khin, Lieth, Tarbell and Gardner 1998) Uveitis (Luna, Chan, Derevjanik, Mahlow, Chiu, Peng, Tobe, Campochiaro and Vinores 1997)

cations whereas the permeability of anions and neutral solutes was unaffected (Yu, Enck, Lencer and Schneeberger 2003).

The cell also determines the recruitment and maturation of the TJs. This is a complex process and involves several kinases and signaling molecules. These include protein kinase A (PKA), protein kinase C (PKC) and G-proteins (Balda, Gonzalez-Mariscal, Contreras, Macias-Silva, Torres-Marquez, Garcia-Sainz and Cereijido 1991; Denisenko, Burighel and Citi 1994). The exact role of each kinase in the process of TJ assembly is still controversial. However, it is thought that E-cadherin mediated adhesion is one of the first steps in the initiation of TJ assembly and that after initial cell-cell contact, TJ and AJ proteins segregate from each other.

On the other hand, TJ proteins also have a "regulatory function" on the proliferation and differentiation of epithelial cells. This function has been identified only recently and is mediated by the transcription factors associated with TJs (Matter and Balda 2003). ZONAB (ZO-1 associated nuclear binding protein) is one such transcription factor recently identified (Balda and Matter 2000; Balda, Garrett and Matter 2003). ZONAB interacts with CDK4 in the nucleus and stimulates cell proliferation. Its nuclear levels have been shown to be high in proliferating epithelial cells. The levels gradually fall off as the cells start becoming confluent and this correleates with the sequestration of ZONAB by ZO-1 to the tight junctions.

4 Association of TJ proteins and TJ function with various human diseases

Quantitative and qualitative changes in tight junction proteins are associated with numerous human diseases either as a primary cause or more importantly, as a contributing factor to the progression of the disease. Almost all the organ systems in the body are affected by changes in these proteins (Table 1).

4.1 Central Nervous System

The blood-brain-barrier (BBB) is a highly selective diffusion barrier that plays a very important role in protecting the brain against toxic substances, infections, tumor cell invasion and other environmental insults. Basically the BBB is made up of two components: cells (endothelial cells, astrocytes and pericytes) and proteins (TJs, adherens junctions).

Several degenerative diseases, autoimmune diseases, malignancies and infections of the central nervous system have been shown to be associated with quantitative and/or qualitative changes in TJ proteins. For example, it has been shown that serum from patients suffering from multiple sclerosis down regulates the expression of occludin and VE- -cadherin in cultured epithelial cells. Similarly, disruption of TJ proteins by the human immunodeficiency virus (HIV) is possibly an important step in the invasion of the central nervous system by the virus.

4.2 Gastro Intestinal System

The gastro intestinal (GI) tract is responsible for the selective absorption of food and solutes and at the same time prevents the entry of toxic substances and pathogens into the body. It is beyond doubt that TJ proteins of the lining epithelium play an important role in maintaining this barrier. Their importance is clear from the fact that several bacterial toxins act by disrupting the TJs. Furthermore, TJ protein abnormalities also seem to be an important contributor towards the pathogenesis of inflammatory bowel diseases. Similarly, bile ducts (constituting the hepatobiliary system) show TJ disruption when they are obstructed and mutations in genes coding for TJ proteins are associated with leakage of bile acids into the serum.

4.3 Respiratory system

The respiratory tract is continuously exposed to numerous environmental irritants present in the inhaled air such as dust, smoke, carbon particles as well as pathogens. The tract is highly impermeable to these substances and prevents the mast cells and other inflammatory cells in the sub mucosa from coming into contact with these stimuli. TJ proteins contribute significantly towards maintaining this barrier. Disruption of the TJs by these irritants (e.g. dust mite) can lead to sensitization and hyperactivity of the inflammatory cells and is probably one of the important mechanisms of developing asthma.

4.4 Renal System

Excretion of waste products and maintaining electrolyte homeostasis by the kidneys requires the renal cells to maintain a high osmotic gradient in the lumen of the renal ductal system. This requires that the cells lining the renal ducts should form a strong ion selective barrier to

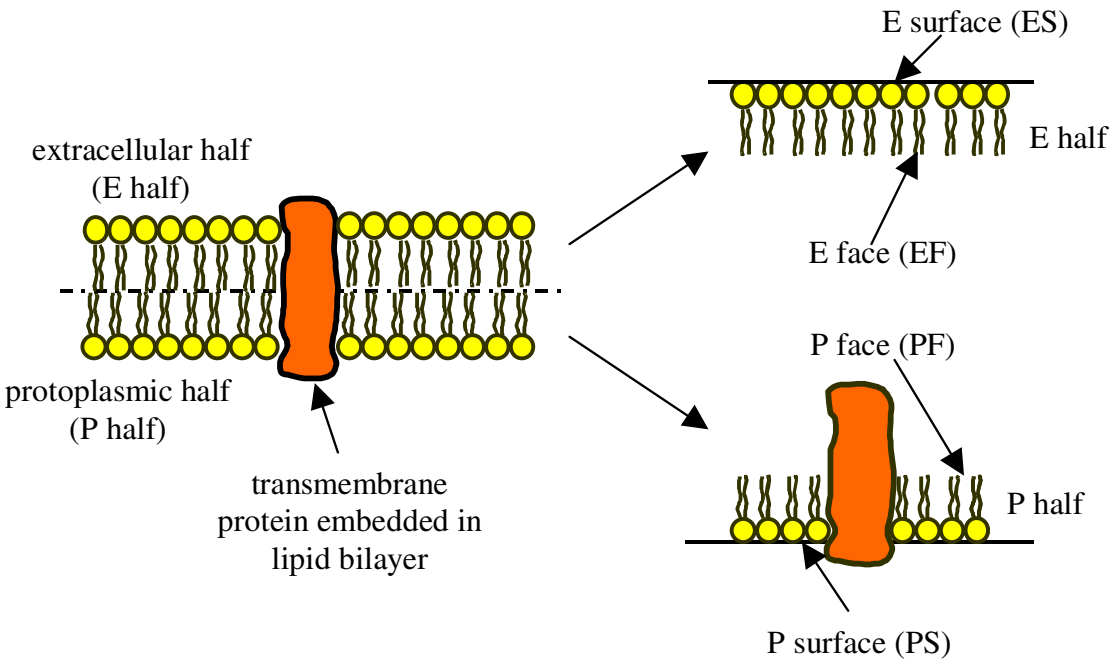

Figure 2 : Schematic depiction of freeze fracture along the hydrophobic plane. The lipid bilayer is fractured along the hydrophobic plane leading to the formation of the protoplasmic half (P half) and the extra cellular half (E half). Each half in turn has a face (PF &EF) and a surface (PS & ES).

the back diffusion of solutes. The importance of TJs in maintaining this barrier is clear from genetic diseases in which mutations in the gene of TJ proteins leads to excessive loss of calcium and magnesium in urine.

4.5 Carcinomas

Metastasis, which is the characteristic feature of carcinomas, requires that the cancerous cells should not only be able to detach themselves from neighbouring cells, but also be able to disrupt TJs of endothelia which form a barrier to their movement into and out of the blood stream. It has been seen that many cancerous cells indeed show altered expression of different TJ proteins. This probably helps them in dissociating themselves from the primary location and metastasizing.

5 Assays for studying integrity and function of tight junctions

As can be seen, significant changes in the localization and properties of TJ proteins can accompany a number of diseases and gene mutations. Hence, there is a need to develop methods and assays that can help to assess the integrity of TJs (Matter and Balda 2003). Monitoring these changes can assist us in better understanding the role of tight junctions in various human diseases. Conventionally, these experiments are performed *in vitro* using epithelial monolayers and more recently, cells grown as three-dimensional cysts and tubules in collagen gels (Yu, Datta, Leroy, O'Brien, Mak, Jou, Matlin, Mostov and Zegers 2005). No single method is conclusive by itself and hence, a combination of several methods is usually necessary in order to draw any meaningful conclusion.The methods available for studying the integrity of TJs can be classified into two categories:

5.1 TJ morphology and protein localization

(a) Freeze fracture analysis of TJ strands. Direct visualization of the TJ proteins has been traditionally done by freeze fracture microscopy. The process basically involves four steps(Nicholas J. Severs 1995): *freezing, fracturing, replica making, and microscopy*. The tissue or cell sample of interest is first frozen by immersion into a liquid cryogen such as Freon or propane. The frozen tissue is then fractured physically using a microtome. The fracture line tends to pass through the lipid bilayers separating the two. The various transmembrane

proteins end up in one of the layers leaving corresponding pits in the other layer. The separation of the bilayers results in the formation of two fracture halves (P half or protoplasmic half & E half or extracellular half) and four fracture sides (PF, PS, EF, ES) (Fig. 2). A metal-carbon replica of the surfaces is made and visualized by electron microscopy. This method can be combined with immunogold labeling to better appreciate the morphological details. Important morphological features to note include the average number, continuity, distribution, and width of the strands.

(b) Localization of TJ proteins in fixed cells

Staining of cells by fluorescence tagged antibodies to various TJ proteins has been used for studying the localization pattern of these TJ proteins. For example, the effect of various TJ disrupting agents can be visualized using this method. Disruptions of the TJs can be seen as discontinuities in the fluorescence staining pattern. Antibodies to ZOs, occludin, and claudins have been used for this purpose. With the use of two-photon confocal microscopy, the changes in the distribution of the TJ proteins can even be estimated quantitatively based on the intensities of the fluorescence. This was not possible to obtain accurately with conventional confocal microscopy as the laser would have "bleached" the proteins which are not in focus thereby introducing errors during observation.

(c) Localization of TJ proteins in living cells

The disadvantage of the above staining methods is that we need to fix the tissue. This means that we cannot get any real time information as to how the TJ proteins are behaving in the cell. Recently, TJ proteins like ZO-1 (Riesen, Rothen-Rutishauser and Wunderli-Allenspach 2002), occludin and claudin (Matsuda, Kubo, Furuse and Tsukita 2004) have been tagged with GFP so that, when these are introduced into the cells, they can be observed in real time. It has been shown that these proteins tagged with GFP are still targeted to the TJs. Furthermore, these proteins have also shown to fold properly and maintain their functionality. In our experiments, we have also used L-fibroblasts stably transfected with GFP tagged occludin and claudins for studying adhesion forces between these cells.

5.2 Permeability

(a) Trans-epithelial electrical resistance (TER)

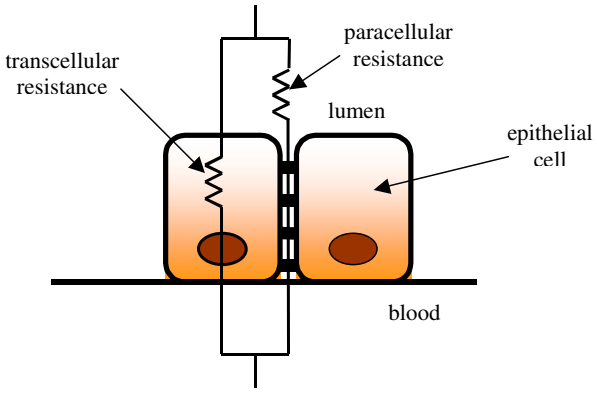

Figure 3 : Simplified circuit diagram of epithelia depicting trans and para-cellular limbs. The total resistance of a layer of cells can be considered as a parallel combination of paracellular and transcellular resistances. Changes in the transepithelial resistance are attributed to the changes in paracellular resistance.

This is probably the oldest and simplest method to measure the TJ permeability of a monolayer of cells. The TER of epithelial cells layers actually reflects the overall permeability of tight junctions to different ions (primarily of Na^+ ions when tissue culture medium is used). Selective ion diffusion across epithelial layers, on the other hand, is determined by measuring dilution potentials (Cereijido, Meza and Martinez-Palomo 1981). Selectivity of ion diffusion is an important function of claudins. Some types of claudins increase the permeability of ions whereas others decrease the permeability. In fact in certain renal diseases (Table 1), mutations in certain claudins can abolish this selectivity leading to ion imbalance.

TER for assaying epithelial permeability assumes simplified circuit models of epithelia (Fig. 3) (Madara, Parkos, Colgan, Nusrat, Atisook and Kaoutzani 1992). A detailed review of impedance analysis of epithelia can be found in (Clausen 1989). The resistance offered by the epithelial layer can be considered to be having two parallel limbs: transcellular and paracellular. However, as the resistance of the transcellular limb is much larger than that of the para cellular limb, it is assumed that the changes that are seen in the TER measured across the epithelia account for the changes in the para cellular resistance. Moreover, as the TJs are considered the rate limiting factor in deciding paracellular resistance, it is further assumed that the changes in the TER reflect the TJ

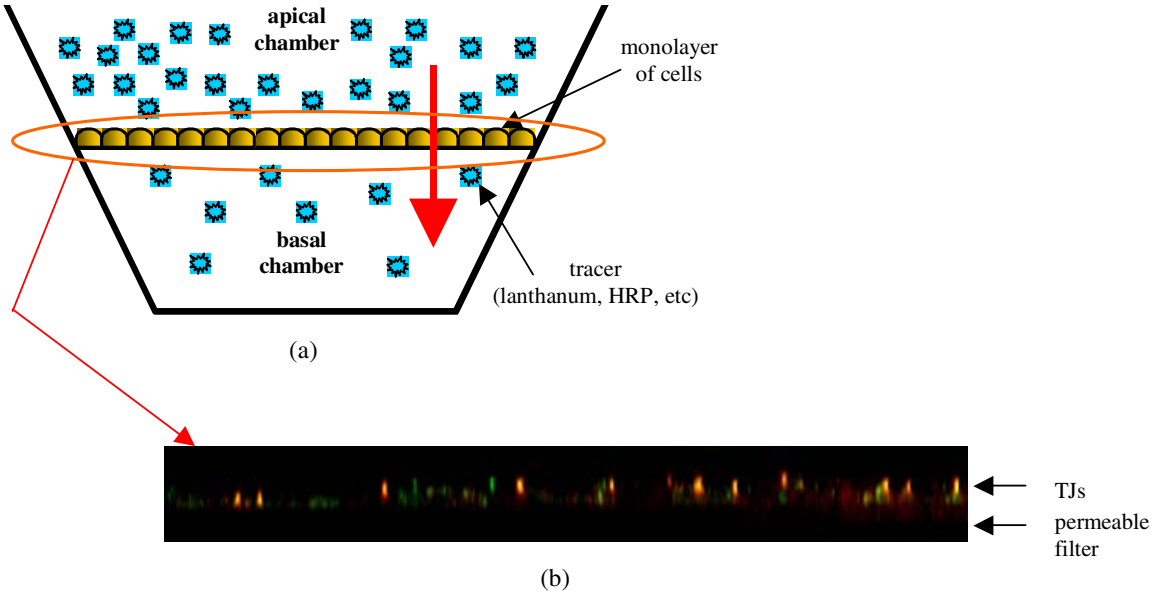

Figure 4 : (a) Schematic depiction of solute permeability across a monolayer of cells (top). Tracer added to the apical chamber can diffuse to the basal chamber only through the intercellular junctions. The concentration of the tracer in the basal chamber measured at different time intervals gives an idea of the parcellular resistance. (b) Confocal z-section image of a monolayer of cells grown on permeable filters immuno stained for Claudin-16(green) and ZO-1 (red) (below). Yellow regions indicate co-localization.

permeability. However, it has to be kept in mind that in many cases, the changes in transcellular resistance may contribute significantly to the total change in epithelial resistance. Hence, interpretation of TJ permeability using TER should always be done in conjunction with other experiments, especially solute flux studies.

(b) Solute flux across monolayer

These methods involve measuring the passive diffusion of various substances across a monolayer of cells. These substances are known to selectively diffuse by the para cellular route.These methods have been used both *in vitro* and *in vivo* for studying the permeability of the tight junctions. Various solutes that have been used include lanthanum (Revel and Karnovsky 1967), HRP (Karnovsky 1967), FITC or radio isotope (^{14}C, ^{3}H) labeled dextran, mannitol and inulin. In case of *in vitro* experiments, the basic experimental design involves culturing of a monolayer of cells on a permeable support till confluence. The solute is added to the upper compartment and the concentration in the basal compartment is measured at different time intervals (Fig.4). In case of in vivo studies, the tracers (lanthanum and HRP) are injected intravenously. The animals are then sacrificed at different time intervals and the tissues of interest fixed and embedded for microscopy.

6 Techniques for studying cell-cell adhesion mediated by TJ proteins and E-cadherins

Different methods have been used to study the intercellular adhesion forces imparted by specific proteins to cells or between different types of cells. These studies have been mostly carried out for AJ proteins and little information is available on the adhesion forces contributed by TJs or individual TJ proteins on overall cell-cell adhesion. Broadly, these techniques can be divided into qualitative (or semi-quantitative) and quantitative methods. Here we will briefly review the basic principle and application of these techniques with particular emphasis on two quantitative methods - the step pressure technique and AFM.

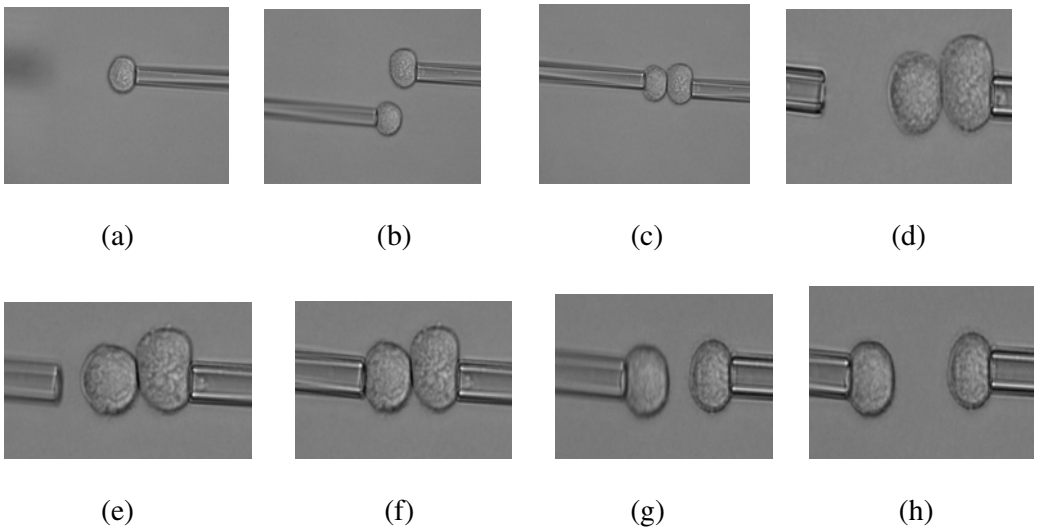

Figure 5 : Step by step depiction of the processes of cell adhesion and separation using the step-pressure technique (Lim, Zhou, Li, Vedula and Fu 2005) (a) a cell is aspirated into the right pipette with a large pressure; (b, c) a second cell is moved close to the first till a contact is made; (d) the cell– cell adhesion is allowed to form for a specified amount of time; (e– h) pressure in the left pipette is increased stepwise till the two cells are detached from each other. (Reprinted from (Lim, Zhou, Li, Vedula and Fu 2005) with permission from Elsevier)

6.1 Qualitative Methods

These consist of traditionally used methods based on re-aggregation of cells that have been transfected with the protein of interest. One method is to monitor the number and size of cell aggregates formed over time.(Moscona 1961; Hausman and Moscona 1975) Based on this method, cells with and without the protein of interest are allowed to aggregate for a specified period of time. The size and number of cell aggregates formed in a fixed time interval for the different groups is then compared.

The second method is to use centrifugation force to disrupt cell re-aggregation. Here, the cells are firstly mechanically shaken and suspended in a flask so that almost all the cells remain separate and single initially. The initial number of single cells (N_0) is counted using a Coulter counter (which can only count single cells). The flask is then rotated at a specified rate (about 80 to 100 rpm) for a fixed period of time. Sample suspensions are taken at specified time intervals and the number of single cells counted (N_t). Cells which are more 'sticky' form more clumps and consequently, the number of single cells will decrease with time (Urushihara, Takeichi, Hakura and Okada 1976; Takeichi 1977). The ratio N_0/ N_t monitored over a specified time interval would reflect the adhesiveness of the cells.

Another method which is also based on the use of centrifugation force is McClay's method (McClay, Wessel and Marchase 1981). Here a set of cells is labeled (with radioactive phosphorous) and incubated with a monolayer of cells in a microtitre plate. The plate is then inverted and centrifuged at different speeds. Following this, the bottom of the plate is cut and the radioactivity measured using a scintillation counter. The centrifugal speed needed to separate 50% of the bound cells gives a rough estimate of the force of adhesion between the cells.

The third method is to use shear force to disrupt cell aggregates. The shear force can be applied by forcing a suspension (remove line break) of cells through a narrow syringe at a specified pressure or by using a flow cytometer as a hydrodynamic focuser. The basic idea is to measure the number of cell aggregates before and after passing through a narrow syringe at different pressures (Bongrand and Golstein 1983; Amblard, Cantin, Durand, Fischer, Sekaly and Auffray 1992). The stronger the cell adhesion, the more the percentage of cell conjugates after the application of the shear stress.

Using centrifugation methods, it has been shown that transfection of these proteins (both TJ proteins and E-cadherins) into cell lines which inherently do not produce these proteins (remove line break) (e.g. L-fibroblasts),

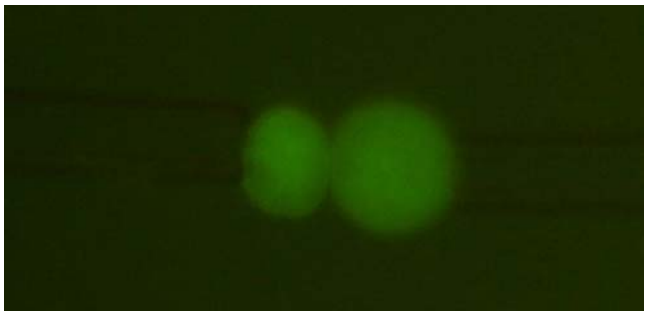

Figure 6 : L-fibroblasts, transiently transfected and expressing GFP & E-cadherin , brought together using micropipettes.

can lead to an increased adhesion between the transfected cells (Van Itallie and Anderson 1997; Kubota, Furuse, Sasaki, Sonoda, Fujita, Nagafuchi and Tsukita 1999). However, these adhesion forces have been objectively quantified only recently using the step pressure technique and the AFM (Baumgartner, Hinterdorfer, Ness, Raab, Vestweber, Schindler and Drenckhahn 2000; Chu, Thomas, Eder, Pincet, Perez, Thiery and Dufour 2004).

6.2 Quantitative methods

In contrast to the above methods, these techniques can give an objective and quantitative estimation of the adhesion force between two cells. The development of AFM has even made it possible to resolve forces at the level of a single molecular interaction. Here we describe two important techniques for objective estimation of intercellular adhesion - the step pressure technique and AFM.

6.2.1 Step pressure technique using micropipettes

This technique was first introduced by Sung et al. (Sung, Sung, Crimmins, Burakoff and Chien 1986; Sung, Saldivar and Phillips 1994) for studying adhesion forces between a cytotoxic T-cell and its target cell. The system consists of glass micropipettes (with a diameter of a few microns) connected to a column of water with the height controlled precisely. This, in turn, regulates the pressure applied across the tip of the micropipette. A pressure transducer converts the pressure applied into voltage. The micropipettes are mounted on micromanipulators to allow the control of fine movement of these pipettes. With a heating stage, one can also perform the experiments at the physiological temperature of 37^oC.

The actual procedure consists of holding a single cell with a pipette by applying a large pressure (Fig. 5). A second cell is then moved close to this cell by a second pipette using a smaller suction pressure. After a specified period of contact, the left pipette is pulled away. If the adhesion force between the two cells is stronger than the applied pressure, the cell slips away from the pipette. The pressure in the left pipette is then increased step wise until it is sufficient enough to pull the left cell away from the right one. The final pressure is multiplied by the area of the pipette tip to obtain the force needed to separate the two cells. This technique has recently been used to objectively quantify the adhesion forces mediated by E-cadherins and nectins (Martinez-Rico, Pincet, Perez, Thiery, Shimizu, Takai and Dufour 2005).

We have also quantified the adhesion forces in L-fibroblasts transfected with TJ proteins using this technique. We have observed that the adhesion force between two transfected cells is indeed greater than that between two wild cells. Tagging GFP to the protein of interest or expressing them together in the same vector can help us in identifying those cells which are actually expressing the protein of interest. This can help in eliminating the tedious process of obtaining stable cell lines (Fig. 6). The computation of adhesion force, in case of stiff cells (like fibroblasts) which do not undergo significant deformation is straight forward. However, in case of highly deformable cells (e.g. RBC) or liposomes, the geometry of the deformation needs to be considered for computing the force (Evans and Leung 1984; Pincet, Le Bouar, Zhang, Esnault, Mallet, Perez and Sinay 2001).

To conclude, the step pressure technique is a simple and easy method to quantify cell-cell adhesion forces.The biggest advantage of this technique is that it is simple to perform. However, the disadvantage is the inability to control the intial contact force as well as the contact area between the cells. It is also not easy to determine the actual number of adhesion points formed between the proteins during contact which actually affects the cell-cell adhesion force induced. However, this can be overcome with the use of AFM which will be discussed in the next section. Finally, large adhesion forces that cause significant deformation of the cells and tether formations can introduce significant errors in the force computation.

6.2.2 Force spectroscopy using AFM

Atomic force microscopy was initially developed to image surfaces at a very high resolution. The basic principle

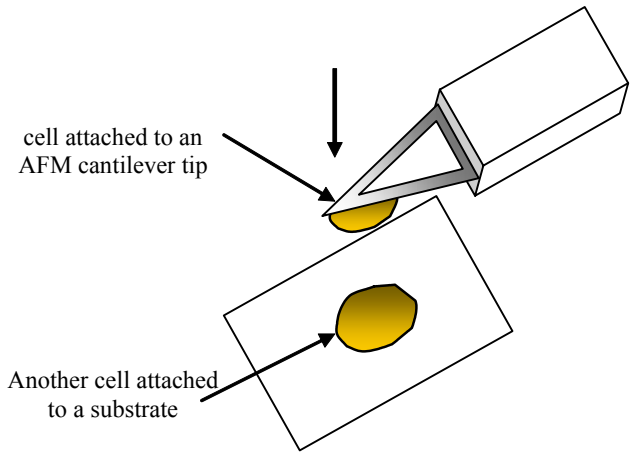

Figure 7 : Schematic drawing showing how AFM is used to probe interaction between two cells.

is the use of a soft deflecting cantilever to probe a surface. Based on the deflections of the cantilever, a map of the topography of the surface can then be obtained. Recently, the use of AFM has been further advanced from imaging to measuring forces at the molecular level - also called "force spectroscopy". Here, the cantilever deflection due to its interaction with a substrate is converted into force (using the spring constant of the cantilever).

The use of force spectroscopy in biological research can be broadly divided into three groups: protein-protein interactions, protein-cell interactions and cell-cell interactions.

A number of methods are available to functionalize the AFM cantilever with the protein or cell of interest. Here we will review the interpretation and basic analysis of force plots obtained using AFM for cell-cell interactions. We are currently studying and characterizing the interaction between L-fibroblasts transfected with different TJ proteins using AFM.

A typical force curve is a plot of the cantilever deflection (or force) on the y-axis versus the z-position of the cantilever on the x-axis. It has two curves- an approach curve and a retrace curve. The approach curve (blue) represents the deflection of the cantilever when it is approaching the substrate and retrace curve (red) represents the cantilever deflection when it is moving away or retracting from the substrate. An adhesion event or interaction between the cantilever and the substrate is observed as a negative deflection in the retrace curve. The rupture of bonds is observed as "jumps" or force peaks in the retrace or retract curve (Fig. 7, 8).

The important parameters that affect the force plots are the contact force, the contact time, the pull velocity and the temperature. The first two are most important when dealing with cells attached to the cantilever as very large contact forces or prolonged contact times can easily detach the cell from the cantilever.

Multiple "jumps" or force peaks in the retrace curve are characteristically obtained when the contact force is high (typically several hundred piconewtons) or contact time is long (several hundred milliseconds).These "jumps" can be due to sequential rupture of a single or multiple bonds. Under these conditions of large contact force and time, it is not possible to obtain the single bond strength. To analyze these plots, two parameters- maximal de-adhesion force and work of de-adhesion have been introduced (Fig. 8). An average of these parameters over several plots can be used for comparing interactions between two cells (Zhang, Chen, De Leon, Li, Noiri, Moy and Goligorsky 2004; Puech, Taubenberger, Ulrich, Krieg, Muller and Heisenberg 2005).

Alternatively, the contact force and contact time between two cells can be lowered to such an extent that the retrace curves predominantly show single de-adhesion events and the number of curves actually showing de-adhesion events are less than 30% of the total curves. A frequency of adhesion events less than 30% gives us a greater than 85% probability that the rupture is a single bond event (Benoit and Gaub 2002; Wojcikiewicz, Zhang and Moy 2004; Zhang, Chen et al. 2004).

During analysis of force plots to resolve interaction forces at the molecular level, we have to keep in mind that bond formation and rupture is a stochastic event and hence, a large number of force curves (several hundreds or more) need to be analyzed for characterizing a particular interaction. Furthermore, there cannot be a single fixed value for a given interaction. We can only obtain a distribution and the values reported are usually peaks of these distribution curves.

Finally, the importance of a proper negative control cannot be over emphasized, especially when studying cell-cell interactions. Cells have a number of surface proteins that may interact either specifically or non-specifically. Without an appropriate control, we cannot attribute a force peak to a particular protein-protein interaction on

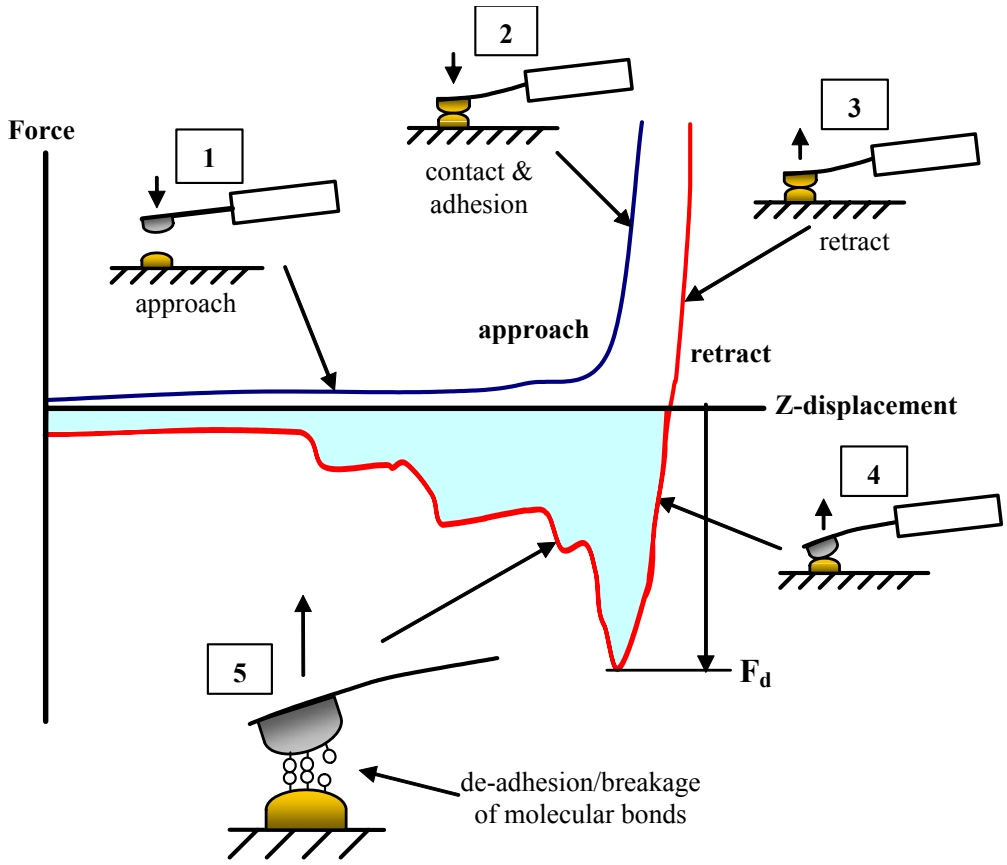

Figure 8 : Typical force-displacement plot of interaction between two cells under conditions of large contact force and contact time. Fd represents the maximal adhesion force and the shaded area is the total work of de-adhesion. The multiple jumps represent single or multiple bond ruptures

the cell. In the context of studying cell-cell interactions mediated by specific proteins, a good negative control can be that of blocking antibodies or mutated cells either lacking or expressing the protein of interest (Benoit and Gaub 2002; Wojcikiewicz, Zhang et al. 2004; Zhang, Chen et al. 2004).

6.2.3 Merits and limitations of the different techniques

In a number of cases, qualitative methods may be sufficient to demonstrate the adhesion properties of a specific protein. The qualitative methods can be quick and easy to perform without the need for sophisticated equipment. On the other hand, the quantitative methods can be very sensitive and as discussed above, can resolve adhesion forces to the level of single cell or even molecule.

The greatest disadvantage of all the above techniques is that they cannot measure the adhesion forces in the natural state. For example in the case of tight junctions and E-cadherins, transfected L-fibroblasts have been used as a model system for studying adhesion forces. However, it is not possible to establish how these forces correlate with the adhesion forces beween tight junctions in epithelial cells in their natural state. Thus, it is important that we develop techniques which can hopefully measure the adhesion forces between two proteins or cells in their natural or physiological state.

7 Conclusions

It is becoming increasingly clear that TJ malfunction is a key factor in the cause as well as progression of numerous human diseases. Three major areas need to be further investigated. Firstly, the possibility of using substances that can reversibly increase the permeability of TJs has to be explored. This would help in the delivery of drugs that are otherwise poorly transported across cell layers. This could be of high clinical significance especially for

delivering drugs to the brain across the blood brain barrier. Further applications could include the delivery of insulin through inhalation (Johansson, Hjertberg, Eirefelt, Tronde and Hultkvist Bengtsson 2002). Secondly, antibodies or peptides have to be developed that can target TJ proteins that are selectively expressed in different carcinomas (Offner, Hekele, Teichmann, Weinberger, Gross, Kufer, Itin, Baeuerle and Kohleisen 2004). Thirdly, the possibility of tight junction proteins as mechanotransducers has to be looked into. The strategic location of tight junction proteins at the intercellular adhesion site, their close association with the cytoskeleton and important transcription factors makes them ideal candidates for acting as mechanotransducers. Techniques for objectively quantifying cell-cell adhesion and protein-protein interaction like AFM and step pressure technique described above can play an important role in exploring these areas.

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