STUDIES OF RECRUITMENT AND MIGRATION OF MESENCHYMAL STEM CELLS

By

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A thesis submitted to the University of Birmingham for the degree of DOCTOR OF PHILOSOPHY

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October 2016

Abstract:

Mesenchymal stem cells (MSC) are used in therapy, often by injection into the blood. Adhesion and migration from flowing blood may be critical steps for their recruitment in the microvasculature. We aimed to understand how MSC from different sources might 'home' to injured tissue. MSC from Wharton's jelly (WJMSC), bone marrow (BMMSC) or trabecular bone (TBMSC) were suspended in culture medium or added to whole blood, and perfused through capillaries coated with matrix proteins (collagen or fibronectin) or P- or E-selectin. Initial comparisons showed that none of the isolated MSC adhered to selectins even at low shear rate, while endothelial progenitor cells showed weak interactions. All of the different cells were able to adhere to collagen or fibronectin at wall shear rates up to about $70s^{-1}$, with adhesion in the order WJMSC>BMMSC>TBMSC. Although BMMSC spread more efficiently than WJMSC, the WJMSC migrated faster through 8μm pore filters. In whole blood, MSC failed to bind to fibronectin, while the fibronectin itself became covered in a single layer of spread platelets. When perfused over collagen, only WJMSC were found to attach, forming aggregates with platelets on the surface. However, all isolated MSC adhered to a surface coated with platelets. Platelets binding to MSC in flowing blood may have formed a shield affecting their attachment. WJMSC appeared to activate those platelets, and could aggregate with platelets activated on collagen. Adhesion of MSC to matrix proteins and to platelets involved both β1- and β3-integrins. Platelets used glycoproteins GpIb and GpIIbIIIa to adhere and aggregate on collagen, and GpIIbIIIa to adhere and spread on fibronectin, but these receptors did not support the interaction between MSC and platelets. These results show intrinsic differences in adhesion and migration of

different MSC, including interaction with platelets, that are predicted to influence their behaviour in vivo and therapeutic effectiveness.

Acknowledgement:

First of all I need to thank my supervisor Professor Gerard Nash for his continuous support, this thesis would not have been possible without his generosity and his sense of humour when time are difficult. I feel very lucky and blessed to have him as my supervisor.

Secondly, I like to thank all the members of our lab for their cooperation and support throughout my work.

This Thesis is dedicated to my delightful husband Mohammed for his support and help. Special thanks to my family members. Especially to my Mom and to my kids Lana, Maalik and Dana.

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Chapter 1 : INTRODUCTION

INTRODUCTION

This thesis considers the ability of circulating progenitor cells – endothelial progenitor cells (EPC) and especially mesenchymal stem cells (MSC) – to attach to vessel wall components from flow in isolation or in blood, and to migrate afterwards. It is based on the possibility that infused EPC or MSC might be used for therapy in vascular disease.

1.1 Introduction – EPC, MSC and the need to study their recruitment

Endothelial progenitor cells (EPC) are circulating nucleated cells, distinct from leukocytes, which are derived from the bone marrow and exist within the adult circulation. Asahara et al, were the first who called a population of circulating cells that exhibit vascular regenerative characteristics EPC in 1997 (Asahara et al., 1997) EPC may be defined as circulating cells that carry cell surface markers that are the same as those expressed by vascular endothelial cells (EC), e.g. CD34, VE-Cadherin and vascular endothelial growth factor receptor2 (VEGFR2), but are distinct from mature EC (Yoder et al., 2007). Hematopoietic and endothelial progenitor cells are thought to have a common precursor because they share many cell surface markers (Ingram et al., 2004). A number of markers have been used for EPC, but there are no unique markers. Functionally, the most important feature of EPC is their capacity to differentiate into mature endothelial cells (Padfield et al., 2010, Harred et al., 1972). EPC thus have attracted great interest because of their potential uses in angiogenic therapies, or as having roles in repair or as biomarkers for cardiovascular disease (Ingram et al., 2004).

Mesenchymal stem cells (MSC) represent a group of progenitor cells existing in tissue stroma with pluripotent potential to give rise to different cells types,

especially adipocytes, chondrocytes and osteoblasts (Meirelles et al., 2006). They carry markers similar to those on tissue fibroblasts and are difficult to distinguish from them. Functionally they are defined as plastic adherent property and able to differentiate into chondrocytes, adipocytes and osteocytes (Lu et al., 2006). The differentiation potential of fibroblasts varies based on their source, for example fibroblast which are prepared from skin (hSDFs) have better differentiation potential than terminally differentiated fibroblasts. MSCs are discriminated from fibroblast (WI38) by two specific properties which are the capacity to form colonies and the differentiation potential. Other conventional properties for MSCs such adhesion and the expression CD44, CD90, CD105 are considered unspecific for stem cells (Alt et al., 2011).

MSC have features which make them potential therapies for a number of diseases (Wei et al., 2013); For example, the ability of MSCs to differentiate into organ specific cells, makes them useful for regenerative therapy and there have been many clinical trials e.g., to treat spinal cord injury and myocardial infarction (Wei et al., 2013). MSC are also able to suppress immune responses (Teo et al., 2015, Karp and Teol, 2009). MSC may be used in allogenic transplantation because there is little risk of immune rejection due to the lack of immunogenicity. Moreover, the isolation of MSCs is well established and quite simple, and there are few ethical objections to their use, which makes them attractive as therapies for the future (Meirelles et al., 2006, Lennon and Caplan, 2006).

MSCs may be mobilized from the host or infused systemically (exogenous MSC). There is no agreement on whether they naturally circulate and the homing process for injected or naturally circulating cells is uncertain. The fact that there are

no universal criteria for MSCs phenotype, makes MSC homing studies challenging. MSC in blood are very rare at least, and the fact that there are many different protocols for MSC culture means that there are no standard studies of their adhesive abilities or migration from peripheral blood into damaged tissue for regeneration

In general, well-characterised paradigms are lacking for 'homing' of EPC or MSC, compared to the well studied adhesion cascades for leukocytes and platelets. While parallels are often drawn (Teo et al., 2015) there is not enough data to clearly define the steps by which progenitors adhere locally (within the vessel) or transmigrate across the endothelium. Thus the abilities of different progenitors cells to bind to intact endothelium or to exposed matrix in damaged vessels is of interest. In this thesis, we set out to investigate MSC and EPC recruitment and migration, but concentrated on MSC after initial comparisons of adhesion from flow.

In the Introduction, the structure of the vascular system and the characteristics of blood flow are described first, before considering adhesive behaviour and recruitment of circulating cells, and of stem cells in particular.

1.2 Blood vessels: structure, function and endothelial regulation.

1.2.1 The vascular system

The vascular system is divided into arteries, capillaries and veins. Arteries carry oxygenated blood from the heart to the tissue. Their wall consists of three layers which support the high arterial blood pressure: tunica intima, tunica media and tunica adventitia (Anne and Allison, 2014). Tunica intima is the innermost layer made of endothelium having an underlying basement membrane which contains elastic fibres. The tunica media is made of primary smooth muscle and it has the highest thickness among the three layers. In addition to its support function, it assists

in regulating vessel blood flow and pressure. The outermost layer consists of connective tissue which contains different concentrations of elastic and collagen fibres. The density of this layer is high at its junction with tunica media, but at the peripheral region its morphology changes to lose connective fibres. The diameter of larger arteries is >10mm and the smaller ones range from 0.1 to 10mm, the smallest being arterioles (Tortora, 2011). In relation to cell adhesion, damaged arteries are sites for platelet attachment to prevent blood loss, but leukcytes rarely adhere (Luu et al., 2010).

The capillaries link arterioles to post-capillary venules. The walls of capillaries are composed of endothelial cells overlaying a basement membrane. Vascular pericytes provide support to them but do not form a continuous sheet. Capillaries are the sites for exchange of nutrients, metabolites and gases between the interstitial space and blood. They are permeable to small molecules such as water, oxygen and carbon dioxide, hormones and electrolytes, but not proteins. They range in diameter typically between about 5-10μm (Tortora, 2011).

Deoxygenated blood is taken towards the heart through the venous system. When blood moves from capillaries, sub-branches of veins called venules receive it (diameter between 7 to 50 μm) and these enter gradually larger veins. Venous walls are multi layered. There are three layers with higher connective tissue content and less smooth muscle than arteries. In general, walls of veins are thin in comparison with arteries and they only have to support a low blood pressure (Tortora, 2011). In relation to cell adhesion, post-capillary venules are the main site for leukocyte adhesion in inflammation (Adams and Nash, 1996).

1.2.2 Endothelial cells - function and roles in cell recruitment

The whole vascular system, including the heart and capillaries, is lined by a single layer of cells, endothelial cells, which are responsible for regulating the exchange of substances between the adjacent tissues and blood (Cines et al., 1998). A network of extracellular substances including heparin sulphate proteoglycan, laminins, thrombospondin, fibronectin, elastin and collagen constitute a basement membrane underneath the endothelial(Brown et al., 2006). Endothelial cells bind with the help of integrin adhesion molecules (see section 1.3.2) to the extracellular matrix proteins present in the basement membrane. In most tissues, the endothelium forms a physical boundary separating the bloodstream from the surrounding environment. In endocrine organs and the liver, fenestrated or discontinuous endothelial layers allow more free exchange of substances (Consigny and Vitali, 1998, Shirota and Matsuda, 2003)

Endothelial cells perform a number of different functions including regulation of vascular permeability (Hutter et al., 2004) control of adhesion of leukocytes and platelets, modulation of vascular wall remodelling by controlling the movement of smooth muscle cells, secretion of bioactive molecules which regulate vascular tone and hence flow of blood, serving as a biochemical and physical barrier and provision of a non-thrombogenic surface (Consigny and Vitali, 1998, Shirota and Matsuda, 2003). In the context of this thesis, their roles in control of blood flow and of cell adhesion are the most relevant

1.2.3 Regulation of blood flow

Endothelial cells secrete substances which influence vascular hemodynamics. Vasodilators like prostacyclin (PGI2) and nitric oxide (NO), and vasoconstrictors

like platelet-activating factor (PAF) and endothelin (ET) are released by the endothelial cells in order to regulate local blood flow and also blood pressure (Pique et al., 1989). Endothelial cells secrete nitric oxide constitutively; however, a number of extracellular physical and chemical stimuli such as shear stress can also up regulate the production of nitric oxide. Besides nitric oxide, PAF, ET and PGI2 are also mainly produced in response to environmental changes such as oxygen tension , blood flow, endothelial stretch, circulating cytokines and growth factors (Haworth, 2006, Parenti et al., 1998).

1.2.4 Regulation of interactions of platelets, leukocytes and stem cells with the vessel wall

In response to infection or tissue injury, and during the process of immune surveillance, platelets and white blood cells may interact with one another or with particular portions of endothelium or with exposed components of endothelium. These interactions are essentially involved in physiological processes of hemostasis and inflammation. However, unchecked attachment of platelets and white blood cells with the endothelial wall leads to thrombotic and inflammatory diseases. Under the influence of shear forces, both white blood cells and platelets interact with the surface of blood vessels via a multi-step mechanism which involves (a) development of initial attachment which is mostly reversible; (b) activation of attached cells; (c) formation of stronger bond which is resistant to shear-forces and (4) spreading and aggregation (for platelets) or migration through endothelium (for leukocytes). (Springer, 1995). Endothelial cells control these adhesive interactions by actively regulating their expression of adhesion molecules and presentation of activatory or

inhibitory agents (including NO and PGI2 mentioned above). The molecular mechanisms of the different steps are described in section 1.3.3.

1.3 Circulation of blood and blood cells

1.3.1 Concepts of blood flow

1.3.1.1. Introduction to blood and blood flow in the circulation

The blood is a suspension of cells (red blood cells, white blood cells, platelets) in plasma, which is a solution containing many inorganic ions and a high concentration of proteins (~70g/l) (Hoffbrand, 1991).The red blood cells occupy 40-45% of the volume of the blood (i.e., haematocrit = 40-45%) and outnumber platelets by about 10:1 and white blood cells by about 1000:1. The blood may also contain very small numbers of circulating endothelial cells and endothelial progenitors, haematopoetic stem cells and mesenchymal stem cells (Roufosse et al., 2004). The main purpose of the cardiovascular circulation is to transport these contents around the body.

Red blood cells (RBC) are non-nucleated discs, biconcave in shape, with diameter ~8 μm and thickness ~2 μm. They are composed of an oxygen transporter protein called haemogloblin which is shielded by a flexible membrane. RBC deliver oxygen to the tissues and carry carbon dioxide from the tissues to the lung for gas exchange. Under physiological conditions, there is no receptor-ligand interaction between RBC and the endothelium, but such interaction may occur in pathological states such as sickle cell disease (Shiu and McIntire, 2003).

White blood cells (WBC or leukocytes) are classified based on their granularity into granulocytes and agranulocytes (or mononuclear cells).

Granulocytes include basophils, eosinophils, and neutrophils. Mononuclear cells include monocytes and lymphocytes (Hoffbrand, 1991). WBC are spherical in the blood and composed of a plasma membrane, cytoskeleton, nucleus and a cortical layer rich in actin (Popel and Johnson, 2005). Their size and physical properties vary; for example, the most numerous are neutrophils (about $5x10^6$/ml blood) with diameter ~8 μm. White blood cells are major components of the immune system, and protect against infection and assist in tissue repair (Hoffbrand, 1991) When circulating WBC are confronted by inflammation or foreign antigens , a multi-step adhesion cascade is used to recruit them to the site of injury (see Section 1.3.3.1).

This cascade starts with leukocytes capture and rolling, followed by activation, firm adhesion and finally transmigration across the endothelium to the inflamed tissue.

Platelets are not considered true cells, as they are discoid, non-nucleated particles with diameter of ~2 μm released by megakaryocytes in the bone marrow. They play roles in preventing blood loss (haemostasis) by adhering to damaged vessels and promoting blood coagulation (Hoffbrand, 1991). They express receptors and adhesion molecules which facilitate platelet-matrix and platelet-platelet interactions (see Section 1.3.3.3).

Blood flow is the consistent blood movement through the cardiovascular system driven by the pumping action of the heart. Blood flow maintains adequate supply of O_2, nutrients and hormones; at the same time it helps the body to remove CO_2 and metabolic wastes. These processes are essential to maintain the cell-level metabolism, pH control, osmotic pressure and the body temperature (Tortora, 2011). Blood flow also delivers cells of the immune system.

Under most conditions, the blood flow can be described as 'laminar' when it travel in layers down the blood vessels. Under steady conditions, the flow profile for long and straight vessels becomes parabolic, with highest velocity in the centre of the vessel. In major arteries, at branches, sharp bends or where there is disease, complex patterns of flow occur and at very high flow rate turbulence may interrupt the laminar (Stein and HN., 1976).

The average blood flow velocity and the vessel diameter are inversely related (see Figure 1-1) (Silverthorn, 2007, Tortora, 2011). Average velocities are highest in the aorta and lowest in capillaries because the total cross-section area of the former is lower than the combined latter. The flow passing over endothelial cells that line vessels generates a friction force against the blood flow which is known as shear stress (Traub and Berk, 1998). Shear stress is a critical hemodynamic force because it modulates the function of endothelial cells and also influences the ability of circulating cells to adhere to the vessel wall (Chien et al., 1998, Topper and Gimbrone, 1999).

The flow parameters which influence delivery of cells are explained in greater detail in the next section.

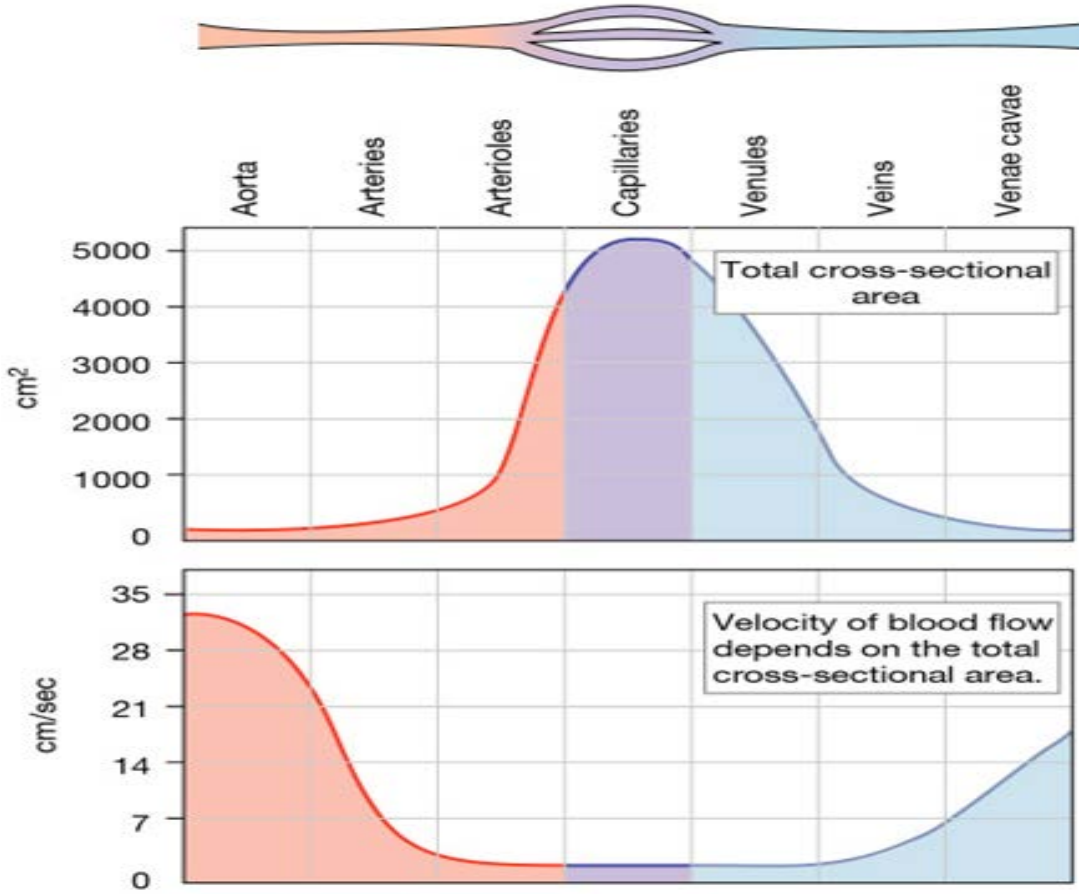


Figure 1-1: The relation between velocity of flow and different cross-sectional area In Aorta.

Area is responsible for the high blood velocity adapted from (Silverthorn, 2007).

1.3.1.2 Haemodynamics and flow in model vessels

The study of the physical factors that control blood flow is called Hemodynamics. The first key factor is the pressure drop (ΔP), equal to the difference between arterial pressure (P_A) and venous pressure (P_V), which is the driving force of blood flow (Q) through the circulation. In general, blood flow is calculated by dividing pressure drop by vascular resistance (R):

$$Q = \frac{\Delta P}{R}$$

This is analogous to Ohm's law, where current is calculated by dividing voltage difference by resistance . The equation can be rearranged to calculate resistance to flow:

$$R = \frac{\Delta P}{Q}$$

Applying the equation to the whole cardiovascular system gives the relation between cardiac output (CO), total peripheral resistance (TPR) and the difference between mean arterial pressure and central venous pressure:

$$CO = \frac{P_A - Pv}{TPR}$$

In order to understand circulatory flow, the different factors which affect pressure, flow and resistance must be studied. For example, usually, changes in vascular resistance or changes in pressure generated by the heart lead to changes in blood flow systemically or locally. In this thesis, we will study blood flow and cell behaviour in individual model 'vessels'. Therefore, the relation between resistance, pressure, and flow in simple tubes is described here.

The relation between volumetric flow rate and pressure drop in a cylindrical vessel is described by the Poiseuille equation (see Figure 1-2A), where flow is

proportional to the fourth power of the radius, and inversely proprotional to the vessel length and viscosity of the fluid (blood). The equation can be rewritten to show that the resistance ($\Delta P/Q$) has two components, one geometrical (dependent on vessel radius and length) and one rheological (dependent on blood viscosity). The most important factor for physiological control of flow is vessel radius because resistance changes inversely to its fourth power, and arterial radius can be actively changed in vivo. The viscosity of the blood also varies depending on its contents, as discussed in Section 1.3.1c.

The Poiseuille equation applies for steady flow of a fluid with constant viscosity (i.e., a Newtonian fluid) in a rigid, straight tube, where there is laminar flow, which can be pictured as the flow of a fluid in layers parallel to each other without any disturbance between the layers. The flow in a small region is illustrated in Figure 1-2B. The velocity of each fluid layer is different. The shear rate is equivalent to the velocity gradient or difference in velocity divided by the distance between layers. The relative motion is resisted by a force generated by the friction between the layers, called the shear stress (force per unit area). The friction depends on the fluid viscosity, which is defined as the ratio of shear stress to shear rate (Figure 1-2B).

In the case of the cylindrical tube, values for velocity, shear rate and shear stress are shown in Figure 1-3A. The fluid velocity at particular position depends on the radial distance from the centre, is zero at the vessel wall and reaches a maximum at the vessel axis. The shear rate (rate of change of velocity) starts from zero at the axis and is maximum at the wall. Shear stress follows the same pattern as it is equal to the shear rate multiplied by the viscosity. When studying behaviour of

cells which may adhere to the vessel wall, the wall shear rate and wall shear stress are particularly important (see section 1.3.4.2).

In the flow assays carried out in this thesis, capillaries with rectangular cross-section (microslides) are used, because they have better optical qualities (see Chapter 2). The equations for flow in rectangular microslides are shown in Figure 1-3B. Practically, the flow rate required to give the desired wall shear rate or stress can be calculated from the dimensions of the microslide and the viscosity of the flowing fluid (Cooke et al., 1993).

The equations used for experiments here are:

$$\text{wall shear rate, } \gamma_W = \frac{6.Q}{w.h^2}$$

$$\text{wall shear stress, } t_W = \frac{6.\eta.Q}{w.h^2}$$

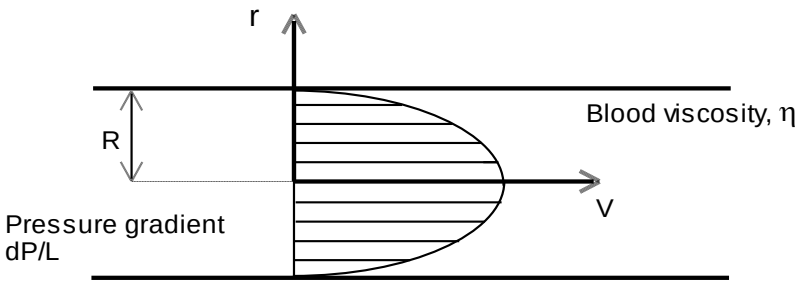
where (in SI units), t_w= wall shear stress (Pa), γ_w= wall shear rate (s^{-1}), η = fluid viscosity (Pa.s; e.g. $0.7x10^{-3}$ Pa.s for aqueous buffers at 37°C), Q = flow rate (m^3/s), w = internal width of the microslide ($3x10^{-3}$m), h = internal height of the microslide ($3x10^{-4}$m).

1.3.1.3 Blood viscosity and effects of RBC

Blood viscosity basically means the thickness of the blood, dependent on its contents. Thin blood with low viscosity gives less resistance to blood flow. The viscosity of plasma depends on its protein concentration and is about 1.8 times that of water at 37°C. However, due to the presence of red blood cells, viscosity of blood is about four times higher than water. Physiologically, the viscosity varies with any alteration of haematocrit. For example, if haematocrit increases from 40% to 60%, it

doubles the blood viscosity. Plasma viscosity will increase, for example, in the acute phase response following injury or inflammation, because of a rise in fibrinogen concentration. In addition, for a drop in body temperature of 1°C, the viscosity increases by about 2% (Barbee, 1973).

A. Flow in a cyclindrical tube



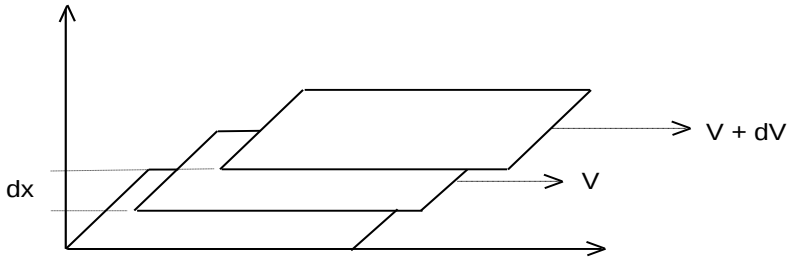
Pouiseuille's equation

Volumetric flow rate, $Q = \frac{dP}{L} \cdot \frac{\pi R^4}{8\eta}$

Resistance $= dP/Q = \eta \cdot \frac{8L}{\pi R4}$

Rheological component *Vascular component*

B. Shear rate, shear stress and viscosity in uniform laminar flow



Planes slipping over each other:

Shear Rate, γ = velocity gradient = dV/dx (s^{-1})

Frictional drag experienced:

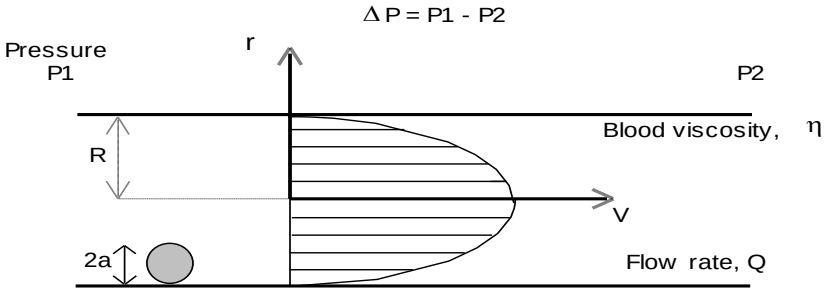
Shear Stress, t = force per unit area (Newtons. m^{-2} = Pascals, Pa)

Viscosity = Shear Stress/Shear Rate (Pa.s)

e.g., water ~1mPa.s at room temp., 0.7mPa.s at 37°C

Figure 1-2: Characteristics of laminar flow.

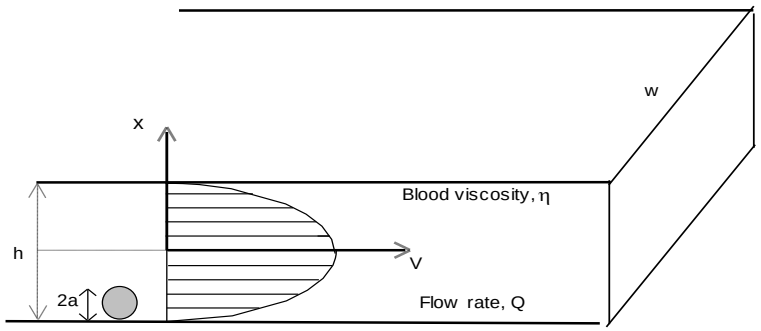
A. Pattern of flow and equations for relations between pressure drop, flow, resistance, vessel geometry and fluid viscosity for a cylindrical vessel. B. Definition of shear rate, stress and viscosity. (Adapted from Lecture Notes of Gerard Nash, University of Birmingham; with permission.)



A Velocity at any radius, $V_r = \frac{2Q}{\pi R^2}(1-r^2/R^2)$
(parabolic flow profile)

Wall shear rate, $\gamma_w = \frac{4.Q}{\pi R^3}$

Wall shear stress, $t_w = \frac{4.\eta.Q}{\pi R^3}$



B Velocity at any distance x off centre

$$V_x = \frac{6Q}{wh^3}.(h^2/4 - x^2)$$

Wall shear rate, $\gamma_w = \frac{6.Q}{w.h^2}$

Wall shear stress, $t_w = \frac{6.\eta.Q}{w.h^2}$

Velocity of particle by wall ~ $\gamma_w.a$

Figure 1-3: . Patterns of flow and equations for relations between flow rate, vessel geometry, shear rate and shear stress for A. cylindrical vessel, or B. vessel with rectangular cross-section. (Adapted from Lecture Notes of Gerard Nash, University of Birmingham; with permission).

Another factor that affects blood viscosity and the resistance to flow is the shear rate. At low shear rate (or stress) the viscosity increases significantly due to the increase in cell to cell adhesion, called rouleaux formation or RBC aggregation (Nash, 1991). As shear increases, the rouleaux divide and scatter Thus in the circulation, RBC undergo aggregation and then disaggregation under the effect of varying shear in different vessels (Nash, 1991, Nash et al., 1992).

The decrease in blood viscosity with increasing shear rate is called shear thinning. In addition to changes in RBC aggregation, RBC deformation plays a role because the cells elongate and line up with each other with increasing shear (Nash, 1991). The RBC can alter their shape while staying unbroken with the help of the spectrin network underlying their membrane that maintains the cells intact and enables them to deform and also to pass through small vessels down to 3μm in diameter (Nash and Dormandy, 1989).

The decline in blood viscosity a companying increasing shear rate or stress means that the blood is non-Newtonian, and that the flow pattern in vessels may not be the same as that described above for Poiseuille flow (Goldsmith and Spain, 1984). The variation in blood flow behaviour in different vessels is described next.

1.3.1.4 Red cell aggregation

Rouleaux are tube-like aggregates of red blood cells which are stacked with the sides of highest area adjacent to each other. It has been established that fibrinogen together with some other macromolecules present in blood induce the RBC aggregation (Fahraeus, 1929). Rampling and Sirs for instance demonstrated that reduction in plasma levels of fibrinogen caused reduction in tendency of the RBC to

form rouleaux (Rampling and Sirs, 1972). Normally, the concentration of fibrinogen in plasma is 0.2-0.3g/100ml (Chien et al., 1971). The correlation between elevated concentration of fibrinogen in plasma and augmented RBC aggregation is well recognized (Rampling and Sirs, 1972). In disease conditions such as chronic inflammation and hypertension and in diabetes, RBC aggregation is increased owing to elevated plasma levels of fibrinogen (Babu and Singh, 2004).

During blood flow, the level of RBC aggregation in a blood vessel is governed by the balanced between forces i.e. fluid shear stress acting on cells to disintegrate the RBC aggregates and the force of temporary aggregation between RBCs which is influenced by the plasma fibrinogen levels (Barshtein et al., 2000). Rouleaux will typically form in an area of a blood vessel where shear rate is lower than ~$50s^{-1}$ (Goldsmith, 1986). This suggests that rouleaux can be formed close to the centre of blood vessels even when shear rates at the wall are higher than the level which allows rouleaux formation (Korotaeva et al., 2007).

Contribute to shear dependence of blood viscosity. However, aggregation also causes an increase in migration of red blood cells towards the centre of vessels in tube (Goldsmith et al., 1999). This inward motion can cause an increasing cell-depleted layer near the wall as flow decreases. In addition, this inward motion of red cells may displace other smaller objects towards the wall, a process called margination (see section 1.3.4.1)

1.3.2 Blood flow in different types of vessels

1.3.2.1 Blood flow in large vessels

The behaviour of the blood inside large blood vessels (> 300 μm)is described as that of a Newtonian viscous incompressible fluid (Whitmore, 1967). Thus the Poiseuille equation and flow equations described above are usually applied. The velocity across the tube reaches its maximum on the axis and reaches its minimum at the wall, whereas the shear rate and stress are maximal at the wall and minimal on the tubes axis. The simple laminar flow may be disturbed at sharp bends and junctions of vessels (Goldsmith, 1986).

1.3.2.2 Blood flow in intermediate sized vessels

The rheological features of the blood are very important in smaller blood vessels. It is not accurate to describe the blood as a homogenous fluid, as the red cells tend to move inward and travel faster down the centre of the vessel with a slower moving plasma sheath (Whitmore, 1967). Lift forces cause the RBC to move from the wall of capillaries towards the centre, especially when shear rate is low and red cell aggregation occurs. As a result, a relatively cell-free plasma layer develops near the wall, and the RBC mean velocity is greater than the mean blood velocity (McWhirter et al., 2012). This causes two hydrodynamic effects known as the Fahraeus and Fahraeus–Linqvist effects which take place in the flow of blood when the diameters of micro-vessels in the vasculature are decreased from about 0.3 mm to 10μm. The first effect is the decrease in tube (small vessel) haematocrit compared to the systemic (large vessel) haematocrit because of the unequal average flow rates of the RBC and plasma in small tubes. The second is the decrease in apparent

viscosity of the blood as the tube diameter decreases, because of the decrease in haematocrit (Goldsmith, 1986).

1.3.2.3 Blood flow and cell deformation in capillaries

The apparent viscosity increases again when the diameter decreases further (<10μm). This phenomenon is called inversion Fahræus-Lindqvist effect (Whitmore, 1967). In these capillaries, the cells flow essentially in single file and their ability to deform and enter the vessel determines resistance to flow, along with the plasma viscosity. The deformability of RBC is mainly determined by its membrane characteristics, and in pathological condition, a severe decline of RBC deformability can lead to raised flow resistance (Nash and Egginton, 1993). While viscosity of blood in large vessels is not usually affected by WBC because of their low number, in capillaries WBC can hold up flow because they are much more rigid than RBC (Nash, 1991). Thus WBC enter capillaries about 1000 times slower than RBC (Bagge, 1983).Activated WBC undergo changes in their cytoskeleton and become even more rigid, so that they may block capillaries in disease (Ernst and Matrai, 1986)

1.3.3 Adhesion of leukocytes and platelets from flowing blood

1.3.3.1 The Multi-step adhesion paradigm

In order of leukocytes to carry out their immune functions, they need to travel from the blood through the endothelium into tissue. Platelets must adhere to the walls of damaged vessels to play their role in prevention of blood loss. In both cases, the cells must first adhere and then obtain signals which change their behaviour (Figure 1.4).

For leukocytes a multi-step adhesion cascade was first described in the 1990s (Butcher, 1990, Springer, 1994)(see Figure 1-4 A). The process consists of overlapping steps: step 1: Capture, where leukocytes tether to the endothelial cells via specific fast-acting adhesion receptors; step 2: Rolling, where leukocytes tumble slowly on the EC until they are activated by chemoattractants on the endothelial surface; step3: Firm adhesion of the activated leukocytes to the EC luminal surface; step 4: Transmigration, where leukocytes travel over then through the EC and the basement membrane into the tissue. Two pathways are possible when crossing EC; either between cell-cell junctions (paracellular pathway) or through the cell body (transcelluar pathway) (Ley et al., 2007). Later studies showed that platelets also used a multi-step process where they first bind unstabley and flip over along the collagen surface (Varga-Szabo et al., 2008). (see Figure1-4 B). They then become activated and firmly adhered. Finally, they spread and start to bind other platelets, but do not migrate. Molecular mechanisms underlying adhesion and activation are described in the following section.

However, before adhesion can happen in the circulation, the leukocytes and platelets must come into contact with the vessel wall. Both travel mixed with the more numerous RBC inside the blood vessels but are forced toward the vessel wall in a phenomena called margination (Goldsmith and Spain, 1984). This margination depends on cell size and the blood shear rate, and is described further in Section (1.3.4.1) The capture after margination is also dependent on the shear rate and shear stress at the wall, factors considered in Section(1.3.4.2)

1.3.3.2 Mechanisms of leukocyte adhesion

EC activation is the starting point of the leukocytes adhesion cascade which occurs in post-capillary venules in inflammation (Springer, 1995, Butcher, 1990, Carlos and Harlan, 1994, McIntyre et al., 2003). Cytokines or inflammatory agonists such as thrombin upregulate expression of specialised receptors on the endothelial surface, including the selectins, P-selectin (CD62P) and E-selectin (CD62E). E-selectin is expressed exclusively on endothelial cells, stimulated by TNF-α, interlukin -1 beta or bacteial endotoxin (Bevilacqua et al., 1987)(. P-selectin is expressed on platelets and EC, where it is stored in Weibel-Palade bodies and can be rapidly mobilised by thrombin (Bonfanti et al., 1989). At the vascular wall, leukocytes are captured by these specialized 'rolling' receptors, which have a rapid reaction rate with their glycoprotein ligands on the leukocytes (Lasky, 1992). The bonds also have rapid reverse rates, so that the leukocytes do not adhere firmly, but roll along, making and breaking bonds. L-selectin expression is restricted to leukocytes and it has a significant role in the recruitment of naive lymphocytes to lymph nodes expressing specialised glycoprotein ligands (Vonandrian et al., 1993).

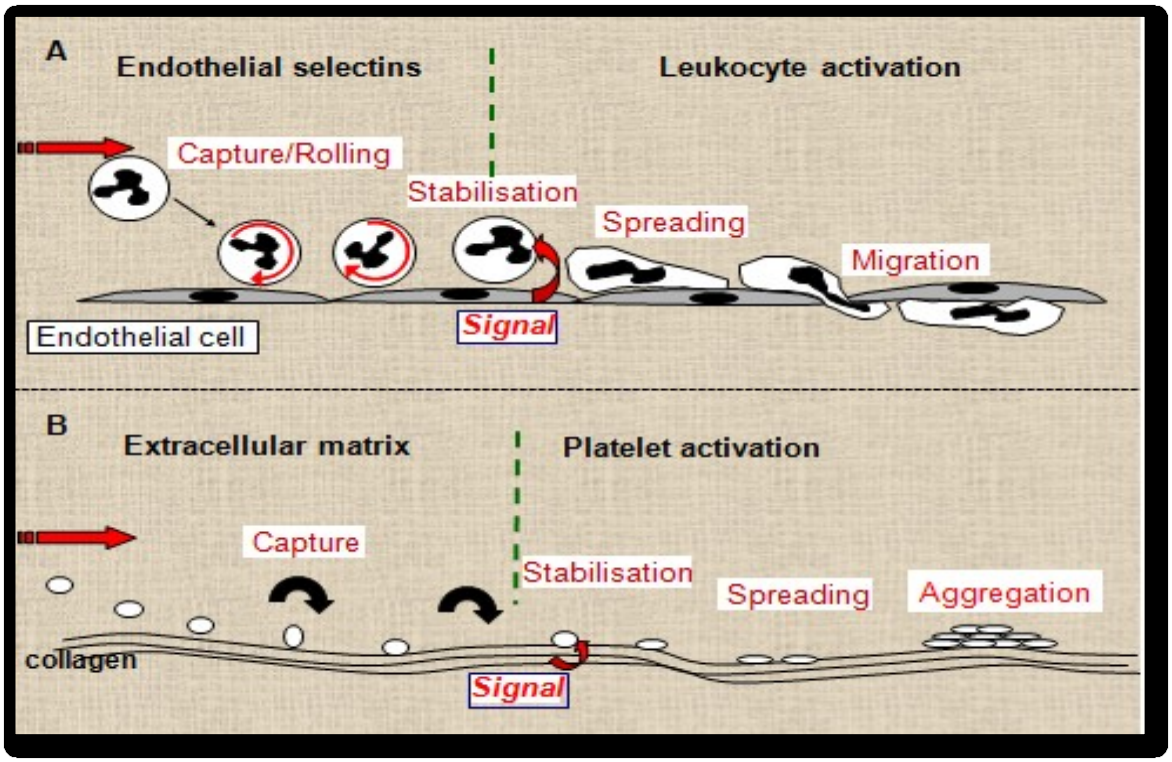


Figure 1-4: Leukocytes adhesion cascade (Adapted from Lecture Notes of Gerard Nash, University of Birmingham; with permission)

Rolling leukocytes contact chemoattractant agents such as chemokines also presented by the activated EC (e.g., IL-8 which activates neutrophils) (Springer, 1995). This leads leukocyte integrins to be activated in turn by "inside-out" signalling, which changes the affinity of the integrins which moves the cascade to the next stage of firm adhesion. The major leukocytes integrins at this stage are of the β2-family (αLβ2 and αMβ2; (see Section 1.3.3.5) and also α4β1-integrin. The endothelial ligands to which leukocyte integrins bind are immunoglobulin gene superfamily (IgSF) members; β2-integrins particularly bind intercellular adhesion molecule-1 (ICAM-1; CD54); α4β1-integrin binds vascular cell adhesion molecule-1 (VCAM-1; CD106) (Phillipson et al., 2006). When leukocytes adhere firmly to the endothelium, they migrate over, then through endothelium via intercellular junctions

by the interaction between integrins and their IgSF ligands in a process called transmigration. Several junctional proteins work together to complete this process such as CD31, CD99, and junctional adhesion molecules (JAMs). Leukocytes may also penetrate into extravascular tissue by passing through EC instead of moving in between the junctions (Ley et al., 2007).

A dynamic interaction between cell surface integrins and leukocytes actin cytoskeleton is required for the migration step. Leukocytes spread on the surface and transmigrate by lamellipodia formation at the leading edge. The friction force generated at the front of the cell is adequately strong to pull leukocytes forward, while new attachments are made at the front and old ones reversed at the rear (Ley et al., 2007).

These processes are similar for different types of leukocytes, although subsets may respond to different chemokines and utilise different integrins. Notably, mononuclear cells (but not neutrophils) can use the interaction between α4β1-integrin and VCAM-1 to be captured from flow, as well as for firm adhesion after activation.

1.3.3.3 Mechanisms of platelet adhesion

When collagen is exposed due to endothelial injury, platelets adhere and accumulate at the site of the injury to form a plug in the injured vessel and facilitate the coagulation cascades so that a stable clot is formed. During physiological conditions, platelets do not adhere to endothelium because the collagen and fibronectin are not exposed. Platelet

adhesion is a coordinated process and happens in stages that are similar to leukocyte adhesion except that platelets adhere to the arterial subendothelium where shear forces are higher than in venules (see Section 1.3.4).

Stages of tethering, rolling, activation and firm adhesion are seen. To start, von Willebrand Factor (vWF) binds to collagen and then binds to platelet glycoprotein (Gp) GPIb in an "on-and-off" manner which results in the formation of tethers which are multiple extensions of the platelet membrane (Dopheide et al., 2002). The platelet roll or flip along. In this step collagen and GPVI interact together in a low affinity bond (Varga-Szabo et al., 2008), which gives an activation signal so that adhesion is strengthened by the GPIa/IIa receptor (α2β1-integrin) (Rivera et al., 2009). The next step in activation is stimulated when platelets release thromboxane A2 and adenosine diphosphate, and /or when the vessel wall secretes tissue factor which activates thrombin (Varga-Szabo et al., 2008). As a results the activation step is completed and firm adhesion is supported by the contact between GPIIb/IIIa (αIIbβ3-integrin) with fibrongen and vWF (Jackson, 2008). At this stage, platelets also spread on the surface and bind to incoming platelets via αIIbβ3-integrin on each forming a sandwich with fibrinongen. Large platelet aggregates may build up on the surface.

1.3.3.4 Leukocyte-platelet interaction

During inflammation, both platelets and leukocytes may become activated and adhere to each other either in the blood or on damaged vessels. The first adhesive contact between neutrophils or lymphocytes and platelets on a surface happens when P-selectin glycoprotein ligand 1 (PSGL1) on the leukocytes identifies

P-selectin on activated platelets (Lalor and Nash, 1995, Piccardoni et al., 2001, Buttrum et al., 1993).

Conformational changes in αMβ2-integrin may take place as a result of neutrophil activation and also platelet-derived chemokines, to encourage firm neutrophil adhesion (Sheikh and Nash, 1996, Stone and Nash, 1999). For monocytes, ability to adhere is increased because of the platelets binding to β1- and β2-integrins on monocytes (Martins et al., 2006).For lymphocytes, platelets may help tethering to peripheral lymph nodes in high endothelial venules for homing during adaptive immune responses (Elzey et al., 2003). Platelets and leukocytes may also form aggregates in the blood when platelets are activated (Rinder et al., 1991). The first step is again mediated by P-selectin interacting with PSGL-1 (Maugeri N, 1994).

The adhesion processes described above are of interest here because stem cells are widely believed to adhere from flow using similar mechanisms and receptors (Teo et al., 2012). Also, stem cell-platelet interaction has been suggested as a mechanism for capturing cells from the blood (Teo et al., 2012). This is considered further in Section(1.5.4.4) after some aspects of stem cell biology have been described. Another common theme is the use of integrins for adhesion. Leukocytes have specialised β2-integrins and platelets have αIIbβ3-integrin. Most other cells do not have these integrins but do have other integrins used for adhesion to matrix. Thus the next section briefly reviews characteristics of integrins.

1.3.3.5 Integrin adhesion molecules and their ligands

In 1986, the expression “integrin” was used to described a compound surface protein which was an essential element for the transmembrane link between the extracellular matrix (ECM) and the cytoskeleton of surface adherent cells

(Tamkun et al., 1986). After that, integrins were realised to be a family of cell-surface receptors that are structurally homologus transmembrane proteins. The molecules are identified as heterodimeric receptors that consist of non covalent linked α- and β-subunits (Hynes, 2002) (Figure1 5).

There are 18 known α-subunits and 8 known β-subunits in the family of human integrins, with an I-domain which is the main ligand-binding site found in many of the α- subunits (Takagi et al., 2001). They are involved in cell-matrix binding as well as cell-cell binding, with different specificities arising from the different α- and β-subunit combinations. The ligands of the different integrins are summarised in Figure 1-6.

Different cell types express different integrins, although many cells share expression of the common matrix-binding integrins. Nonetheless, some cells types have specific integrins which are not expressed on other cells; for instance, as noted above, leukocytes express unique β2 integrins (Kilshaw, 1999). The first discovered integrin in humans was αLβ2 (Sanchez-Madrid F, 1982) which is expressed on different immune cells including neutrophils, monocytes, lymphocytes and dendritic cells (Smith, 2008) and binds mainly to ICAM-1, ICAM-2 and JAM-A (Ostermann et al., 2002).

A characteristic of integrins on leukocytes and on platelets is that they may exist in non-active, non-binding states on unstimulated cells. When the cells are activated by agonists, inside out signalling causes change in the conformation of the integrins which increases their affinity (Luo et al., 2007). Integrins are not only considered adhesion molecules but also involved in a range of signalling functions (Danen and Sonnenberg, 2003). On binding to ligand there is 'outside-out'

signalling, which modifies cell responses such as spreading in platelets and migration in neutrophils.

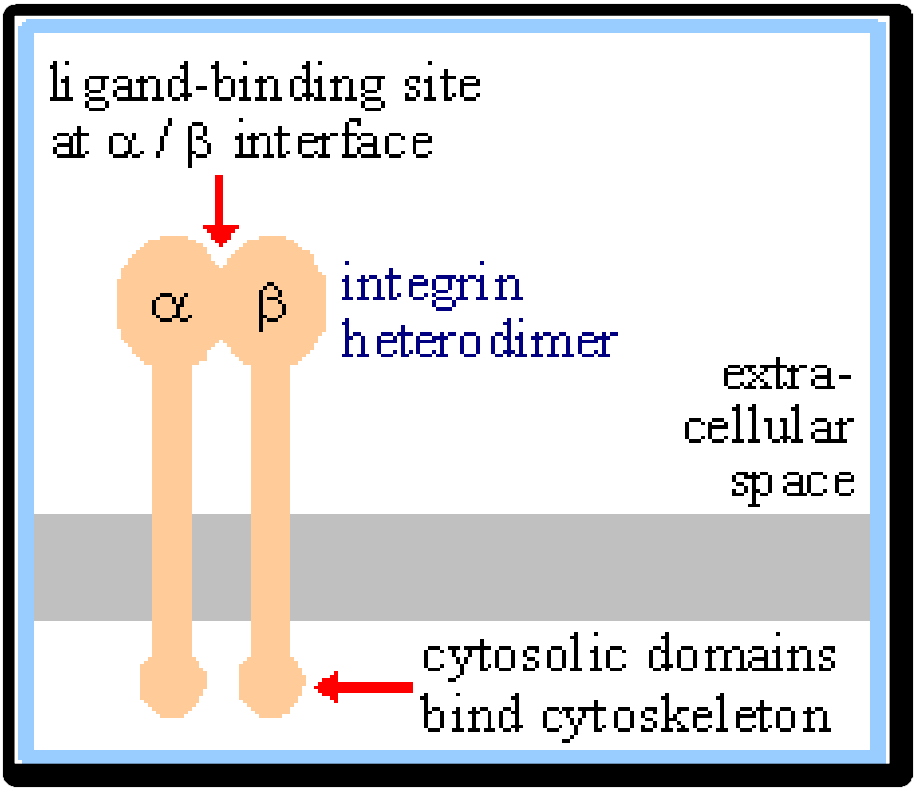


Figure 1-5: Schematic of integrin structure adapted from (Diwan, 2007).

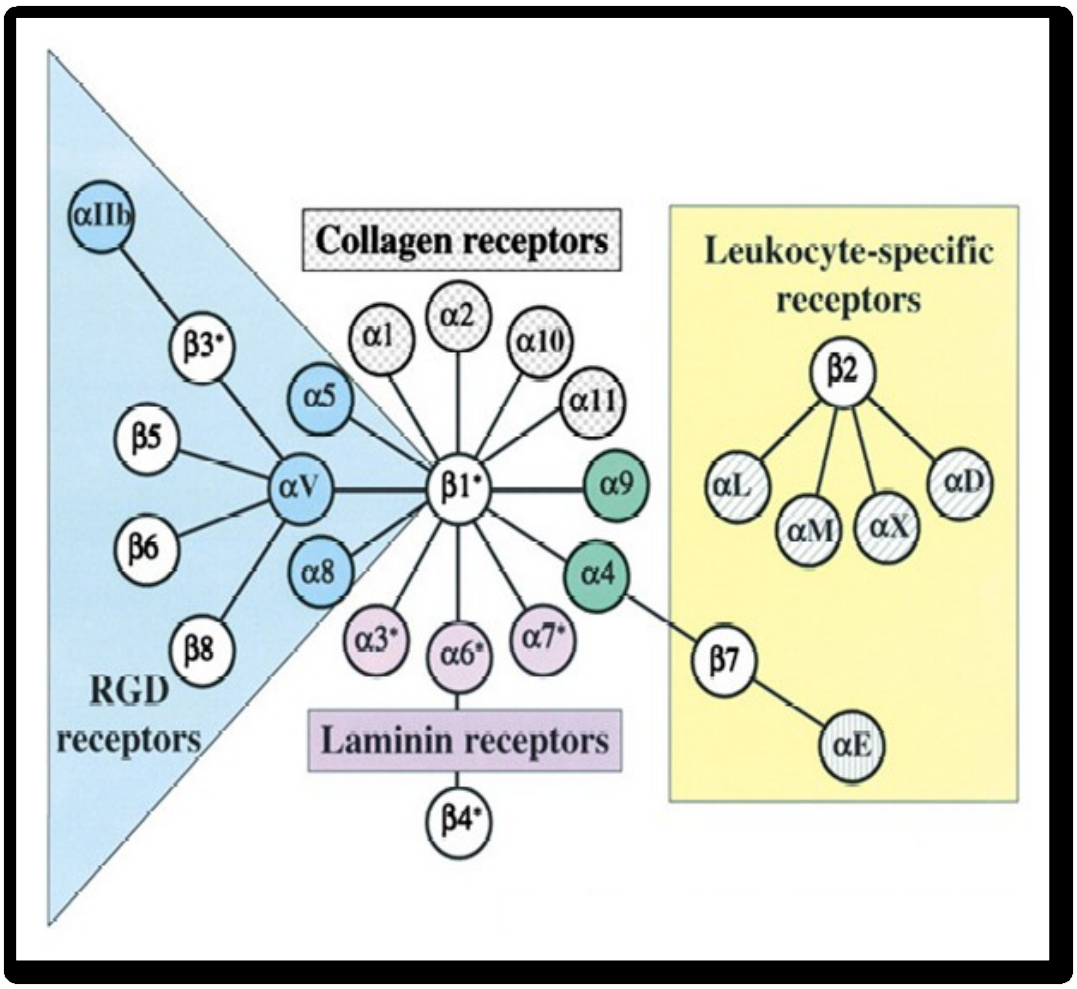


Figure 1-6: Integrin family. Grouping of the integrin subunits in cells acccording to their matrix affinity or cell specific expression (Hynes, 2002).

1.3.4 Effect of haemodynamics on adhesion

The multi-step adhesion processes described above are adapted for recruitment of different cells from the blood (Watts et al., 2013). Several of the steps are influenced by the blood flow and blood rheology described earlier. Adherence of platelets and leukocytes with the walls of blood vessels is controlled by three main factors i.e. margination of these cells allowing them to come close to the wall of vessel, and shear rate and shear stress at the vessel wall which affect the effectiveness and stability of adherence. This section considers how the initial steps of margination and initial attachment are influenced by the above factors and their link to cell and blood rheology: cell size and aggregation, haematocrit and blood viscosity. This will enable later discussion of the adhesion of stem cells.

1.3.4.1 Cell margination in blood - effects of cell size and shear rate

Margination refers to a state during which platelets or leukocytes are displaced radially towards the walls of blood vessels by the RBCs flowing in the centre. Variation is observed in the degree of margination in different regions of the circulation and between margination of platelets and leukocytes (Palmer, 1967). In suspensions flowing in tubes, particles tend to move away from the wall towards the centre. In blood, different cellular blood components move away from the vessel wall at varying rates because of differences in their relative sizes. In increasing order, the tendency of different blood components to move away from the wall towards the vessel centre was found to be platelets < single RBC < leukocytes < RBC rouleaux (or aggregates) (Palmer, 1967). Distribution of different blood components is controlled by the rate with which they move towards centre. For

instance, due to their smallest tendency, platelets are more likely to be marginated (present on the periphery) than others and rouleaux are mostly found in the centre (Aarts, 1985).

Margination also depends on shear rate because of its effects of RBC rouleaux which dissociate as shear rate increases. Leukocytes are only marginated at low shear rate in blood, and if red cell aggregation is abolished using washed RBC instead of whole blood, their margination is lost even at low shear rate (Goldsmith and Spain, 1984). Platelets are however smaller than individual red cells, and even without rouleux formation and at high shear rate, they are marginated (Watts et al., 2013).

The presence of red cells is essential for margination, but margination of leukocytes and platelets occurs over a wide range of haematocrits, and similar numbers are found adherent to the upper or lower surfaces of adhesive tubes (Abbitt and Nash, 2003, Watts, 2015). However, at very low haematocrit (10%) in horizontal tubes, sedimentation of red cell aggregates and leukocytes did occur and few leukocyte were seen or adhered at the upper surface, although platelets did adhere (Watts, 2015). This was presumably because sedimentation rate is strongly dependent on particle diameter. If suspensions of leukocytes or platelets alone are perfused without red cells, the leukocytes sediment towards the lower surface and can adhere there, but platelets do not adhere efficiently presumably because they sediment slowly (Watts, 2015).

Cells larger than leukocytes in the blood might fail to marginate even at low shear rate, if they are larger than rouleaux. At the same time, the high sedimentation rate of large cells could make them to settle away from the upper surface but towards

the lower surface, to give an unequal distribution even when red cells are present. This has never been tested to our knowledge.

1.3.4.2 Effects of shear rate and shear stress, and cell size on cell attachment from flow

The leukocyte adhesion cascade takes place mainly in post capillary venules, at low shear rates. Platelet adhesion can occur in all vessels including arteries with high shear rate. Apart from margination described above, these findings appear to arise from the effects of shear rate and of shear stress on cells of different size (Watts et al., 2013).

Shear rates at the wall of a vessel affect the velocity of a cell before it is adherent and the wall shear stress affects the force on a cell if it adheres (see Figure 1-7). The velocity of the cells is proportional to its radius and the wall shear rate, while the force on an adherent cell is proportional to its radius squared and the wall shear stress (Goldsmith and Spain, 1984). The higher the velocity the lower the likelihood of the cell formaing an adhesive receptor-ligand bond, while the higher the force, the less likely that the bond will remain (Chen and Springer, 2001). Thus one can expect decreasing formation and survival of adhesive bonds in high shear regions of the circulation, and for any region of the circulation, smaller cells will travel slower and experience less force.

In blood flow, wall shear rate and stress vary greatly. In particular,across the circulation shear rates lie in the range of around 100-5000s^{-1} and shear stresses lie in the range of approximately 0.2-10Pa (Tangelder et al., 1988, Lipowsky, 1988). Moreover, leukocyte adhesion can be seen only in regions where shear rates are low, mostly located in post capillary venules. Conversely, platelet adhesion is mostly seen

in areas where there are high shear rates and stresses i.e. on the arterial side of circulation. This variation between the adhesion demonstrated by platelets and leukocytes can be linked to the difference in their sizes (Watts et al., 2013). Due to their small size, platelets move along the wall of vessel with near-wall flow velocity ~four times lower in comparison with leukocytes. Additionally, the force exerted on the adhesive bonds is also lower by a factor of 16 due to the small size of platelets. Hence, it can be stated that although the adhesion receptors of platelets demonstrate similar intrinsic kinetics, they are able to adhere with walls of vessels over a broad range of shear rates and stresses (Doggett et al., 2002). If a cell were twice the diameter of a leukocyte, it would be expected to travel twice as fast and experience four times more force for a given shear rate and stress.

Researchers have carried out both in vitro and in vivo studies to study the influence of shear rate on adhesion. Isolated leukocyte suspensions have been used to study the influence of shear rate on leukocyte adhesion in vitro. Two comparable studies have been conducted to analyse adhesion behaviour of leukocytes on human umbilical vein endothelial cells (HUVEC) which were activated by cytokine- and subjected to different shear rates. A parallel plate flow chamber was used to develop shear rates (Lawrence et al., 1987, Lawrence and Springer, 1991). It was demonstrated by these two studies that the number of adhering cells reduced by a factor of 30 with the increment in shear rate from 280 to 430s^{-1}. Abbitt and Nash studied the influence of shear rate on leukocyte adhesion in whole blood using a rectangular glass capillary coated with P-selectin as the surface for adhesion (Abbitt and Nash, 2003). It was found that there was a reduction in the number of adhering leukocytes with the increase in shear rate and few cells demonstrated adhesion when

the shear rate was above $280s^{-1}$. Increased adhesion of rolling leukocytes with reduction in shear rates in mesenteric venules (diameter 25-40μm) of a cat has been demonstrated in vivo (Bienvenu and Granger, 1993, Perry and Granger, 1991). These researchers used an adjustable screw clamp on the arterial side of the circulation to alter the wall shear rate. It was also demonstrated that decrease in shear rate reduced the rolling velocity of the adherent leukocytes (Perry and Granger, 1991).

Adhesion of leukocytes and platelets in blood was compared by Watts et al. (Watts et al., 2013), over a range of shear rates, for capillaries coated with P-selectin or collagen. As expected, they found that platelets adhered up to higher shear rates than leukocytes. Also, changes in red cell aggregation had opposite effects. Aggregation increased leukocyte margination and adhesion, but decreased platelet adhesion. The differences between leukocytes and platelets were attributed to differences in their size affecting velocity and force applied to cells at the wall, and also the effects of the cell-free layer that develops at the wall at low shear when aggregation is present.

In summary, studies on flowing leukocytes and platelets have described multi-step adhesion processes, identified key receptors for adhesion, and shown the importance of flow parameters and blood rheology. Comparable studies have been fewer for stem cells, and especially mesenchymal stem cells.

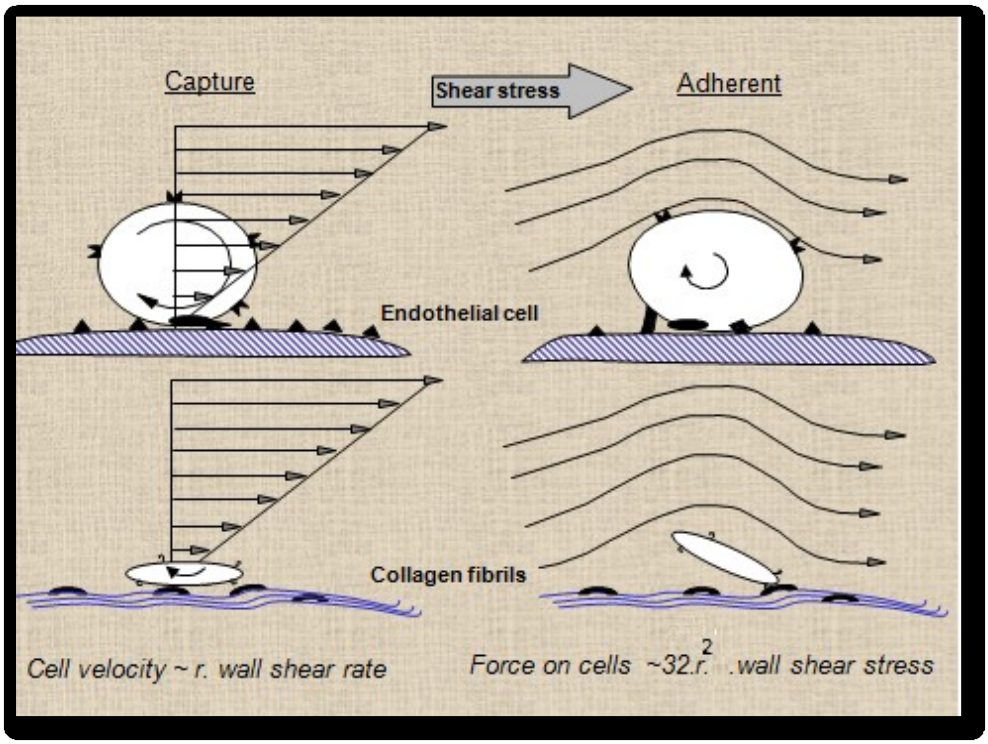


Figure 1-7: Schematic of effects of wall shear rate and shear stress on a platelet or leukocyte adhering to a vessel wall.

(Adapted from Lecture Notes of Gerard Nash, University of Birmingham; with permission).

1.4 Characteristics of EPC

1.4.1 Isolation, culture and identification of human EPC

Two methods have been used for isolating and culturing EPC from human blood, which result in early EPC (eEPC) and outgrowth endothelial cells (OEC). In the first approach, mononuclear cells (MNC) from human peripheral blood or cord blood are collected and plated in dishes that are coated with fibronectin in a cell culture medium that contains fetal calf serum along with various endothelial growth factors (VEGF ,hEGF, R3-IGF-1, hFGF-B) (Ito, 1999, Hill and Goldspink, 2003). After 48 hours, the non-adhered cells are collected and subsequently cultured on another fibronectin-coated surface with the same medium, generating round cells in colonies after 5 days. The cells isolated by this method are identified as early outgrowth EPC or colony-forming unit-EC (CFU-EC) (Yoder et al., 2007). This method is simple but does not encourage the appearance of an EPC that can generate mature EC. A number of proteins classically reserved for endothelial cells (von Willebrand factor, endothelial nitric oxide synthase, CD31, CD144, and vascular endothelial growth factor 2 receptor [KDR]) (Hassan et al., 1986, Schmeisser et al., 2003) are expressed by monocytes which are derived from peripheral blood MNC when cultured on plates with fibronectin in media that contain endothelial growth factors. However, this method cannot be recommended for isolation of EPC since the resulting cells express CD45 suggesting they are of haematopoeitic lineage (Fadini et al., 2012).

In the second method, isolated MNC are seeded onto plates coated with collagen I. The non-adherent cell population is removed within the first 24 hours in order to remove contaminating cells of leukocyte lineage, and the adherent

population is cultured. After about two to three weeks, colonies arise from these cells and are recognized as endothelial colony-forming cells (ECFC) or late outgrowth EPC (Yoder et al., 2007). These cells express CD31, VE-cadherein, and vWF in the absence of CD14 or CD45, and are able to bind and internalize DiI-acetylated-LDL. They thus express EC-markers and absence of monocyte features (Yoder et al., 2007). Late outgrowth EPC were also found to have similar characteristics to cultured arterial endothelial cells (Brown et al., 2009). Human EPC used in the current study were derived by the second method.

1.4.2 EPC Mobilization and Recruitment

When the body suffers from hypoxia and trauma, EPC mobilizing factors are produced such as colony stimulating factor, VEGF and basic fibroblast growth factor. In the bone marrow this leads to increased enzymatic activity of matrix metalloproteinases (Francisco and Dias., 2012). Activation of matrix metalloproteinase-9 promotes the transformation of membrane-bound Kit ligand to a soluble Kit ligand, leading to diversion of the hemangioblast to either hematopoietic precursor cells or EPC (Urbich and Dimmeler, 2004) . During ischemic injuries such as a heart attack, EPC are mobilised and play a role in neovascularization and repair of endothelium by their capability to incorporate incorporate in new blood vessels and/or to produce proangiogenic factors (Cao, 2010). It is proposed that the signal leading to EPC egress from bone marrow comes from a peripheral blood signal arising from chemoattractants from the site of injury (Hutter et al., 2004). In animal studies, increased circulating EPC number following ischemic limb or endothelium damage was also associated with the high levels of endogenous VEGF (Szmitko et al., 2003).

EPC need specific molecules to adhere and migrate from the blood. Adhesion molecules including P-selectin glycoprotein ligand (PSGL)-1 and β1-integrins on EPC may bind to endothelial receptors to allow capture and then firm adhesion (Langer et al., 2006). When EPC interact with tissue-specific chemokines, they become activated and start integrin-mediated adhesion to endothelial vascular cells and finally transendothelial migration into sites of vascular and tissue remodeling (Francisco and Dias., 2012). Usually, the concentration of chemokines is greater in regions of tissues undergoing active remodeling (Francisco and Dias., 2012).

A number of studies suggest that platelets play a role in the recruitment and differentiation of EPC. (Langer et al., 2006) found that platelets could affect both EPC adhesion and chemotaxis. In mice, surface-bound platelets used PSGL-1 and VLA-4 to capture EPC under high-shear flow conditions and promoted their differentiation, judged by development of endothelial markers vWF and Weibel Palade bodies. Later studies identified stromal cell-derived factor 1α as a platelet-derived chemokine promoting capture and differentiation of EPC in damaged arteries (Massberg et al., 2006, Stellos et al., 2008). Human platelets also captured EPC from flow in vitro, and when P-selectin or P-selectin glycoprotein ligand-I (PSGL-I) was blocked, the platelets-EPC interaction was inhibited, but not when glycoproteins Ib-IX-V or IIb/IIIa was blocke (Lev et al., 2006).

1.4.3 Differentiation of EPC

EPC contribution to new vessel formation and remodeling, depends on three processes: differentiation into mature EC, direct incorporation into neovessels, and/or production of paracrine and/or juxtacrine signals which encourage

interactions with pre-existing EC (Francisco and Dias., 2012). EPC differentiation into EC can be subdivided into three steps: direct interaction between integrin α5β1 and fibronectin is essential in the initial steps of EPC differentiation (Urbich and Dimmeler, 2004); to regulate EPC proliferation and survival, growth factors including the VEGF family are required; in order for differentiated EPC to function as mature EC, they must acquire the specific phenotype which depends on the regulation of the transcription factor HoxA, which regulates the expression of endothelial nitric oxide synthase, VEGFR-2 and vascular endothelial cadherin (Urbich and Dimmeler, 2004). Currently, there is no exact characterization of when EPC turn into a mature EC in vivo, although the loss of CD133 followed by von Willebrand factor expression has been defined to the mark the formation of mature EC (Hristov et al., 2003).

1.4.4 EPC function

Bone-marrow derived endothelial cells are believed to be involved primarily in angiogensis, by direct generation of cells incorporated in the vessel wall and by releasing pro-angiogenic factors. The release of the angiogenic factor VEGF by EPC was first document by Rehman et al. (Rehman et al., 2003). Pro-inflammatory cytokines of varying profiles are also secreted by early growth EPC as well as late growth EPC (or ECFC), and inhibition of such release is reported to be caused by statin treatment (Zhang et al., 2009). The role of mononuclear cells in neo-angiogenesis induction and localization was investigated in-vivo by Anghelina et al. (Anghelina et al., 2006). These cells took part through released angiogenic factors, and also produced enzymes which were responsible for matrix degradation. According to Krenning et al. (Krenning et al., 2009), EPC could act in a similar

way. While formation of new blood vessels occurs when existing endothelial cells start sprouting and replicating, blood derived EPC were considered as facilitators and stimulant of this process. Bone-marrow derived cells were also found to be involved in angiogenic repair processes and they may be the source of CD34 positive and CD41 negative cells which circulate in the bloodstream. The role of reconstituted genetically marked bone marrow was supported by four different studies on animals: on collateral vessel formation in response to ischemia, on VEGF-stimulated angiogenesis and on tumour-induced angiogenesis. Presence of bone-marrow derived cells as perivascular cells was documented in all of them (Tibor Ziegelhoeffer, 2004, Rajantie et al., 2004, Zacchigna et al., 2008, Wickersheim A, 2009).

1.5 Characteristics of MSC

1.5.1 Isolation, characterisation and differentiation of MSC from different tissues

Isolation of MSC most commonly uses aspiration of bone marrow which is a relatively simple approach that can be applied in experimental models including mice. For initial MSC isolation, researchers have typically relied on the plastic adherence method, although minor modifications in the basic technique can lead to substantial differences in the phenotype and behaviour of the MSC obtained (Dominici et al., 2006).The minimum criteria to be fulfilled by a cell to be called as a human MSC (hMSC) was stated in a position paper published by the International Society for Cellular Therapy (ISCT) in 2006 (Dominici et al., 2006). As per this statement, cells need to satisfy three conditions to be recognized as hMSC. These are: (a) expression of certain antigens on the cell surface, (b) demonstration of plastic

adherence and (c) ability to demonstrate tri-lineage differentiation i.e. osteogenic, chrondrogenic and adipogenic. The antigenic markers which are present on the surface of hMSC include CD73, CD90 and CD105. On the other hand, the markers CD11b, CD14, CD19, CD45, CD79α and human leukocyte antigen-D related (HLA-DR) must not be expressed by cells to be characterized as hMSC. However, a cell is not identified as an hMSC only on the basis of expression of these markers; fulfilment of the other two conditions is also necessary as these conditions prove to be the most suitable for distinguishing hMSC from other extracted cells (Dominici et al., 2006).

Thus, MSC must be capable of differentiating into different cell lineages including adipogenic, chondrogenic and osteogenic cells (Dominici et al., 2006). It has been reported that human MSC differentiate into osteogenic lineages when they are incubated in growth medium containing fetal bovine serum (FBS), ascorbic acid, dexamethasone and β-glycerophosphate which amplify activity of alkaline phosphatase and deposition of calcium (Jaiswal et al., 1997, Pittenger et al., 1999). Alternatively, they differentiate into chondrongenic lineage when they are grown at high cell-density, exposed to transforming growth factor TGF-β in serum free medium. This treatment causes generation of greatly sulphated, cartilage-specific proteoglycans and type II collagen. Finally, when MSC grown in FBS containing medium supplemented with indomethacin, isobutyl methyl xanthine, insulin and dexamethasone differentiated into adipogenic cells as indicated by the appearance of lipid vacuoles with red O staining. However, all clonal populations are not capable of differentiating into these three lineages since certain MSC clones have been found to be unable to differentiate into at least one particular lineage (Pittenger et al.,

1999).

Although researchers first discovered MSC in bone marrow, these cells were later identified in connective, adipose and muscle tissue of adult human beings (Friedenstein et al., 1974, Nathanson, 1985). Studies were carried out to find alternative sources of MSC since there occurs reduction in number and differentiation potential of MSC with age (D'Ippolito et al., 1999). Human umbilical cord blood (UCB) and veins, placenta, and amniotic fluid have been shown to contain MSC (Anker et al., 2004) (Panepucci et al., 2004). Besides these, numerous fetal tissues like spleen, lung, blood, liver and bone marrow are also sources of MSC (Campagnoli et al., 2001, Anker et al., 2003). MSC have also been obtained from synovium as a population of adherent cells which demonstrated ability to differentiate into adipocytes, osteocytes and chondrocytes (De Bari et al., 2001). Those cells were able to make a contribution in regeneration of skeletal muscle in a nude mouse model (De Bari et al., 2003).

Research in this thesis is based on comparison between MSC from three different sources i.e. MSC obtained from Wharton's Jelly, MSC obtained from trabecular bone and MSC obtained from bone marrow.

1.5.1.1 MSC and Bone Marrow

Friedenstein et al (Friedenstein et al., 1970) described a bone marrow-derived fibroblast-like cell for the first time which later became the most extensively studied MSC which are sometimes regarded as the 'gold standard. Bone marrow serves to be the main source of haematopoetic stem cells (HSC) but non-HSC are responsible for sustaining the microenvironment required by HSC for their development and differentiation (Prockop, 1997). This bone marrow microenvironment, which is

known as hematopoietic niche, is largely supported by MSC (Pittenger and Martin, 2004). Physiologically, migration of MSC from BM to periphery is rare, and there are no protocols to induce the translocation. Therefore, direct MSC isolation from BM is the most successful method for MSC preparation., and researchers have isolated MSC from several different species (Pittenger et al., 1999, Hatzistergos et al., 2010, Nardi NB, 2011). Isolation of MSCs from other cells in bone marrow aspirates involves three main steps: separation of non-nucleated RBC from nucleated cells using density gradient centrifugation; adherence of cells for culture onto plastic; utilization of trypsinization for separating monocytes from MSC. Procedures for isolating and culturing MSC from different species are usually more or less same. Culturing of plastic adherent MSC has proven to be the most extensively used procedure for their isolation.

While bone marrow has been accepted as the chief source of MSC (Pittenger et al., 1999, Haynesworth et al., 1992), utilization of bone marrow-derived MSC is not recommended in all cases because of increased level of viral exposure and considerable reduction in number of cells and their ability to differentiate and proliferate with donor age. Moreover, the method of obtaining a sample of bone marrow is invasive and painful. For that reason, alternative sources of MSC can prove to be beneficial for improved clinical efficacy and greater accessibility (Stenderup et al., 2003, Zhang H, 2005).

1.5.1.2 Trabecular Bone MSC

Trabecular bone can also be considered as a source of MSC as clinicians may use fragments obtained from femoral heads as bone grafts. These bone fragments have been shown to contain mesenchymal progenitor cells, and usually

taken from individuals subjected to elective surgery for orthopaedic problems (Sakaguchi et al., 2005, Tuli et al., 2003). MSC collected from trabecular bone from femoral heads that are fragmented mechanically demonstrate the characteristics which are required for therapeutic purposes i.e. ability to differentiate into multiple lineages (chondrogenic, osteogenic and adipogenic) and immuno-modulatory properties (see below).

Initially, MSC were obtained from trabecular bone by digesting it with collagenase (Tuli et al., 2003). These MSC were collected from individuals subjected to surgery because of an osteoporotic hip fracture or individuals suffering from osteoarthritis, which could have been changed inherently or due to the disorder and hence have different properties as compared to the cells taken from healthy iliac crest. Such a comparison has been done by Sanchez-Guijo et al. (Sanchez-Guijo et al., 2009). It was found that both sets of cells were similar in their multilineage differentiation potential, proliferation and immunophenotypes. However, the MSC obtained from trabecular bone demonstrated increased expression of immature marker CD90, shorter expansion time through different passages and a greater proportion of cycling cells. In another study washing MSC from trabecular bone gave a reduced differentiation capability compared to aspiration of MSC from the bone marrow in the same elderly patients (Sanchez-Guijo et al., 2009). Culture expansion of cells under adipogenic conditions, osteogenic or chondrogenic conditions, all showed impaired differentiation form the former cells. Greater capability for differentiation was demonstrated if MSC were cultured with 1 ng/mL fibroblast growth factor 2 (FGF-2), which was taken to imply that MSC obtained

from trabecular bone of elderly individuals are not recommended for use as cell therapies for regeneration and bone repair unless they are grown with FGF-2.

1.5.1.3 MSC from Umbilical Cords

One vein and two arteries are present in the umbilical cord. Mucoid connective tissue termed Wharton's jelly surrounds these vessels and contains a network of collagen fibrils and glycoprotein microfibrils (Frank et al., 1983). An outer epithelium derived from the surrounding amnion wraps the cord. Since 1988, the umbilical cord blood has been employed as a source of MSC (Gluckman et al., 1989). Large numbers of hematopoietic stem and progenitor cells are found in the blood that remains in the umbilical vein after birth, which have been used as an allogeneic source for the treatment of several different genetic, oncologic, immunologic, hematologic and pediatric diseases (Gluckman et al., 1989, Kim et al., 2002). Non-hematopoietic stem cells are also present, including MSC (Kogler et al., 2004, Greschat et al., 2008). However, Wharton's jelly is a source of numerous stromal cells which may further differentiate to give rise to adipocytes, chondrocytes and osteoblasts (Wu et al., 2009, Baksh et al., 2007). A distinct cell population of Wharton's jelly mesenchymal stromal cells showed stemness phenotype and may have been depositied during fetal migration (Wang et al., 2008). Alternatively, these MSC may indeed be primitive stem cells generated from mesenchyme, embedded within the matrix of the cord. These MSC may play roles during gestationt through the release of proteins such as mucopolysaccharides, extracellular matrix proteins and glycoproteins which form a gelatinous ground substance to prevent the cord vessels from strangulation (Bongso and Fong, 2013). MSC isolated from young donors tend to be more proliferative and immunosuppressive that those isolated from

adult donors and this may be attributed to the robust stemness and immune properties of fetal MSC (Kim et al., 2013).

Isolation of MSC can be achieved by explant culture of small pieces of cord or by enzymatic digestion (Salehinejad et al., 2012, Seshareddy et al., 2008). The latter method generated nearly three times more cells per 1g of cord compared to the former method (Yoon et al., 2013), although the time taken by cells to double their number was longer for WJMSC obtained through enzymatic digestion (Han et al., 2013). Additionally, MSC obtained from explants showed greater viability, perhaps because growth factors are discharged from tissue pieces in the culture (Yoon et al., 2013, Sobolewski et al., 2005). Among these growth factors, bFGF is of significance since it controls self-renewal and accelerates chondrogenic and osteogenic differentiation of MSCs if present in the culture media (Tsutsumi et al., 2001, Auletta et al., 2011).

1.5.1.4 Comparison of MSC from different sources

In comparison with bone marrow, the umbilical cord offers benefits for use as a source of human stem cells (HSCs). The umbilical cord is typically viewed as medical waste, and unlike bone marrow aspiration, it is collected through a painless, safe and simple technique after delivery. Additionally, cord collection does not involve technical and ethical issues and cord MSC are more primitive as compared to cells obtained from other sites and may thus be a preferable source of MSC for therapy (Wu et al., 2009, Lu et al., 2006, Wu et al., 2007). On the other hand, it has been claimed that the rate of successful isolation of MSC from cords is smaller (63%) than from bone marrow (100%) (Kern et al., 2006). Moreover, allogenic transplantation of cord cells does not require a perfect match of the human leukocyte

antigen type (HLA), since there are less chances of immunological reactions to be induced by these cells as compared to cells taken from BM (Park et al., 2007).

Large numbers of cells are usually needed in transplantation for clinical applications. Regrettably, the number of MSC that can be collected from BM is very small. Reports indicate that the yield generated from bone marrow is only 0.001-0.01% of mononuclear cells (Pittenger et al., 1999). On the other hand, yield obtained from 1g of adipose tissue was $5x10^3$ stem cells, about 500 times higher than for bone marrow (Fraser et al., 2006). According to another report, isolation efficiency demonstrated by Wharton's jelly was as high as $1\text{-}5x10^4$ cells/cm of cord (Weiss et al., 2006). Comparing MSC obtained from bone marrow, adipose tissue and Wharton's jelly, the last demonstrated greatest potential of proliferation (Amable et al., 2014). It has also been demonstrated that MSC obtained from different tissues exhibit variable potential for differentiation into different cells. For instance, MSC from adipose were better more capable of differentiating into adipocytes than those from skin (Al-Nbaheen et al., 2013). In addition, MSC collected from cords were more capable of differentiating into chondrocytes as compared to the MSC collected from bone marrow (Sakaguchi et al., 2005, Wang et al., 2009). In comparison with MSC obtained from adult tissues, those obtained from fetal tissues demonstrated greater capability of differentiation into endothelial cells and cardiomyocytes (Kim et al., 2013).

The above comparative studies are pre-clinical, in vitro investigations. Clinical trials to analyse efficacy and safety of the therapeutic agent must employ a particular kind of MSC, but tyopically it is not known which type of MSC is most suitable for a particular therapy. For that reason, research studies continue to

investigate different characteristics and potentials of MSC from different sources. For example, Hsieh and co-workers conducted a study to compare the ability of MSC collected from BM and WJ for regenerating infarcted myocardia (Hsieh et al., 2013). According to them, WJ-MSC were more neurogenic, neuroprotective and angiogenic because of differences in their secretome. In addition, a pre-clinical trial of myocardial infarction in rats was conducted by Naftali-Shani and colleagues during which they used human stromal cells collected from different origins, particularly right atrium, subcutaneous fat, pericardial fat and epicardial fat (Naftali-Shani et al., 2013). It was found that the greatest quantities of inflammatory and trophic cytokines were discharged in vitro by hMSC obtained from right atrium and pericardial fat. These hMSC also demonstrated increased levels of heart recovery in vivo. Such studies continue to be important for identifying the most suitable tissue-derived mesenchymal stromal cells for a specific regenerative treatment.

1.5.2. Functions of MSC

1.5.2.1 MSC for Tissue Regeneration

MSC can be of great significance for healing tissue damage owing to their distribution in a wide range of tissues, their differentiation potential and the reparative effects noticed when MSC are infused in pre-clinical and clinical models (Wei et al., 2013). It is widely accepted that there are roles for MSC in tissue growth, healing of wounds and maintenance of the cell supply to compensate for the cells lost due to apoptosis and pathology. Due to these roles, researchers and clinicians have used MSC for treating degenerative disorders and tissue injuries. Clinical indices of liver function have been shown to improve after infusion of autologous bone marrow mesenchymal stem cells in individuals who are suffering

from liver failure or liver cirrhosis due to hepatitis B (Chapel et al., 2003, Kharaziha et al., 2009). Bone marrow MSC are also capable of bringing about significant therapeutic outcomes in musculoskeletal system. Reports indicate their efficiency in healing of burn-induced skin defects, bone damage due to osteonecrosis, diabetic critical limb ischemia and periodontal tissue defects (Lu et al., 2011, Yamada et al., 2006, Rasulov et al., 2005). Pre-clinical studies indicate that hMSCs can be used for treatment of myocardial infarction and damaged cornea (Lee et al., 2009, Roddy et al., 2011). Utilization of MSC for treatment of damaged tissues such as spinal cord, brain (Zeng et al., 2011) and lung (Ortiz et al., 2007), involves similar activity . Moreover, the engraftment of haematopoietic stem cells can be complemented by co-transplantation of MSC (Chapel et al., 2003).

1.5.2.2 MSC for Immunomodulation

Prevention of graft versus host disease by MSC in transplanted patients indicated that MSC have the potential for immunomodulation, and it has been found that they are also capable of acting against innate immunity (Sohn and Gussoni, 2004, Fiorina et al., 2009). Accordingly, MSC are capable of reducing inflammatory responses, decreasing generation of ROS and thereby delaying apoptosis of activated neutrophils (Huang et al., 2009), and of causing suppression of NK cell responses (Zuk et al., 2002). Monocytes are also prevented from differentiating into dendritic cells by MSC as they amplify secretion of anti-inflammatory cytokines (IL-10) and decrease generation of pro-inflammatory cytokines like TNF- α (Tomar et al., 2010, Houlihan et al., 2012). It has also been shown that MSC influence the survival, proliferation and effector functions of T cells thereby modulating development of adaptive immune responses connected to chronic inflammatory disorders (Krampera

et al., 2013, Morikawa et al., 2009). MSC also demonstrate cell-to-cell contact leading to modulation of immune responses, through several different proteins are expressed on the surface of MSC. For instance, Jagged-1, the notch ligand present on hMSC plays a role in suppressing activation of T cells (Liotta et al., 2008).

In our laboratory, we showed MSC and endothelial cells communicate with each other via soluble mediators to up-regulate production of IL-6 by MSC (Luu et al., 2013). This caused a reduction in the response of the endothelial cells to inflammatory cytokines and recruitment of leukocytes. These studies suggest that MSC delivered in the blood and coming into contact with endothelial cells would enagage in 'cross-talk' that would be anti-inflammatory.

There are thus a number of reports which indicate therapeutic use of MSC in pre-clinical animal models of immune diseases. MSC have proven to be effective in inhibiting graft-versus-host disease in individuals subjected to bone marrow transplantation (Muller et al., 2008, Prasad et al., 2011). These are of great significance especially for those patient with steroid resistance (Kebriaei et al., 2009, Wu et al., 2011, LeBlanc et al., 2008) Moreover, MSC reduced inflammation in patient with Crohn's disease and systemic lupus erythmatosus (SLE) thereby decreasing damage to bowel and kidneys via inducing regulatory T cells (Sun et al., 2010, Carrion et al., 2010, Ciccocioppo et al., 2011). Due to their immediate immunomodulatory effects (Honmou et al., 2011), bone marrow MSC have been shown to be capable of improving amyotrophic lateral sclerosis, multiple sclerosis (Karussis et al., 2010, Connick et al., 2011, Choi et al., 2010, Honmou et al., 2011) and multiple system atrophy (Lee et al., 2008).

1.5.3 Therapeutic administration of MSC - local vs. systemic delivery

The site used for administration of MSCs for therapeutic purpose can influence the route taken by cells to reach the desired destination (Boltze et al., 2015). For therapy, MSCs can be administered through intracardiac (IC), intra-arterial (IA), intraperitoneal (IP) or intravenous (IV) injection. Although intravenous administration is least invasive, greater engraftment rates were demonstrated by IA and IC administration as compared to IV administration in models of myocardial infarction (Barbash et al., 2003, Freyman et al., 2006, Walczak et al., 2008). They administered radiolabelled cells in models with brain injury and found that IA injection in the extracranial right internal carotid artery (near target) led to greater homing of cells in the brain as compared to IV injection in the femoral vein. Walczak, et al, demonstrated that IA injection near the desired organ gave better results than IV injection at a distant point (Walczak et al., 2008). In cases of IV administration, MSC accumulated in filtering parts of the body such as the spleen, liver or lung, but this accumulation was reduced in cases of IA injection (Barbash et al., 2003, Kraitchman et al., 2005, Sackstein et al., 2008). However, there was a greater chance of microvascular occlusions with IA injection (Walczak et al., 2008), a condition known as passive entrapment. In cases of IA and IC administration, greater number of MSC were able to reach and engraft at an ischemic site as the cells bypassed the lungs.

IP administration of MSC is occasionally used. It was used it to administer MSC to foetuses in mice with muscular dystrophy as IV injection was considered to be inappropriate for this particular case (Chan et al., 2007). The donor cells were detected in muscular as well as non-muscular tissues. Finally, one can also use the

method of local delivery by injection of MSC directly into the target site. Beggs et al. administered Dil-labelled MSCs into baboons through IV injection but could not detect cells in limb muscles (Beggs et al., 2006). On the other hand, when they injected the cells directly into the muscle, DiO labelled MSCs could be detected there (Beggs et al., 2006). However, Muschler et al.reported that this method is not feasible in most clinical cases because it is too invasive, particularly in the brain or heart (Muschler et al., 2004). Moreover, locally injected cells may die prior to their role in healing because of limited supply of oxygen and nutrients.

Since intravascular infusion is the most common form of therapy, it is important to understand the mechanisms by which MSC might be delivered to the microcirculation, become adherent to the walls of blood vessels and subsequently migrate through them. It is also useful to consider whether endogenous MSC can circulate 'normally' in the blood.

1.5.4. MSC circulation and recruitment to tissue

1.5.4.1 Circulation of endogenous MSC

The ability of MSC to circulate in the blood under physiological steady-state condition is controversial since the available literature contains reports with quite contrasting results. There are some reports which indicate existence of MSC in blood; though only very low levels of circulating MSC have been mentioned in these reports (Kuznetsov et al., 2001). A number of other studies indicate absence of any circulating MSC (He et al., 2007). It is quite difficult to harvest adequate quantities of circulating MSC at steady-state conditions owing to the requirement of obtaining blood through venepuncture. This process, theoretically, might discharge small numbers of connective tissues cells or pericytes into the collected blood or into the

circulation. da Silva Meirelles et al. (Meirelles et al., 2006) failed several times in deriving a long-term culture of MSCs from blood accessed through the portal vein, which would have a lower chance of contamination of blood with pericytes and other connective tissue cells. Isolation of MSCs will be significantly influenced by the technique used to mobilize MSC in the peripheral blood, culturing methods and methods used to avoid and get rid of contamination. Heterogeneous expression of markers has been demonstrated by MSCs taken from peripheral blood. In particular, fibroblast-like stem cells isolated from blood of four different mammals proved to be adherent and demonstrated adipogenic and osteogenic potential (Kuznetsov et al., 2001). These isolated stem cells were different from hMSC isolated from bone marrow in the sense that they lacked endoglin and Stro-1 (Kuznetsov et al., 2001). MSCs have also been isolated from peripheral blood during a study carried out by Tondreau et al. (Tondreau et al., 2005). They used preselection methods for CD133+ cells in G-CSF-mobilized peripheral blood. According to Rochefort et al. (Rochefort et al., 2006), such stem cells were capable of differentiation into neuronal/glial cells, chondrocytes, osteoblasts and adipocytes.

Perhaps of greater interest here is that greater levels of MSCs were detected in peripheral blood cells isolated from injured mice (injury causing intimal hyperplasia) in comparison with the controls i.e. mice without injury (Wang et al., 2008). This finding is in accordance with the observation that peripheral blood levels of G-CSF and VEGF are also increased in the case of injury. MSC obtained from injured mice demonstrated greater potential of differentiation as compared to those obtained from healthy mice: when MSC from injured animals were cultured up to

ten passages, they demonstrated capability of trilineage differentiation in vitro (Karp, 2009).

1.5.4.2 Administration of MSC to the blood and subsequent fate

If exogenous MSC are injected into the blood, they may get arrested non-specifically in microvessels or get recruited inside the vasculature of the desired tissue and then transmigrate across the endothelium in a process termed MSC homing by analogy to the behaviour of leukocytes (Karp, 2009). However, in contrast to the well-established mechanisms of adhesion and migration which characterize leukocyte homing, a discrete mechanism for MSC homing is not well established. In practice, the available literature is deficient in data elucidating the final position of MSC after administration, so that it is difficult to determine whether the cells have been localized (captured inside vessels) or homed (subjected to targeted adhesion and transendothelial migration (Karp, 2009). Nevertheless, there are a number of studies evaluating the adhesion molecules expressed by MSC and the cells' adhesive and migratory capabilities, described below.

Non-specific localisation or capture in arterioles or capillaries may occur because of the size of the MSC which are larger than leukocytes (for human cells, diameter about 20μm vs <10μm) (Luu et al., 2013). Indeed, when rat MSC (diameter 23μm) were infused into rats iliac artery, >90% became trapped in the first pass through microvessels of the rat cremaester observed directly by intravital microscopy (Toma et al., 2009). This study also showed that they blocked 10μm pore filters in vitro at pressures when mononuclear leukocytes cells did not. In other animal models, MSC infused into systemic veins have been found to locate in large numbers in the lung (Fischer et al., 2009, Kang et al., 2012). Mechanically-trapped

cells might still adhere to endothelium and migrate through it, and so adhesive and migratory behaviour would still be important in this situation.

1.5.4.3 Adhesion molecules and mechanisms supporting MSC recruitment

If the homing concept is correct, tissues would need to recruit circulating MSC from the flow to ensure effective delivery to damaged sites. For this purpose, MSC have on their surface a number of different adhesion molecules shared by leukocytes. These adhesion molecules include CD24, CD29 (β1-integrin), CD44 and CD49a-f (α1-α6-integrin) (Chamberlain et al., 2007), although other studies found no CD24 (Ruester et al., 2006). Adhesion molecules which are found on endothelial cells are also expressed by MSC. These molecules include vascular cell adhesion molecule-one (VCAM-1), intercellular adhesion molecule-one (ICAM-1) and intercellular adhesion molecule- two (ICAM-2) (Majumdar et al., 2003).

It seems that the number and type of adhesion molecules found to be present on MSCs may be influenced by the source of MSCs and method used for their isolation and culture. For example, adhesion molecules expressed by hMSCs at passage 4 and passage 6 were found to be different (Aldridge et al., 2012). There was a linear relationship between passage number and the expression of CD49, but a decrease in the expression of CD44 was noted at passage 6. However, other reports indicated no difference between the molecules expressed by hMSCs at passages 3, 5 and 7 e.g. CD73, CD90, and CD105 (Lo Surdo and Bauer, 2012). In relation to the origin of hMSCs, it was found that adhesion molecules expressed by hMSCs isolated from bone marrow and those isolated from adipose tissue differed. Differences in expression were noted for cell adhesion molecules CD49d (Integrin alpha4), CD54 (ICAM-1), CD34, and CD106 (VCAM-1) with large variation in CD106 (VCAM-1)

and CD54 (ICAM-1) (De Ugarte et al., 2003). It is therefore likely that the source and methods of isolation and expansion must be taken into consideration while when evaluating adhesive properties of MSC adhesion. The comparison becomes even more complicated if it is made between cells isolated from different species (such as humans, rats and mice) as different species demonstrate different profiles of some adhesion molecules (Chamberlain et al., 2007). The substantial heterogeneity of MSC within an isolated population adds to the above-mentioned complications. The potential for differentiation, for example, varies between different cells of the same population (Pevsner-Fischer et al., 2011) While our knowledge about the mechanisms giving rise to this intrapopulation variation is still deficient, it has been established that intrapopulation variation exists in terms of expression of adhesion molecules. For instance, only 50% of the MSC population from hMSC was found to express CD49d (Aldridge et al., 2012).

Several mechanisms involving different adhesion molecules have been proposed for recruiting flowing MSC to the vasculature. During a study on hMSC recruitment to the vasculature in mice, Rüster and colleagues (Ruester et al., 2006) found that P-selectin and the α4β1-integrin/VCAM-1 played a major role in recruitment in venules. In comparison with the wild type controls, the P-selectin-/- mice demonstrated a lesser degree of MSC rolling in the ear venules. The function of other adhesion molecules was also investigated through in vitro studies that made use of endothelial cells as substrate for the adhesion. During a flow-based assay, the number of MSC demonstrating adherence decreased considerably when P-selectin was blocked on the TNFα-treated endothelial cells (Ruester et al., 2006) . However, it was found that MSCs neither expressed P-selectin glycoprotein ligand-1(PSGL-1;

CD162) nor the alternative P-selectin ligand–CD24 on their surface. This implied that MSC expressed an alternative P-selectin ligand (Ruester et al., 2006) . In the same study, adherence of MSC to the TNFα-treated endothelial cells was found to be reduced after blocking α4β1-integrin or VCAM-1 to a similar degree to each other, showing a role for this pathway (Ruester et al., 2006). It should be noted that in these studies, flow was reduced to a very low shear tress (-) to allow attachment followed by an increase in flow to 'washout'. In another study, low numbers of MSC adhered to cytokine-treated EC after prolonged perfusion at 0.1Pa, also through VCAM-1 (Segers et al., 2006).

In a recent study from our laboratory (Luu et al., 2013) MSC were also perfused over EC treated with TNF. It was found that MSC adhesion was negligible at a wall shear stress of 0.05 Pa, which resembles the low end of venular shear. If the flow was decrease to 0.01 Pa to allow attachment, then washed out at 0.05 Pa, adhesion could be detected on stimulated, but not unstimulated EC (Luu et al., 2013). MSC adhered in large numbers if allowed to remain stationary in contact with EC for 30min before washout at 0.05Pa. Chamberlain et al. (Chamberlain et al., 2011) also found little adhesion of perfused MSC to endothelial cells unless flow was stopped and the cells allowed to settle before washing out. These data suggest that attachment of flowing MSC in intact vessels would be rare under normal circulatory conditions, but that MSC could adhere to endothelium only if already arrested or trapped (Chamberlain et al., 2011).

A wide range of different cells express the glycoprotein CD44 on their surface, which can act as a ligand to allow adhesion via several other molecules including hyaluronan (Aziz et al., 2000). Its role as a ligand for P-selectin has also

been reported (Alves et al., 2008), and it may be the ligand for P-selectin expressed by MSC. Studies indicate that hematopoietic cell E-/L-selectin ligand (HCELL) is capable of binding with E-selectin (Dimitroff et al., 2000, Burdick et al., 2006). HCELL is a specific glycoform of CD44 which is considered to be one of the most powerful ligands for E-selectin (Dimitroff et al., 2000) (Burdick et al., 2006). It has been reported that trafficking of human MSC to murine bone marrow is mediated by HCELL (Avigdor et al., 2004). While MSC express CD44 molecules heavily on their surface, it was found that MSC adhesion was not decreased by blocking E-selectin on endothelial cells (Ruester et al., 2006). However, other researchers have found CD44 on hMSC to interact with E-selectin (Thankamony and Sackstein, 2011).

Herrera and colleagues (Herrera et al., 2007) demonstrated that mMSC were recruited to the renal microcirculation of mice after acute renal failure (ARF), in a process where CD44 was required. They isolated mMSC from CD44−/− or CD44+/+ animals and then administered into mice suffering from ARF. CD44+/+ mMSC were detected in the ARF animal's renal circulation. On the other hand, the renal vessels of mice without ARF did not contain CD44+/+mMSC. Likewise, when the researchers administered ARF animals with mMSC taken from CD44−/−animals, the administered cells were not detected in the renal microcirculation, implying that recruitment of mMSC required CD44 expression (Herrera et al., 2007). The recruitment was specific for CD44 expressed by MSC and when CD44 activity was inhibited through antibodies or treatment of the cells with hyaluronic acid, it also resulted in decreased renal localization of MSC (Herrera et al., 2007).CD44 was probably not the only receptor required for recruiting MSC into the renal

environment, because even in the case of blocked CD44, damaged tissues demonstrate a greater level of MSC recruitment compared to the tissues with no damage (Herrera et al., 2007). The molecular mechanisms involved in the mouse MSC recruitment to the heart were investigated in animals suffering myocardial infarction (Ip et al., 2007). Upregulation of several genes was recorded in the heart after infarct and these included the genes for VCAM-1, ICAM-1 and E-selectin. Recruitment of mMSC in the infracted myocardium decreased considerably when mMSC were treated with antibody against β1-integrin. Blockade of α4β1-integrin (CD49d/CD29) did not affect recruitment, and the particular α-integrin subunit working in this process was not identified, although the presence of α9-, α6 and α8-integrins were demonstrated (Ip et al., 2007).

Given their inefficient adhesion from flow, surface modification of MSC has been used to try to improve homing. Various techniques have been used to modify MSCs membrane, including delivery of sialyl Lewis X (SLeX), a mediator of leukocyte rolling. hMSCs were fused with biotinylated lipid vesicles which enabled streptavidin-linked SLeX to bind. The authors noted an increase in MSCs rolling on P-selectin at a shear stress of 0.05 Pa (Sarkar et al., 2010). Similarly, biotinyl-N-hydroxy-succimide was fused to hMSCs and successfully bound to free amine groups. The authors noted again that this enabled streptavidin-linked SLeX to be bound to the surface which again led to an increase in MSCs rolling on P-selectin (Sarkar et al., 2008). This technique was later used in vivo to improve hMSCrecruitment to inflamed mouse ear (Sarkar et al., 2011). Directing MSC homing by using enzymatic modification techniques has been attempted. For example, the surface receptor CD44 was modified by enzymatic treatment to bind to

E-selectin. This conversion enhanced hMSC bone marrow homing in vivo (Sackstein et al., 2008).

Another technique of surface receptor modification is genetic manipulation where the adhesion and homing receptors can be overexpressed. For instance, genetic modification of CD44 to form the variant HCELL noted above, was found to increase adhesion from flow and transmigration of MSC through endothelial cells (Thankamony and Sackstein, 2011). Overexpression of CCR1 (chemokine (C-C motif) receptor 1) increased chemotaxis by MSC when stimulated by CCL5 (chemokine (C-X-C motif) ligand 5) (Huang et al., 2010). In addition, overexpression of CCR1 enhanced engraftment after intra-myocardial injection in ischemic mouse heart studies. The overexpression of CXCR4 improved MSC homing in myocardial infarction rat model (Bang et al., 2012). On the other hand, overexpression of CXCR4 and CXCR7 did not enhance MSC homing in a mouse renal injury model (Gheisari et al., 2012). These findings suggest that different techniques may be required to enhance MSCs homing depending on the target tissue (Cheng et al., 2008). While genetic modification has the potential to be a tool to promote MSC homing, unpredicted negative effects on cellular function may be problematic. For example, overexpression of survival genes (such as Akt) can lead to risk of tumourgenesis (Phillips and Tang, 2008).

Even though, collagen and fibronectin are considered one of the major proteins in the Extra Cellular Matrix (ECM), their role in human MSC integrin receptors binding is not fully addressed.

Studies of MSC adhesion to collagen and fibronectin are rare. There is only limted study which has addressed the binding between BMMSC and collagen. Study

conducted by Lan CW et al who studied the adhesive behaviour of osteoprogenitor cells isolated from bone marrow (BMSCs). They showed that these cell adhere twice more to the surface when the surface is coated with collagen under flow condition, Also, They noticed when they flush all marrow cells by shear stress of 1.10 dyne/cm, 25% of cells which are adhered to collage coated surface remained attached to the surface despite the flush force which indicate the strength of the adhesion (Lan et al., 2003).

Regarding collagen, a study was conducted to investigate the adhesive behaviour of BMMSC by using Collagen nanofibers scaffold. The authors reported that over 45% of BMMSc adhered efficiently to collagen which coated with nanofibers (Chan et al., 2009). additionally, studies on Murine bone marrow cells (BMC) revelled again higher MSC adhesion to collage coated surfaces (Vandersluijs et al., 1994).

In case of on fibronectin protein, Ogura N et al. (2004), found that fibronectin stimulated adhesion, spreading and growth of human BMMSC (Ogura N, 2004). In addition, Veevers et al. (2011) found a receptor, α5β1-integrin, which support cross talk between growth factor receptor and integrin receptor signals on fibronectin (Veevers-Lowe et al., 2011). This interaction between cell and fibronectin led to a chain of actions which caused greater phosphorylation of PDGFR-β and subsequently promoted the adhesion and migration of the human BMMSC. They noted that collagen types I or IV had little effect on PDGFR-β activity compared to fibronectin.

1.5.4.4 The role of platelets in recruitment of MSC

Platelets have been reported to be involved in recruitment of MSC in both in vitro and in vivo models. In a flow-based adhesion assay, Langer and co-workers (Langer et al., 2009) noticed an increase in the recruitment of hMSC to human arterial endothelial cells when the EC were pre-incubated with platelets. In particular, pre-incubation with platelets caused greater hMSC adhesion in comparison with the activation of EC with IL-1β. In vivo studies generated results which were in accordance with these findings. hMSC adhesion was found to be decreased considerably in a murine model with carotid artery injury after treatment with anti-GPIb and platelet-depleting antibody. It was also demonstrated that αvβ3-integrin blockade decreased the adhesion of platelets to immobilized hMSC (Langer et al., 2009). In a rat model of pulmonary arterial hypertension, infused rat MSC protected again a rise in right-sided blood pressure and cardiac hypertrophy (Jiang L et al., 2012). MSC were found in the lung, and their adhesion there was reduced by blockage of P-selectin and of GpIIbIIIa. The same receptors were found to support attachment of MSC along with platelets to collagen in an in vitro flow assay. It was concluded that platelets mediated MSC homing to the lung. In a recent study, there was preferential trafficking of infused MSC to an inflamed vs control ear, but this was decreased if platelets were depleted from the blood (Teo et al., 2015). Direct observation of microvessels showed MSC adherent along with platelets and neutrophils. The above studies strongly suggest that MSC will interact with platelets in blood and that this interaction will modify their behaviour in vivo.

1.5.4.5 Transmigration of MSC

Transmigration of MSC through the endothelium has not been investigated widely. In a flow assay, adherent murine MSC spread and appeared to migrate across murine aortic endothelial cells over 1-2 hours (Chamberlain et al., 2011). In that study, the MSC could also transmigrate across an endothelial monolayer on a porous filter over a 16 hour period. In a static assay, (Steingen et al., 2008), transmigration of hMSC occurred via non-activated endothelial monolayer through interaction between α4β1-integrin and VCAM-1. Over about 60 minutes MSC embedded in the endothelial monolayer, and after 240 minutes, the endothelial monolayer released the integrated MSC allowing them under the monolayer. Teo et al (Teo et al., 2012) observed transmigration of a proportion of adherent MSC through endothelial monolayer in about 1 hour, with adhesion and transmigration increased if the endothelial cells had been treated with TNF. In contrast , the time for transmigration of leukocytes is 5-20 minutes (Ley et al., 2007). The difference from the transmigratory pattern of leukocytes might be linked to the utilization of non-activated endothelium or the lack of transmigratory potential in the MSC. For instance, considerably decreased transmigratory activity is demonstrated by lymphocytes on non-activated endothelium because relevant adhesion molecules are not present on the surface. However, the available literature indicates that transmigration of MSC is regulated by MSC-endothelium interactions specific to these cell types, and that this process needs to be investigated in detail under conditions which mimics the active inflammatory scenario (Teo et al., 2015).

Migration across filters has also been studied without an endothelial layer. In studies of migration across 8μm pore filters coated with Matrigel, MSC from cord blood migrated slightly more efficiently than those from bone marrow in response to chemokine CXCL12 or hepatocyte growth factor (Son et al., 2006). It was also noted that the number of MSC migrating decreased with increasing passage number. Others found that coating the under-side of an 8μm pore filter with fibronectin promoted migration of human bone marrow MSC (Veevers-Lowe et al., 2011). Migration was further increased by addition of platelet-derived growth factor, and migration was inhibited by blockade of α5β1-integrin.

1.5.4.6 Outstanding questions in MSC recruitment

The studies of EPC, MSC, of leukocyte and platelet adhesion and of blood rheology described above raise various questions related to adhesion of flowing progenitors and the behaviour of MSC if infused into blood.

The leukocyte and platelet adhesion cascades have been well documented, and some reports suggest EPC and MSC follow a similar multi-step process to leukocytes. However, this literature is not extensive or all in agreement in relation to MSC in particular. One of the shortcomings of the literature is the lack of studies which cover the effects of haemodynamic and blood rheological factors on MSC adhesion. It is not clear whether MSC will marginate in blood, over what range of shear rates they can adhere, and how important their large size is compared to leukocytes and platelets. There is doubt whether they can adhere to endothelial cell receptors from flow and little information on whether they can bind to the matrix that may be exposed in a damaged vessels, as platelets do. On such surfaces, it is not known whether they can go through steps of rolling, stopping, spreading and

migrating. Most studies of MSC adhesion, spreading and migration have used static rather than flow assays.

Recent studies suggest MSC may interact with platelets as EPC were reported to earlier, but how they interact in flowing blood is unclear, as are the receptors that may support their interaction. This makes it difficult to predict how MSC might behave if injected into the blood for therapy. In addition, most studies in this area have only used MSC from bone marrow, and is not known how adhesion and recruitment and interaction with blood might vary between cells from different tissues, as other behaviours do.

The above topics need further investigation, comparing MSC from different sources, in dynamic models and including presence of blood. Clarification is also needed as to the receptors MSC may use to adhere to surfaces and to blood cells such as platelets.

1.6 Hypothesis and aims

The hypotheses in this thesis are that the adhesive and migratory behaviour of MSC varies between cells from different tissues, and that their adhesion from flow will be affected by presence of blood and interaction with cells in it. Thus, we hypothesise that different MSC may be vary in how they will behave if injected into the circulation, and these differences may affect their use as a therapy.

Therefore, the main aims of this thesis were:

- To compare the adhesion of EPC to MSC from different sources to different surfaces (such as matrix proteins and endothelial receptors) under different flow conditions in vitro.
- To evaluate the ability of different MSC to spread on different surfaces, and migrate .
- To compare adhesion in blood to adhesion of isolated cells and evaluate interaction with platelets.
- To evaluate receptors supporting cell adhesion in the different circumstances.

Our initial intention to compare EPC and MSC was not extended beyond the studies of dynamic adhesion because of the need to focus on a specific cells and the relative lack of information on MSC behaviour in vivo (where EPC naturally circulate but MSC do not).

The overall aims were to provide better understanding of the impact of the physical factors on the adhesion behaviour of different types of MSC. This understanding will give insights into the behaviour of MSC in the circulation which may be beneficial to the design of their therapeutic uses.

Chapter 2 : METHODS

The study was carried out after ethical approval from the Science, Technology, Engineering and Mathematical Ethical Review Committee of the University of Birmingham.

2.1 Cell derivation and culture

2.1.1 Basic cell culture

Endothelial progenitor cells (murine cells line or primary cells from umbilical cord blood) and mesenchymal stem cells (from bone marrow, trabecular bone or umbilical cord Wharton's jelly) were used in this study. Their sources are detailed below and culture media listed in Table 2.2. All cell cultures were performed under sterile conditions, using a Class II microbiology safety cabinet. Culture media were filtered by Minisart 0.2 um single-use filter units (Sartorius Stedim Biotech, UK) before use. Reagents to be used in the culture were pre-heated to 37 ^{O}C using a heating box (Thermo Scientific, Town, County/State) for minimum of 30 minutes. Cells were retrieved from cryo-preserved aliquots (see below), thawed rapidly, and grown to approximately 80% confluence in tissue culture flasks (25cm² or 75 cm²; Cellstar, Dorset, UK) maintained at 5% $C0_2$ and 37 ^{O}C in a Heraeus incubator (Therm Fisher Scientific, Leicestershire, UK). A 25cm² culture flask (T-25) was used initially for growing cells from cryopreserved stock, and 75 cm² flasks (T-75) were used for expanding cultures.

2.1.2 Subculture and cryopreservation of cells

To detach cells, they were first washed using 3ml ethlenediaminetetraacetic acid solution (EDTA) and then 0.25% trypsin-EDTA solution was added for 4 minutes (both from Sigma-Aldrich Co., UK). The detached cells were diluted and

washed once using culture medium in a 15ml conical tube centrifuged at 400g for 5 minutes. The supernatant was removed and 3ml of fresh medium was added for cell suspension. The cells were either placed in a single T-75 (if taken from a T-25 flask) or divided between 3 different T-75 flasks (if taken from a T-75 flask) and then incubated at 37 ^{O}C 5% C02 until they reached 80% confluence. They were then either passaged again, cryopreserved or used in an assay.

For cryopreservation cells were dettached and washed as above, and re-suspended in ice-cold Cryo-SFM freezing medium (PromoCell GmbH, Germany; 1ml per T-25 flask). This suspension was transferred to an ice-cold cryovial (1ml/vial), placed at -80^{0}C overnight, and then transferred to liquid nitrogen storage.

2.1.3 Origin and derivation of endothelial progenitor cells (EPC)

2.1.3a Murine EPC line

Murine foetal lung mesenchyme-derived mEPC (MFLM-4) were obtained from Seven Hill Bioreagents (Ohio,USA). mEPC were culture in Dulbecco's Modified Eagle Media (DMEM) with supplements (see Table 2.2). They were observed daily by microscope and the culture medium changed every 48h until passage or use.

2.1.3b Primary EPC from human umbilical cord blood

Human hEPC were isolated from umbilical cord blood as described by Ingram et al. (2004). Umbilical cord blood was collected in citrate phosphate dextrose by the Human Biomaterials Resource Centre (University of Birmingham) after informed consent. Blood (6ml) was diluted 1:1 with phosphate buffered saline (PBS) without Ca^{+2} or Mg^{+2} and aliquots of 5ml placed on the top of 5 ml of

Histopaque 1077 and centrifuged at room temperature at 2500 for 30 mins. The mononuclear cells were collected from the plasma-Histopaque interface and washed 3 times with PBS, re-suspended in 15 ml of cEPC culture media see Table 2.2, placed in a T-75 flask coated with 0.1 mg/ml rat tail type 1collagen (Becton Dickinson;UK) and cultured at 37 ^{O}C 5% $C0_2$. The medium was changed after 24hours and then every 2-3 days thereafter. Endothelial cells colonies appeared between 14 to 22 days of culture. The cells were then detached and transferred to a T25 flask and cultured and passaged as described above.

2.1.4 Origin and derivation of mesenchymal stem cells (MSC)

2.1.4a Bone marrow MSC (BMMSC)

Human bone marrow-derived MSC were from Lonza (Lonza Ltd., Basel, Switzerland) who provided instructions for culture which were strictly followed. The recommended basal medium and the supplements needed for the culture are shown in Table 2.2. Cells delivered frozen were thawed and expanded to passage 3 (counting those supplied as passage 1) and then frozen in aliquots as described in Section (2.1.2). Passage 3 aliquots were thawed and expanded, and used between passages 5-7.

2.1.4b Trabecular bone MSC (TBMSC)

Trabecular bone chipping samples were provided by Dr A Thomas, Dr Andrew Filer and Dr Mark Pearson, taken from the femoral heads removed during hip surgery at the Royal Orthopaedic Hospital (Birmingham). Before placing the samples into the T-25 culture flask with LG DMEM growth media Table 2.2, the bone pieces were cut into 2-3mm bits. The flasks were then incubated at 37°C, 5%

CO_2. Depending on the donor, the time for achieving a confluent flask varied, but the average was about one and a half months from when the material was initially separated. After about 2 weeks, the BMSC could be observed adhered to the flasks after migration from the bone fragments. Finally, the bone fragments were removed from the flask, cells were transferred to a T-25 flask and passaged as described above.

2.1.4c Umbilical cord Wharton's jelly (WJMSC)

Umbilical cords were collected by the Human Biomaterials Resource Centre (University of Birmingham) after informed consent. For exposing the blood vessels, the cord was first cut into 5cm pieces and then each of the pieces was sliced longitudinally. After cutting out the vein and two arteries , the remaining tissue was cut into 3 pieces and placed in 50ml falcon tubes with 10ml of PBS (with calcium and magnesium chloride) and 50U/ml hyaluronidase (Sigma) and 1mg/ml Collagenase type 2 (C6885; Sigma). The samples were incubated for 5 hours at 37°C on a rotor.

To remove large pieces, the sample was diluted 1:10 in PBS and passed through a 70µm pore filter (BD Bioscience). The cell suspension was centrifuged, and the cell pellet re-suspended in culture media (DMEM Low Glucose; see Table 2.2) and seeded in T-75-flasks . The medium was replaced every 2 or 3 days. The flask reached 70-80% confluence within two weeks and were then passaged as described above.

2.2 Characterisation of MSC

All MSC were characterized and fulfilled the criteria established by the International Society of stem cells therapy (2006). They expressed CD90, CD73 and CD105 and lack expression of CD34, CD45, CD14 or CD79alpha ,CD19 CD11b and HLA-DR surface molecules, and were able to differentiate in vitro to adipocytes, chondroblasts and osteoblasts,

2.2.1 Surface characterisation

Accutase was used to detach MSCs (Lonza and UC). Then, the mixture was resuspended in 1ml of FACS buffer (PBS awith 1% BSA). MSC were isolated, counted and aliquots of $5x10^4$ cells were transferred to FACS tubes. The FACS tubes were spun at 400g for 5 minutes and the pellet was resuspended by adding 100ul of FACS buffer. MSC phenotyping cocktail, antibodies against chosen integrins or isotype controls (all 2.5µl; Table 2.3) were added for 20 minutes in the dark in the fridge. FACS buffer was used to wash the samples by adding 3ml, and then samples were spun for 5 minutes at 400g. cells were resuspended in 300ml of FACS buffer and the tubes were kept in ice until analysis. Samples were fixed in 1% Formaldehyde in PBS if the analysis was carried out after 24 hours. For comparison, leukocytes were isolated from fresh blood (CD45+, CD20+ and CD14+ cells) and endothelial cells from umbilical cords (HUVEC; gift of Hafsa Munir).

The principle of flow cytometry depends on the light scattering and fluorescence features of single cells. Briefly, inside the flow cytometer chamber; labelled cells pass thorough a beam of laser light. The intensity of light scattered by the cells or of fluorescent light is measured. The data is displayed in histograms

representing the size of the cells and their level of expression of labelled molecules. FACS samples were analysed using a Cyan flow cytometer(Dako) and Summit 4.3 software was used to anlayse the data.

2.2.2 Cell count and size

MSC counting and size measurements were done with several different techniques: cellometer, Coulter counter and light microscopy.

2.2.2a Cellometer

Cells in suspension were analysed using a Cellometer Auto T4 (Nexcelom Bioscience Ltd, Manchester, UK). The device incorporates a microscope and camera system that automatically measures and identifies the cells, considering their morphology, size and brightness using pattern recognition software and bright field imaging.The cell concentration and diameter were determined. The disposable counting chambers of the cellometer enclosed two independent chambers of controlled height. A single channel pipette was used to transfer 20 µl cell suspension into each chamber which was then placed in the device's imaging slot. The data were calculated automatically and displayed on a linked PC screen.

2.2.2b Coulter Counter

The counting and sizing of the cells was also been done using a Z™ Series Coulter Counter (Beckman Coulter, Buckinghamshire, UK). The cells were diluted typically 1:1000 in a conductive fluid (ISOTON II diluent;Beckman Coulter), and 1ml was sucked through a glass aperture with electrodes either side. The change in

conductance registered as an increase in voltage as each cell passed through the aperture. The number and size of the voltage pulses were analysed, to give the concentration and cell diameter distribution, based on calibration with beads of known diameter. Electronic 'gates' were set to count all MSC between 7-27 μm or only those 12-27 μm. The Coutler counter could also be used to count platelets (see Section 2.3.2) in the gate between 1-4μm (i.e platelets).

2.2.2c Microscopy

Images collected by phase-contrast microscopy during and after cell adhesion assays (see Section 2.4.3) were analysed offline using Image-Pro software (Media Cybernetics Inc., Maryland, USA). A computer mouse was used to draw around cell outlines and the programme calculated the surface area and the diameter of the circle with equivalent area as measures of size. The size of images was calibrated using an image of a graticule with lines marked every 10μm. In some cases, MSC were allowed to settle on a glass slide, and microscopic images were captured and analysed in the same way.

2.3 Blood cells

2.3.1 Blood collection

Blood was collected by venepuncture into sodium citrate 3.2-3.8% as anticoagulant in a mixture of 1:9. Blood donors were healthy adult volunteers who gave written informed consent. Blood was handled according to the Health and Safety Policy (UHSP/22/BTVR/14) of the University of Birmingham.

2.3.2 Preparation of platelet-rich plasma (PRP) or isolated platelets

To prepare PRP, blood was centrifuged at 200g for 20 minutes, and the PRP retrieved without disturbing the packed cells. For the isolation of platelets, 25ml of blood was added to 25ml Tyrode's buffer with 5mM glucose. The cells were centrifuged for twenty minutes at 200g at room temperature. A Pasteur pipette was then used to remove the PRP without disruption of the buffy coat and red blood cells. The platelets were washed three times in Tyrodes's buffer with added protacyclin (PgI2)(Cayman Chemical Company, Michigan, USA), 5ul by centrifugation for ten minutes at 1000g. Platelets were finally resuspended in 4ml Tyrode's buffer containing glucose and counted using the Coulter counter (Figure 2.2.1). Platlets were diluted to $2x10^8$/ml in Tyrode's buffer and left for 30 minutes before use.

2.4 Flow-based adhesion assays

Flow-based assays were used to examine the adhesion of cells under different flow conditions in vitro. Cells were perfused through glass capillaries (microslides) coated with different substrates and cell behaviour under different flow rates assessed. The experimental plan is shown in Figure 2. 1 (flow system diagram) and Figure 2.2 (experimental plan).

2.4.1 Preparation of coated microslides

Microslides are open ended glass capillaries (50mm tubes) (CamLab, Cambridges shire, UK) having the dimensions of 0.3mm height and 3mm width and a rectangular cross section. In order to provide a surface for binding of proteins, the

microslides were coated with 3-amino-propyltri-ethoxysilane (APES) as described (rCooke et al., 1993). The microslides were kept in nitric acid solution (70% in distilled water) for one day and then washed with distilled water ten times in a 50ml conical tube Excess water was removed by inverting the tubes over tissue paper. Next, the tubes were filled with 30ml acetone twice and with 4% APES in acetone twice. Next the microslides were covered with 30ml of 4% APES. The conical tubes were inverted three times followed by overnight incubation at room temperature. Microslides were then washed twice with 30ml acetone and twice with 30ml distilled water. After every wash the tubes were inverted 3 times. Finally, the washed microslides were dried on Whatman blotting paper (Whatman Plc), placed in an oven at 37^{o} C for one hour and finally autoclaved.

Microslides were coated with required proteins: recombinant human or murine P-selectin at 10µg/ml in PBS (Sigma); E-selectin at 25µg/ml,50 µg/ml or 100 µg/ml in PBS; 20µg/ml human plasma fibronectin (Sigma) in PBS; 500µg/ml equine tendon collagen Horm collagen (Axis-Shield). As a control, separate microslides were filled with 50ul of PBS. All microslides were incubated for two hours at 37°C. Following incubation, microslides were flushed by drawing up 500µl of 1% bovine serum albumin (BSA) in PBS. Finally, the microslides were filled with 1% BSA to block non-specific protein binding sites and were incubated overnight in the fridge.

To test the quality of the APES coating, PRP (see section 2.3.2) was drawn into a treated microslide and kept for 45 minutes in an incubator at 37^{0}C with 5% CO_2. If a confluent monolayer of platelets was formed at the microslide surface, the APES coating procedures was considered successful.

2.4.2 Flow system

The flow system was set up as in the Figure 2.2 The coated microslide was glued to the centre of a glass microscope slide. Double-sided sticky tape was used to wrap both ends of the microslide. Next, one end of the microslide was connected via silicon tubing to an electronic valve which was connected to two syringe barrels filled with either wash buffer or a cell sample. An electronic syringe pump (Harvard Apparatus) was connected at the other end of the microslide which was then positioned under a phase contract microscope equipped with a CCTV camera linked to a video recorder. A heating box enclosed the flow system to maintain the temperature constant at 37°C.

The syringe pump was set to deliver a chosen flow rate and hence wall shear rate, γ_W, calculated using the equation:

$$\gamma_W = 6.Q/w.h^2$$

where w = width of the microslide , h =height of the microslide and Q = volumetric flow rate (reference). Typically flow rates of 0.048, 0.95 and 0.191ml/min were used, equivalent to wall shear rates of 18, 35,70s^{-1} respectively.

To conduct an adhesion assay, cell free medium was first washed through the microslides and then cell suspension or blood (see below) was perfused through the microslides for four minutes, followed by washout of non adherent cells until the field of view had cleared. Video recordings and measurements were made as described below.

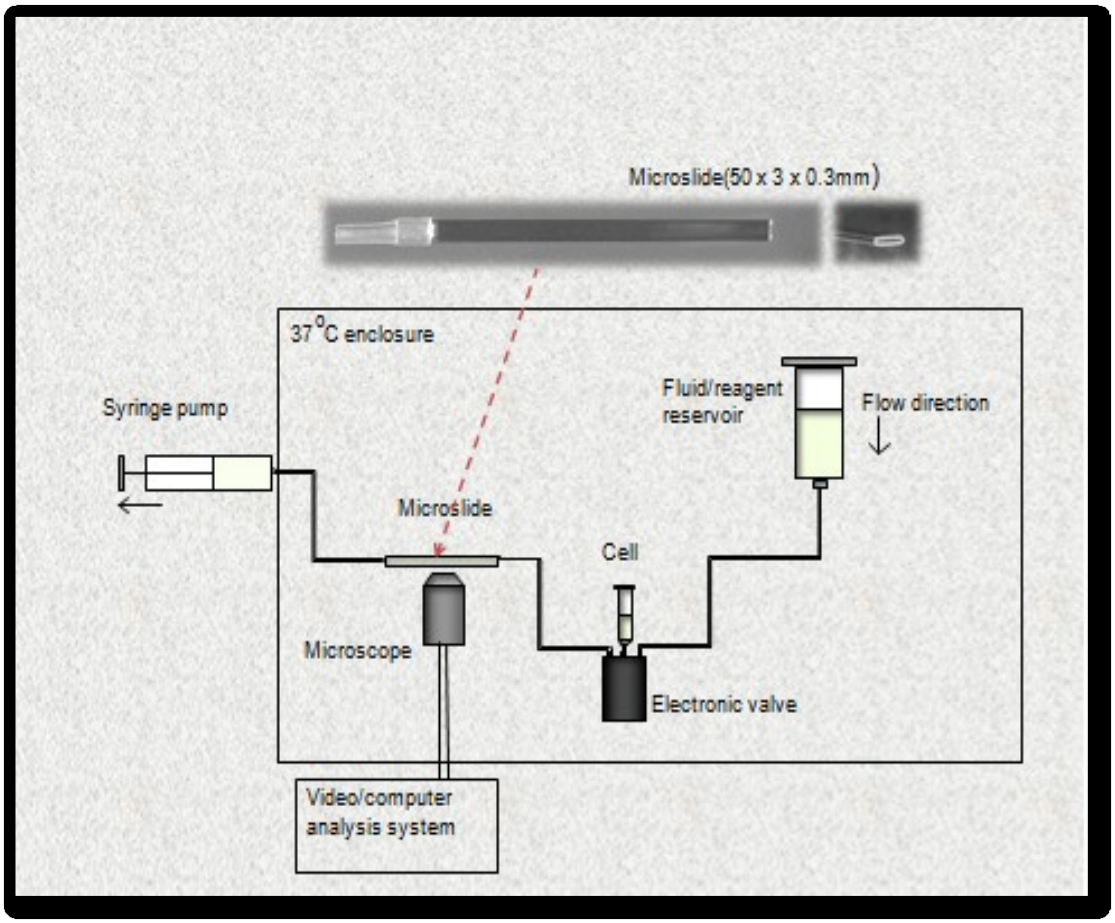


Figure 2-2-1: The flow system: protein-coated microslides

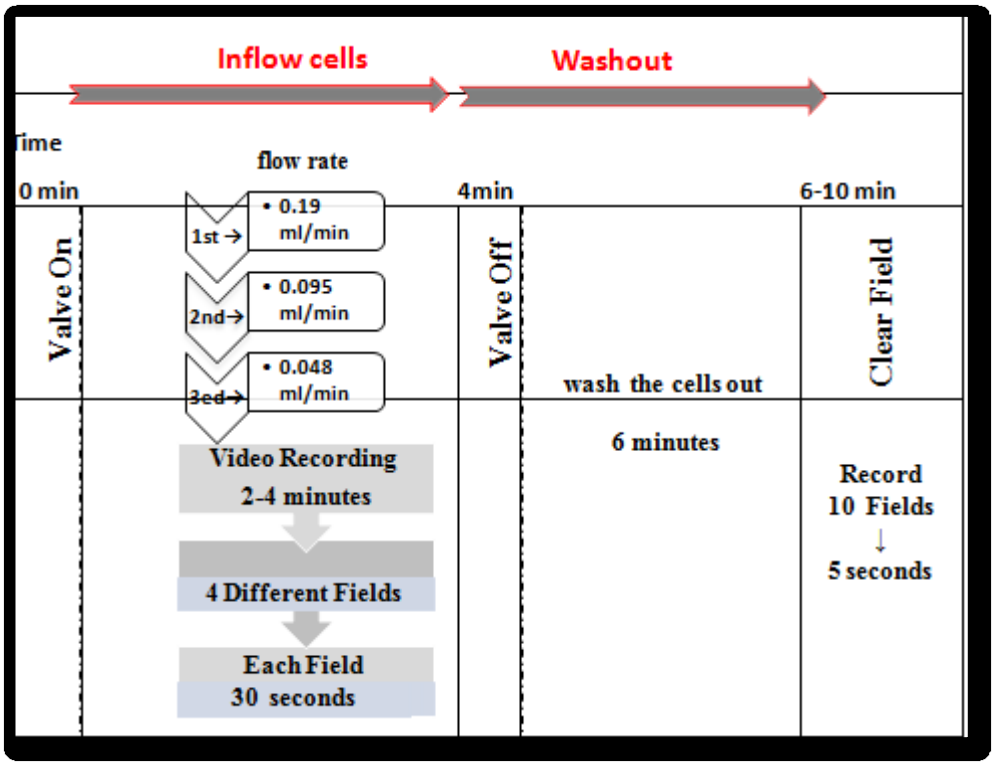


Figure 2-1-2: Experimental design for flow Adhesion assay

2.4.3 Adhesion assay for isolated MSC

Before the MSC isolation, the flow system was set up as described in section 2.4.2.The microslides were coated with BSA, Fibronectin, P-selectin, E-selectin or collagen as described in section 2.4.1.

Trypsin EDTA solution was used to dettach the MSC from the culture flask as described in Section (2.1.2). The cellometer was used for counting the number of cells and they were suspended at a density of $5x10^{5/}$ml in culture medium. The sample was perfused through the microslide for two minutes prior to starting video recording. To allow analysis of the speed of cells passing over the surface, the cells flowing at the lower surface of the microslide were then recorded for two minutes through a CCTV camera which was connected to a time-lapse video recorder. The

field of view was changed every 30 seconds during the recording,.The microslide was washed out after the 4 minute perfusion of cell suspension using cell-free medium, to eliminate remaining unbound cells. Recording of ten random fields of view along the microslide's centreline was done to allow counting of adherent cells. In some experiments, recordings of adherent cells were repeated at intervals to analyse cell spreading. All recordings were analysed offline using Image-Pro v7 software (Media Cybernetics Inc., Maryland, USA).

2.4.3a Cell adhesion

The number of adherent cell was counted in each of the the ten fields recorded after washout. The counts were averaged and normalised per mm² using the known dimensions of the field of view which was calibrated using a micrometer graticle. The counts were then expressed as cells/mm²/10^6 perfused, based on the known concentration and volume perfused in 4 minutes. Finally, adhesion was also expressed as a percentage of all cells perfused, assuming adhesion was uniform over the lower surface of the microslide, with area 150mm^2.

2.4.3b Cell velocity

The video recordings of cells passing over the surface during inflow were played back frame-by-frame; recordings were at 50 frames per second, so each frame advance was 0.02s. The visible area's width was 750µm. The number of frames that a cell took to cross the screen was counted and multiplied by 0.02s to calculate the 'time of flight'. The width was divided by this figure to calculate the speed of the passing cells in µm/s. Typically, for each sample, 10 cells were analysed, and the average calculated.

2.4.3c Cell spreading

Images gathered at the end of washout and at intervals afterwards were analysed as described in Section 2.3.2c so that the area of cells could be calculated as an index of cell spreading. Cells were also counted and divided into those that were phase-bright and rounded and those that were phase dark and 'spread', so that percentage of cells spread could be calculated. In some experiments, at the end of washout a single field was recorded continually for 35min to follow the time course of spreading for individual cells.

2.4.4 Adhesion Assay for MSC in blood

MSC were detached as stated in Section (2.1.2)and re-suspended in 10ml PBSA. Cell Tracker Green (Life Technologies) was added to have 5μM, and the cells were incubated at 37°C in dark for 1h. The cells were then washed with PBSA and re-suspended at a concentration of 1.5 x 10^5 per ml in whole blood in CPDA anticoagulant (Citrate phosphate dextrose adenine (1:9) (CPDA; Sigma). Following this, the in vitro adhesion assay was performed as stated in Section 2.4.3. Washout of blood and non-adherent cells took longer than when using isolated MSC. However, once cleared, adherent cells were counted as above.

In some experiments with microsides coated with fibronectin, whole blood was perfused through the microslid for 4 min at a wall shear rate of $35s^{-1}$. The microslide was then washed out with PBS without Ca+2 and Mg+2 so that a platelet monolayer was left on the surface (see Results). When desired, to fully activate the deposited platelets, 10μM thrombin receptor activating peptide (TRAP); PAR-1 receptor-specific peptide (SFLLRN; Alta Biosciences, Birmingham, UK). was then

added for 30 min. Then isolated MSC at concentration of $5x10^5$/ml were perfused through the microslide coated with platelets for 4 min and adhesion analysed as before.

2.5 Aggregation of MSC with platelets

The principal of the Chrono-log Born Aggregometer is based on changes in light transmission. In brief, when platelets aggregate, the sample light transmission increases. Since there is slight variation in plasma among donors, each donor's Platelets Poor Plasma (PPP) was used to set a reference value. When an agonist is added, the change in the transparency between PPP (platelet poor plasma) and PRP (Platelets Rich Plasma) is tracked by the system.

The variations of light transmission are plotted in graph format: 0% represent no variation in transparency from when the sample began, which means no platelet aggregation. Whereas 100% represents a huge variation in transparency which indicates platelets clump together which in turn allows more light to pass through the sample.

MSC were detached as described in Section (2.1.2) except that the pellet was resuspended in culture medium to achieve the density of $2x10^6$/ml. Washed platelets were isolated from human blood sample and resuspended in tryrodes buffer at $2x10^8$/ml as descrided in Section 2.3.2. 400 µl of platelet suspension was transferred into a glass tube containing a magnetic flea. The tube was then placed in the aggregometer (Chrono-log,Labmedics, Manchester,UK) with a magnetic stirrer maintained at 37°C.

Following two minutes of agitation, 100ul of MSC suspension was added to the platelet and the cells were agitated for a further 10 minutes, during which time the light transmission was recorded. As an agonist, in some experiments Horm collagen was added either alone or with MSC (see Results). As a positive control, 1 unit/ml of thrombin (sigma,poole,UK) were add to platelet suspension to assess the platelet response. Finally, the sample suspension was transferred from the aggregometer and fixed with 1% formaldehyde. Fixed samples were examined under phase-contrast microscope for morphological changes such as clump formation.

2.7 MSC migration Through 8μm pore filters

1% BSA, human plasma fibronectin (20g/ml) or Horm collagen (500μg/ml), was used to coat the surface of 8μm-pore transwell filters (BD falcon) either from the bottom or from the top. In order to coat the top, 50μl of the protein solution was placed inside the filter, followed by incubation for two hours at 37°C. Following incubation, excess proteins were removed and 1% BSA was used to wash the filter. In order to coat the bottom of filters, they were flipped upside down and then the protein solution was pipetted as a 'bead' followed by incubation for two hours at 37°C and rinsing with BSA. Coated filters were placed into 24 wells plate which contained 700 μl of culture media.

MSC were detached as described in Section (2.1.2) and resuspended in freshly prepared growth medium to achieve a cell density of $1.4x10^5$ cell/ml. After adding 200ul of cell suspension to the upper chamber, the system was incubated for 24 hours at 37^oC. After incubation, the medium was collected from above the filters and from the 24 well plate. The top and bottom surfaces of filters were washed once

with 200 µland 700µl PBS respectively, with the wash medium added to the collected samples from above and below the filter. Trypsin-EDTA solution was then used to detach cells from upper and lower surfaces of the filter, and these cells were added to the wash samples to obtain final 'TOP' and BOTTOM' cells in known volumes. These isolated cells were added to 20ml PBS in a Coulter Counter sample cup (Sarstedt) containing 2% formaldehyde. The numbers of fixed cells in the TOP and BOTTOM samples were counted using a Coulter counter (see Section (2.2.2b). Percentage migration was calculated as:

BOTTOM/(TOP+BOTTOM) x 100%.

2.8 Treatment of MSC or blood with function-blocking antibodies

Isolated MSC were treated with function-blocking antibodies (10µg/ml) against integrins or isotype-matched controls for 10min at room temperature. They were then analysed in adhesion assays (Section 2.4), either as isolated cells or after they were added to whole blood. In some experiments, MSC were added to whole, blood which had been pre-treated with antibody against the platelet receptors GPIIb/IIIa or Gp1b for 30 min at room temperature. The antibodies used are listed in Table (2.3)

2.9 Statistical analysis:

Data are shown as mean ± SEM of (n) replicate experiments using different culture samples on different occasions. Statistical analysis was performed using Minitab 17 software (Minitab Inc.). Effects of multiple treatments or conditions were analysed using a general linear model analysis of variance (ANOVA) and where appropriate post hoc comparisons between treatments or to control were made

using the Bonferroni test or Dunnett test respectively. Single treatments were compared to control by paired t test.

Table 2-1-: List of General Reagents

Reagent	Supplier	Application
Histopaque	Sigma-Aldrich Co.	EPC isolation
PBS (Phosphate-buffered saline)	Sigma-Aldrich	Cells Washing
BSA (Bovine serum albumin)	Sigma-Aldrich	Cell washing Buffer
EDTA	Sigma-Aldrich	Cell washing Buffer
M199	Gibco Invitrogen	Culture media
DMEM	Gibco Invitrogen	EPC culture media
10% Fetal Calf Serum (FCS)	Gibco Invitrogen	PBMC, DMEC culture
1%Penicillin/ Streptomycin	Gibco Invitrogen	Cell culture antibiotic
Gentamycin	Sigma-Aldrich	Cell culture antibiotic
25 mg.ml^{-1} Amphotericin B	Sigma-Aldrich	Cell culture antifungal drug
Trypsin	Gibco Invitrogen	Cell Dissociation reagent
0.1 µg.ml^{-1} Basic fibroblast	Gibco Invitrogen	EPC growth factor
2 mM L-glutamine	Gibco Invitrogen	supports the growth of cord EPC
Endothelial basal medium- EBM-2	Gibco Invitrogen	Cord EPC isolation
20 %FBS hyclon	Lonza	Cord EPC isolation
MSCGM(BulletKit)	Lonza	MSC basal medium and growth media
DMEM Low Glucose	Biosera	WJMSC culture media

Table 2-2: List of Culture Media

Cell Type	Medium	Supplier
mEPC	Dulbecco's Modified Eagle Media (DMEM),10% Fetal Bovine Serum, 100µg/ml Penicillin, 250µg/ml. Amphotericin B , 200µmol Glutamine and 10µl Basic fibroblast growth factor	Dulbecco's Modified Eagle Media (DMEM)(Sigma), Fetal Bovine Serum (Sigma) Penicillin (Sigma) Streptomycin (Sigma) Amphotericin B (Invitrogen) Glutamine (Sigma) Basic fibroblast growth factor (Sigma)
hEPC	• LONZA endothelium cells culture medium (2MV) • rhEGF-B, Epidermal Growth Factor Human recombinant in a buffered BSA saline solution, 5µg/ml. • rhFGF-B, rHuman Fibroblast Growth Factor-B, 10µg/ml • rVEGF, Endothelium Growth Factor Vascular Human Recombinant, 0.5ml • Hydrocortisone 0.2µg/ml • GA-1000 Gentamicin sulfate,Amphotericin-B 250µg/ml • Ascorbic acid in aqueous solution, 1µg/ml. • R3-IGF-1 Recombinant R Insulin- Like	In LONZA endothelium cells culture medium 2MV, CC-3156 with supplements of rhEGF Hydrocortisone GA-1000 (Gentamicin, Amphotericin-B) rVEGF Ascorbic acid Insulin R3-IGF-1 20% ES Screened HyClone FBS(Lonza)

	Growth Factor, 20µg/ml. • ES Screened HyClone FBS 0.05ml/ml	
WJMSC	DMEM Low Glucose, 10% Fetal Bovine Serum and 1% Penicillin,1% Streptomycin.	DMEM Low Glucose; (Biosera) Fetal Bovine Serum (Sigma) Penicillin (Sigma) Streptomycin (Sigma)
BMMSC	MSCBM Mesenchymal Stem Cell Basal Medium and MSCGM hMSC SingleQuot Kit (Mesenchymal Stem Cell Growth Supplement (MCGS); L-Glutamine, GA-1000)	MSCBM hMSC Basal Medium and MSCGM hMSC SingleQuot Kit(Lonza)
TBMSC	DMEM Low Glucose, 10% Fetal Bovine Serum and 1% Penicillin,1% Streptomycin.	DMEM Low Glucose, (Biosera) Fetal Bovine Serum (Sigma) Penicillin (Sigma) Streptomycin (Sigma)

Table2-1: List of Monoclonal antibodies.

Antibody	Supplier	Application
β1-integrin/antihuman CD29 (mab13;rat IgG2a;)	BD Pharmingen	Integrin β1 identification
β3-integrin/ anti-human CD61 (SZ21; mouse IgG1)	BeckmanCoulter	Integrin β3 identification
α v-integrin/ anti-human CD51 (mAb L230)	EnzolifeSciences	Integrin α v identification
αvβ3 /(23C6 mouse IgG1)		Integrin αvβ3 identification
Human Integrin α 4 (MAX 68P0)	Gift; Cell Tech, Slough, UK	Integrin α 4 identification
GPIIb		Platelets Integrin(receptor for fibrinogen and von Willebrand factor
GPIIb/IIIa (CD41)	Eli Lilly	Platelets Integrin(receptor for fibrinogen and von Willebrand factor)
IgG1-FITC	Dako	Isotype control
CD44 APC	BD Bioscience	hMSC
CD73 FITC	BD Bioscience	
CD90 Bv421	BD Bioscience	
CD105 PerCp-Cy 5.5	BD Bioscience	
CD166 PE	BD Bioscience	
CD146 APC	BD Bioscience	
CD45		Leukocytes marker
CD20		B cell marker
CD14		macrophages , neutrophils markers
CD34		hematopoietic and vascular progenitor cells marker
Mouse IgG1		Non-specific control
Rat IgG2a		Non-specific control

Chapter 3 : COMPARISON OF ADHESIVE PROPERTIES OF DIFFERENT PROGENITOR CELLS UNDER FLOW.

3.1 INTRODUCTION

Based on the concept that infused EPC and MSC may be used to treat a variety of conditions such as vascular injuries and chronic inflammation, this chapter compared the potential ability to adhere from flow of isolated progenitor cells: mouse EPC cell line (mEPC), primary human cord blood EPC (hEPC), and primary MSC from bone marrow (BMMSC), Wharton's jelly (WJMSC), and trabecular bone (TBMSC). In order to investigate the effect of flow on adhesive behaviour of these cells on various surfaces, we used the flow adhesion assay illustrated in Section 2.4.3. We compared adhesion to surfaces coated with receptors that would be presented by inflamed endothelium (P-selectin and E-selectin) or by damaged vessel wall (collagen and fibronectin), to 'control' surfaces coated with albumin alone. In this chapter, the isolated progenitor cells were perfused over the coated surfaces at the following shear rates; $18s^{-1}$, $35s^{-1}$, $70s^{-1}$. Higher rates were initially tested as well, but no adhesion was seen. The number of adherent cells of each cell type at the different surfaces was counted and expressed as a percentage of all those perfused, as described in Section 2.4.3a. We also measured velocity of non-adherent cells in the flow near the surface of P-selectin and albumin in order to study whether there was weak adhesion or 'rolling' on P-selectin compared to free flow for albumin. Finally, we investigated whether cell size influenced behaviour. As described in Section 2.2.2, the diameter of cells flowing near the wall of a vessel is expected to affect their velocity and forces exerted on them. It is possible that larger cells would adhere less well. Therefore, we measured the size of MSC as whole populations and of the adherent MSC.

These studies thus aimed to improve our understanding of the dynamic adhesion property of different types of progenitors cells from a flowing suspension onto matrix or inflamed vessels.

3.2 RESULTS

3.2.1 Comparison of the adhesion of different flowing progenitor cells to endothelial or matrix receptors

We first investigated the adhesive properties of two different types of EPC, mEPC mouse cell line and hEPC from cord blood. Figure 3.1A shows the behaviour of human cord EPC for different surfaces. We noticed that when we flowed hEPC over collagen at $18s^{-1}$, about 50% of the perfused cells adhered. By increasing wall shear rate to $35s^{-1}$, the percentage of adhesion was reduced to less than 10%, while at $70s^{-1}$, less than 5% adhered. When we perfused hEPC over fibronectin at $18s^{-1}$, around 20% adhered, and the numbers decreased to similar levels to those seen on collagen upon increasing the wall shear rate to $35s^{-1}$ or $70s^{-1}$. When we flowed EPC over P-selectin at $18s^{-1}$, we noticed a just detectable but very low percentage of adhesion. When the shear rate was increased to $35s^{-1}$, the percentage of adhesion was decreased, while at $70s^{-1}$, we completely lost the adhesion. Finally, for BSA (control), no hEPC were observed adhered to the surface at any shear rate.

Figure 3.1B shows the behaviour of mEPC under the same conditions. We noticed that mEPC adhered to collagen or fibronectin at similar levels and with similar effects of varying wall shear rate. When we flowed mEPC over P-selectin, adhesion was just detectable at $18s^{-1}$ or $35s^{-1}$, but not at $70s^{-1}$. Again, for BSA, no mEPC were seen adhered to the surface at any shear rate.

In summary, hEPC or mEPC could adhere to matrix proteins from flow better than to P-selectin, which showed levels just above background for BSA. Adhesion tended to be higher for collagen than fibronectin, but this trend was not statistically significant.

In Figure 3.2, we used the same assay conditions to study the adhesion behaviour of three different MSC types: WJMSC, BMMSC, TBMSC. Figure 3.2A shows that when we flowed WJMSC over collagen at $18s^{-1}$, around 30% adhered. By increasing shear rate to $35s^{-1}$, we lost more than half of the adhesion, and at $70s^{-1}$, the percentage of adhesion declined to under 5%. We noticed that when we flowed WJMSC over fibronectin at $18s^{-1}$, around 20% adhered. By increasing shear rate to $35s^{-1}$ or $70s^{-1}$, adhesion reduced in a similar manner to collagen. Finally, on P-selectin or BSA, no WJMSC were observed adhered to the surface at any shear rate

In Figure 3.2B, when BMMSC were perfused over the same surfaces, we observed similar effects of shear rate and lack of adhesion to P-selectin or albumin. Adhesion to collagen and fibronectin tended to be lower than those for WJMSC but effects of shear rate were similar. We saw occasional cells attached to P-selectin at the lower shear rates.

Figure 3.2C shows adhesion data for TBMSC tested in the same way. The trends were similar to those for the other MSC, but the levels of adhesion to collagen and fibronectin appeared to be lowest overall.

In Summary, all types of MSC adhered to collagen or fibronectin from flow, but not to P-selectin or albumin. Adhesion again tended to be higher for collagen than fibronectin, but this trend was not statistically significant. Adhesion tended to

be in the order WJMSC>BMMSC>TBMSC. To test this more directly, we re-plotted the data in Figure 3.3. to compare the three different cells type.

In Figure3.3A, we compared the adhesive properties of WJMSC, BMMSC, and TBMSC on collagen; we found that WJMSC bound at significantly higher levels than BMMSC or TBMSC, while the difference in binding on collagen between BMMSC and TBMSC was not statistically significant. In figure 3.3B, we compared the adhesive properties of WJMSC, BMMSC and TBMSC on fibronectin; we found that WJMSC bound at significantly higher levels than BMMSC or TBMSC. Again, the difference in binding on fibronectin between BMMSC and TBMSC was not significant.

In Figure 3.4 we have re-plotted the whole adhesion data to compare effects of shear rate on adhesion of all the progenitors cells to the different surfaces. On collagen and fibronectin surfaces, MSC tended to bind better than EPC. However, for P-selectin, we noticed that EPC bound to the surface at higher levels than MSC, where only BMSC occasionally bound to the surface.

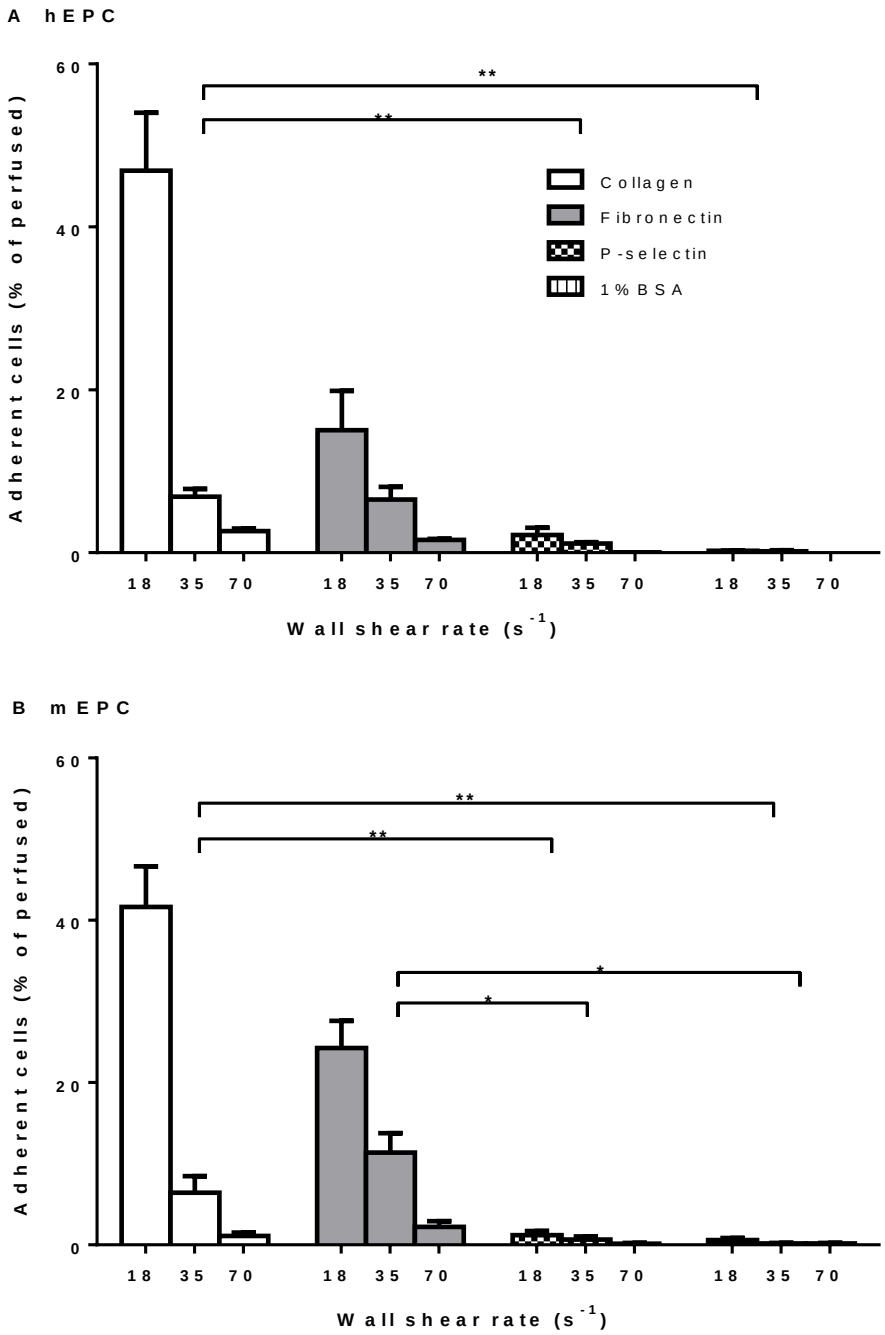


Figure 3-1: Adhesion of A. hEPC, B. mEPC to different surfaces: effects of wall shear rate.

EPC were perfused for 4 min at 37^0C over collagen, fibronectin, P-selectin or albumin at wall shear rates of 18, 35 or 70s^{-1}. Data are the mean ± SEM from three to four experiments. Overall, in A and in B, ANOVA showed significant effects of wall shear rate and of adherent substrate ($p<0.01$ in all cases). *=$p<0.05$; **=$p<0.01$ for post-hoc comparison of adherent substrates including all values for shear rate by Bonferroni test.

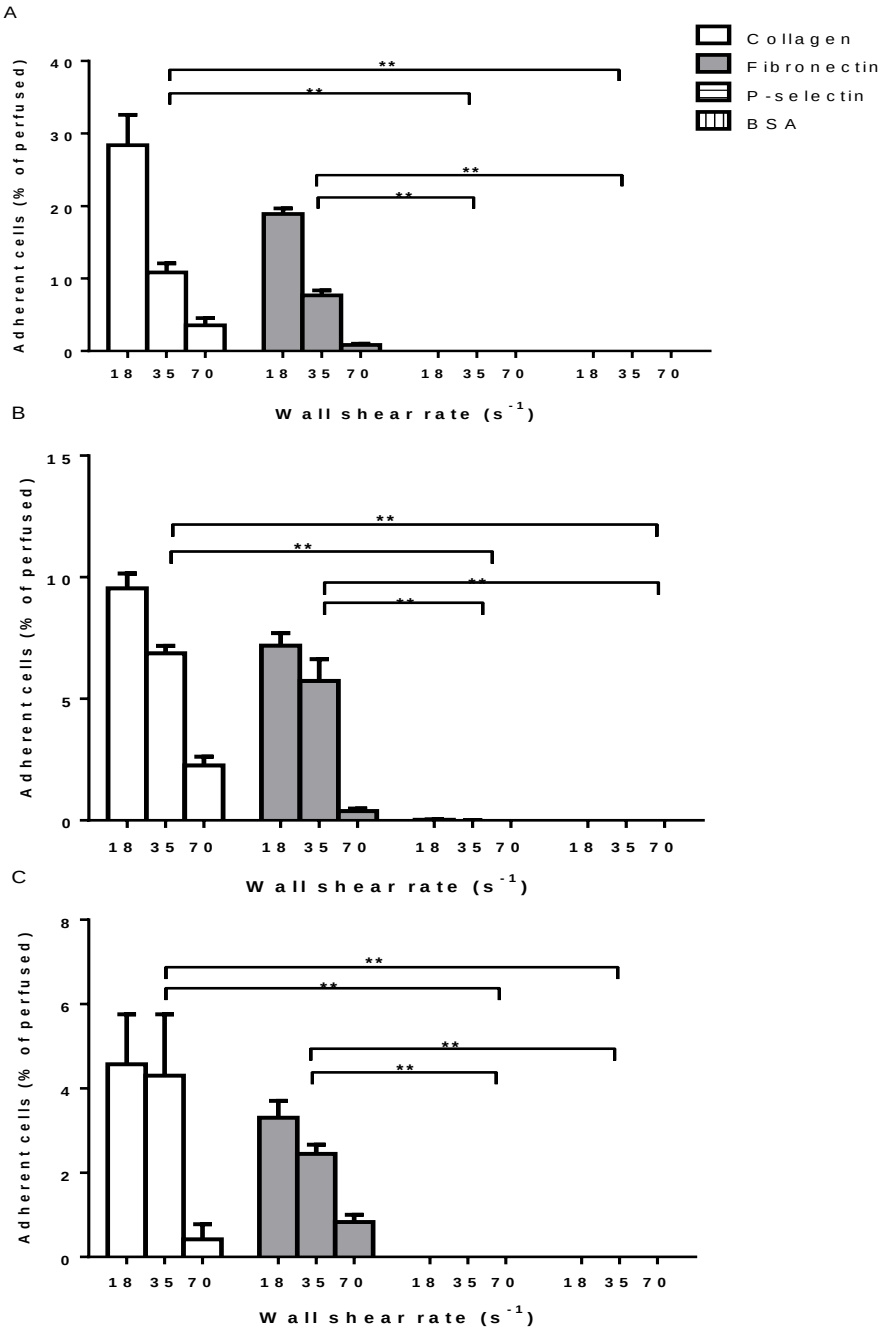


Figure 3-2: Adhesion of (A)WJMSC, (B)BMMSC, (C)TBMSC to different surfaces: effects of wall shear rate.

Wharton's jelly, bone marrow and trabecular bone MSC were perfused for 4 min at 37^0C over collagen, fibronectin, P-selectin or albumin at wall shear rates of 18, 35 or $70s^{-1}$. Data are the mean ± SEM from three or four experiments. Overall, in A, B and C ANOVA showed significant effects of wall shear rate and of adherent substrate ($p<0.01$ in all cases). *=$p<0.05$; **=$p<0.01$ for post-hoc comparison of adherent substrates including all values for shear rate by Bonferroni test.

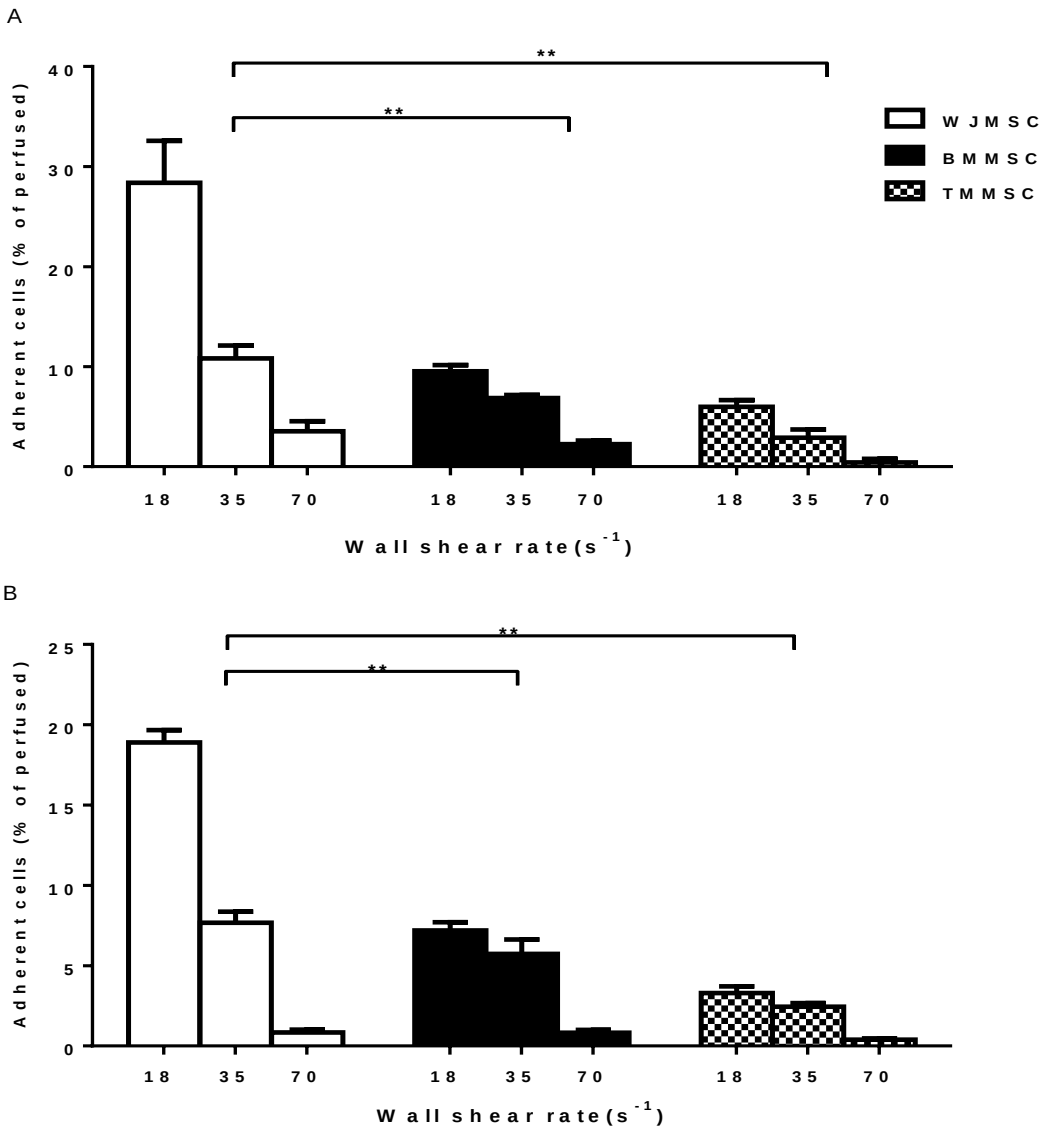


Figure 3-3: Comparison of Adhesion of WJMSC, BMMSC and TBMSC to (A) collagen or (B) fibronectin.

MSC were perfiued for 4 min at 37^0C over collagen or fibronectin at wall shear rates of 18, 35 or 70s^{-1}. Data are the mean ± SEM from three or four experiments. Overall, in A and in B, ANOVA showed significant effects of wall shear rate and of cell type ($p<0.01$ in both cases). *=$p<0.05$; **=$p<0.01$ for post-hoc comparison of cell types including all values for shear rate by Bonferroni test.

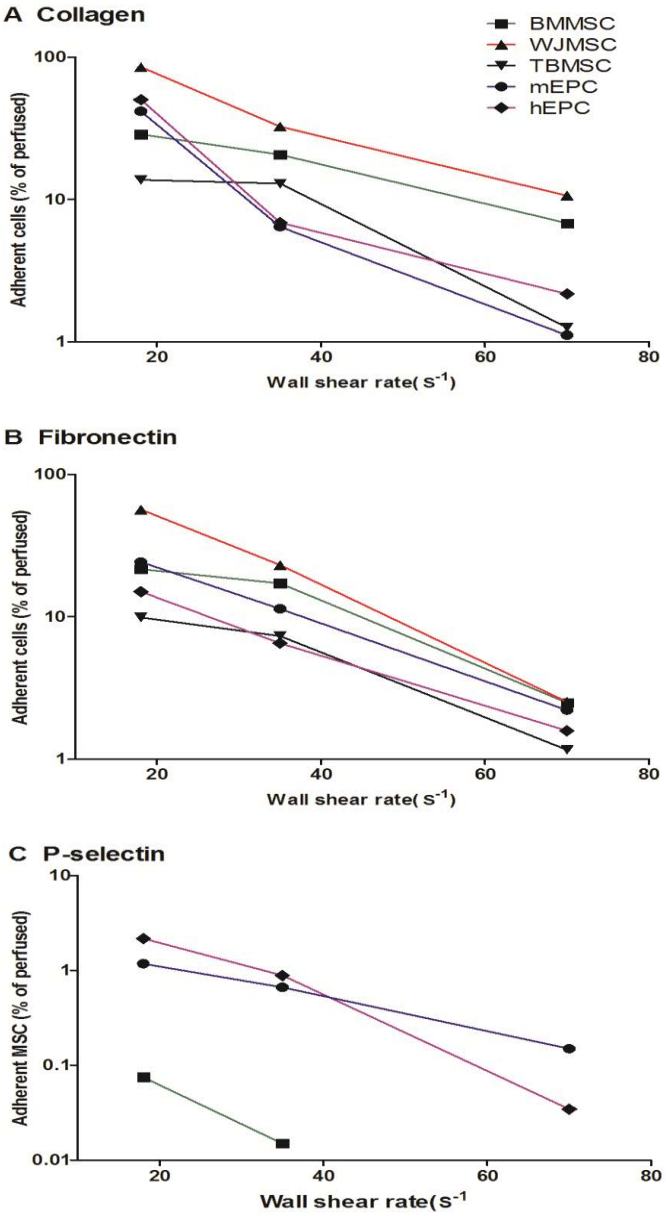


Figure 3-4: Comparison of adhesion of different types of endothelial progenitor cells and mesenchymal stem cells on (A) collagen, (B) fibronectin or (C) P-selectin.

Cells were perfused for 4 min at 37^{0}C over collagen, fibronectin or P-selectin at wall shear rates of 18, 35 or 70s^{-1}. Data are mean values. Numbers of experiments are the same as for Figures 3.1 -3.3.

3.2.2 Near-wall velocities of different progenitor cells

Since the binding to P-selectin was lower than we expected from literature reports, we also measured the velocity of cells near the surfaces to see if we could detect weak adhesion or 'rolling', compared to non-adhesive albumin. Figure 3.5A compares velocities for P-selectin and BSA for hEPC. As expected, cell speed increased with increasing shear rate. The graph also illustrates a significant effect of adherent surface, with flowing cells slowed down slightly for P-selectin compared to albumin, which may indicate there is a weak rolling interaction. Figure 3.5B shows very similar behaviour and trends for mEPC. Overall, the EPC flowed about 15% slower for P-selectin than albumin.

Figure 3.6 shows near-wall velocities of the different types of MSC on collagen, fibronectin, P-selectin and BSA. The velocities of WJMSC, BMMSC, and TMSC increased with shear rate but showed no effect of surface. Since flowing cells were not slowed down, it appears that no rolling occurred.

We also carried out experiments where EPC or MSC were perfused over E-selectin. In 2 experiments with each type we used different E-selectin concentrations 20µg/ml, 50µg/ml and 100µg/ml at the same shear rates as above. However, we did not detect any adhesion.

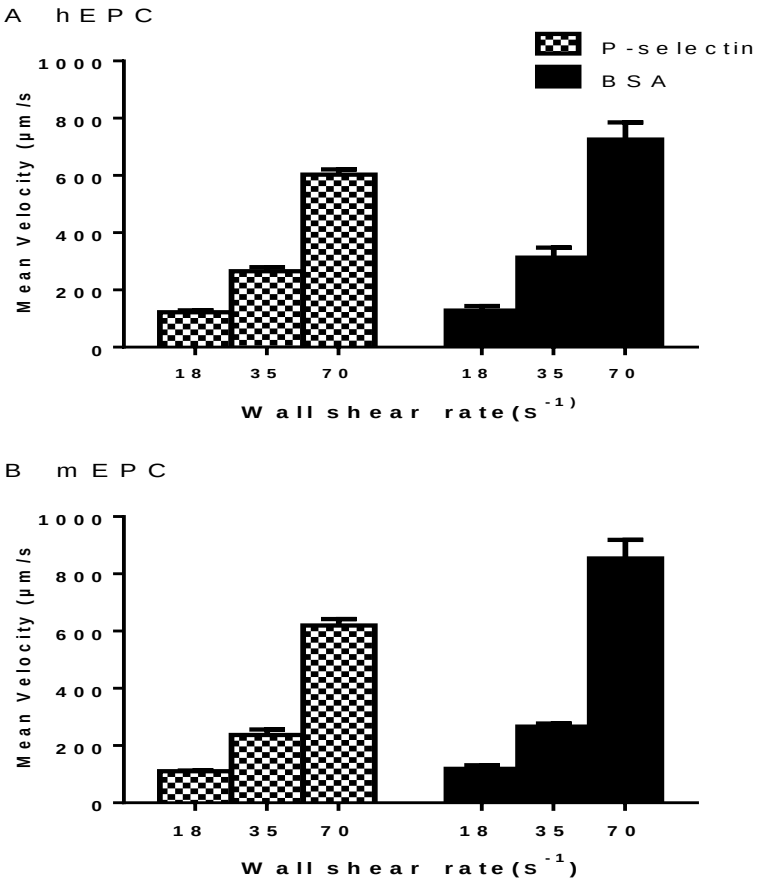


Figure 3-5: Velocity of (A) Human cord EPC (B) mouse EPC perfused over different surfaces: effects of wall shear rate.

EPC were perfused for 4 min at 37^0C over P-selectin or albumin at wall shear rates of 18, 35 or 70s^{-1}. Data are the mean ± SEM from three or four experiments. Overall, in A and in B, ANOVA showed significant effects of adherent substrate and of shear rate ($p<0.01$ for shear rate; $p<05$ for adherent substrate).

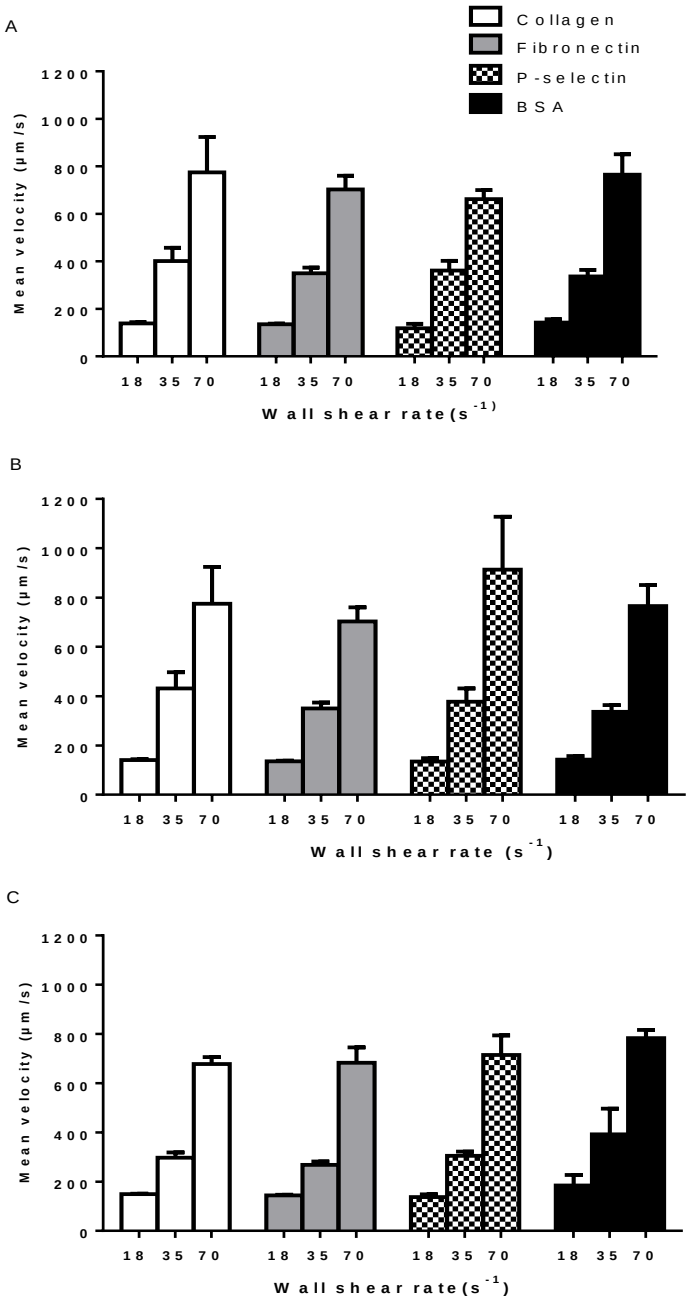


Figure 3-6: Velocity of (A) WJMSC (B) BMMSC and (C)TMSC perfused over different surfaces: effects of wall shear rate.

MSC were perfused for 4 min at 37^0C over collagen, fibronectin, P-selectin or albumin at wall shear rates of 18, 35 or 70s^{-1}. Data are the mean ± SEM from three or four experiments. Overall, in A, B and C, ANOVA showed significant of wall shear rate ($p<0.01$ in each case) but not of adherent substrate.

3.2.3 Comparison of diameters of different MSC and effect on adhesion

Three different methods were used to measure cell diameters during preparation or during adhesion assays : Coulter counter (see Section 2.2.2b); Cellometer (see Sections 2.2.2a); microscopy and image analysis (see Section 2.2.2c). To test whether they yielded the same results, we compared measurements for identical samples of cells. Table 3.1A shows diameters of BMMSC in suspension measured by Coulter Counter or Cellometer. The results were nearly identical. Table 3.1B compares values for cells settled in a microslide, with diameter measured by microscopy, compared to a sample of the same cells measured by Cellometer. The value for the Cellometer was consistently larger than ImagePro analysis by 5±1%.

Based on the above, to investigate the effect of cell size on adhesion, we compared the mean diameters of BMMSC, WJMSC, and TBMSC before perfusion (by Cellometer) and after they had adhered to collagen or fibronectin (by microscopy and ImagePro). Table 3.1C shows the data, with the microscopy values multiplied by 1.05 to allow for the systematic difference between the methods as noted above. From this Table, we found that smaller-size cells were adhered to collagen compared to the original sample, and smaller-size cells were adhered to fibronectin compared to the original sample. Moreover, smaller cells adhered to fibronectin compared to collagen, although this trend was not statistically significant.

We also tested whether cell diameter (measured by Cellometer) was linked to the velocity of non-adherent cells flowing near the wall of microslides for different samples of WJMSC prepared on separate occasions. Figure3.7A shows the average velocity of WJMSC measured at different shear rates on two different surfaces P-

selectin and 1%BSA. At a wall share rate of $70s^{-1}$, we found that cell velocity increased when cell diameter was greater, whereas, at low shear rate of $18s^{-1}$, the trend was not seen. In figure 3.7B, the relation between cell size and velocity is re-plotted for the data pooled from the different shear rates. Figure3.7A showed that velocity was dependent on shear rate and cell diameters.Linear regration showed that velocity averaged over the difference shear rate was not significantly correlated with diameters(figure 3.7B)

Table 3-1: Measurement of cell diameter: comparison of methods, and of adherent cells with original perfused sample.

3-1A. Comparison of cell diameter measured by Cellometer or using Coulter Counter - BMMSC.

	Cellometer(μm)	**Coulter Counter(μm)**
Isolate 1	19	19.1
Isolate 2	17.4	17
Isolate 3	20	20
mean	18.8	18.7
SEM	0.8	0.9

Table 3-1.B: Comparison of cell diameter measured by Cellometer or using microscope and ImagePro analysis of digitised images - WJMSC.

	Cellometer (μm)	**ImagePro(μm)**
Isolate 1	23	21.6
Isolate 2	24	23.2
Isolate 3	24	22.8
mean	23.7	22.5
SEM	0.3	0.5

Table 3-1.C. Comparison of diameters of cells adherent to different surfaces with cells originally perfused

Cell type	**Size of Original sample(μm)**	**Size of Adherent cells (μm) onCollagen***	**Size of Adherent cells (μm) onFibronectin****
BMMSC	22.3 ± 0.9	18.9 ± 4.2	16.8 ± 4.2
WJMSC	20.3 ± 1.2	17.8 ± 1.2	15.9 ± 1.2
TBMSC	19.3±0.3	15.8 ± 1.3	15.4 ± 3.9

Diameter of the original sample was measured by Cellometer. Diameter of adherent cells was measured using ImagePro software and digitised images. Data are mean ± SEM from 3 experiments where different MSC were perfused over collagen or fibronectin.

*ANOVA showed for comparison of collagen adherent and original sample, no effect of cell type but overall adhesion vs. nonadhesion $p<0.05$.

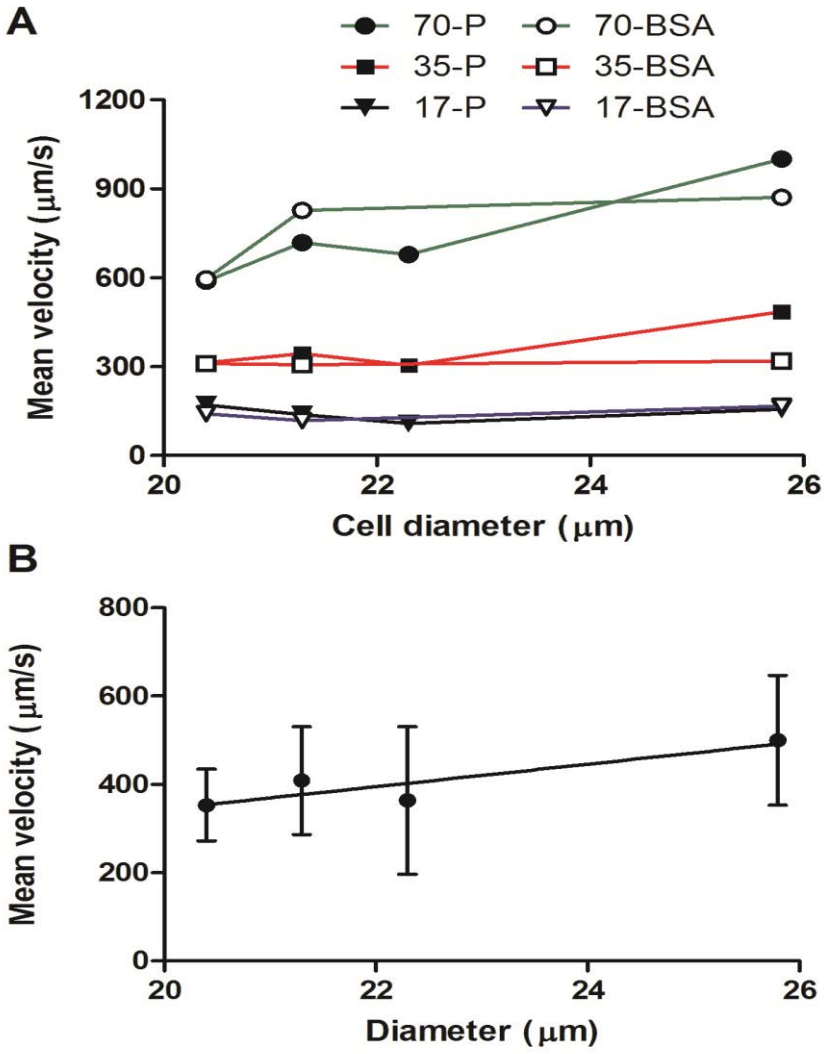


Figure 3-7: Velocity of WJMSC perfused over P-selectin or albumin at different shear rates: effect of mean diameter of the perfused cells.

MSC were perfused for 4 min at 37^{0}C over P-selectin or albumin at wall shear rates of 18, 35 or 70s^{-1}. Data are from a single experiment under each condition for 4 different samples of WJMSC with different mean diameters. In B, data are mean ± SEM for the 3 different shear rates and two surfaces, for each sample. In A, ANOVA showed significant ofwall shear rate ($p<0.01$) and diameter ($p<0.05$) but not of adherent substrate.In B,Linear regretion of mean velocity vs diameters was not statiscally significant (P0.10)

**ANOVA showed for comparison of fibronectin adherent and original sample, no effect of cell type but overall adhesion vs. nonadhesion $p<0.01$.

3.3 DISCUSSION

In this Chapter, we compared adhesive behaviour of flowing EPC and MSC on different surfaces, and then analysed whether the diameter of MSC affected their adhesion. The main new findings were that the cells could adhere from flow to collagen or fibronectin, and surprisingly, that adhesion was more effective on the matrix proteins than selectins. This was particularly the case for MSC, which adhered to collagen better than fibronectin, with very few adhering to albumin. MSC and EPC adhesion decreased the higher the wall shear rate, and they could adhere up to a wall shear rate of $70s^{-1}$ but not above this level. All adhesion counted was stationary. There was evidence of slowing down of EPC flowed over P-selectin compared to albumin, suggesting a very weak 'rolling' interaction. MSC did not slow down on P-selectin, and also either stopped or flowed at full speed on matrix proteins. Once attached, cells adhered firmly, with little subsequent detachment when exposed to $70s^{-1}$ shear rate. The MSC that adhered were on average smaller than the population perfused, suggesting that smaller cells adhered better. The velocity of non-adherent MSC was greater, the bigger the mean diameter of the cell sample perfused.

EPC adhesion from flow has been more widely studied than MSC adhesion. EPC circulate in the blood and need to be captured from flow to carry out repair or angiogenesis functions. They were studied here for comparison to MSC which would not normally be expected to circulate unless injected for therapy. We observed that cord EPC and mouse EPC could adhere to P-selectin at shear rates at

the low end for the venous circulation. Others have found that selectins and β2-integrins can mediate adhesion of flowing EPC to endothelial cells or purified receptors ((Hristov et al., 2003) and to platelets (Langer et al., 2006, Stellos et al., 2008). Surprisingly, we found that the EPC adhered better to collagen or fibronectin than P-selectin. This suggests that these cells might bind to exposed matrix protein better than inflamed endothelial cells in vivo. This might be consistent with the general understanding of EPC involvement in angiogenesis, in damaged tissue at least.

We found that WJMSC, BMMSC and TBMSC adhered to collagen significantly more than to fibronectin, compared to nearly zero adhesion on P-selectin, E-selectin or albumin. In addition, WJMSC adhered the most efficiently, while BMMSC adhered more than TBMSC. However, all showed similar shear rate dependence, with adhesion very low at $70s^{-1}$ and not detectable above that level. Since MSC are naturally stromal cells not found in the circulation, it may not be surprising that they adhered less than EPC to P-selectin. However, they would be expected to bind to matrix proteins in their natural tissue, likely through integrin receptors. This is investigated further in Chapter 6. The cells are very large, about 20μm in diameter, which is larger than most capillary diameters. It is thus likely that MSC would be trapped in small vessels even without adhesion (Karp, 2009). However our results suggest they could adhere to damaged vessels where matrix was exposed. They would then need to spread and or migrate to take part in tissue repair. This behaviour is considered in Chapter 4.

Studies of MSC adhesion from flow to matrix have been rare. To our knowledge only one study has addressed the binding between BMMSC and

collagen. The authors reported that there were no adherent MSC on a collagen surface at shear rate of $1000s^{-1}$ (Jiang L et al., 2012). This is consistent with our finding which show that we lost cell adhesion when we increased shear rate from 70s-1 to 100s-1.

Our data is in agreement of a study conducted by Lan CW et al who studied the adhesive behaviour of osteoprogenitor cells isolated from bone marrow (BMSCs). they showed that these cell adhere twice more to the surface when is surface is coated by collagen under flow condition, interestingly enough they noticed when they flush all marrow cells by shear stress of 1.10 dyne/cm, 25% of cells which are adhered to collage coated surface remained attached to the surface despite the flush force which indicate the strength of the adhesion (Lan et al., 2003). Moreover; another study was conducted to study the adhesive behaviour of BMMSC by using Collagen nanofibers scaffold. they reported that over 45% of BMMSc adhered efficiently to collagen coated nanofibers (Chan et al., 2009). additionally, studies on Murine bone marrow cells (BMC) revelled again higher MSC adhesion to collage coated surfaces (Vandersluijs et al., 1994).

This is the first study to compare dynamic adhesive behaviour of MSC from different sources, and to show they can adhere to both collagen and fibronectin from flow. Variation between the adhesion behaviour of different MSC could be attributed to the source and/or the isolation and culture techniques. This assumption is based on the findings of Aldridge et al. (2012) who suggested that the expression of adhesion molecules on MSC are affcetd by their source and the isolation method (Aldridge et al., 2012). The high WJMSC adhesion compared to BMMSC on collagen and fibronectin may be linked to differences in regeneration ability. WJ

MSC have been shown to be more neurogenic and angiogenic than BMMSC, possibly also linked to differences in their secretomes (Hsieh et al., 2013). Regarding the low adhesion of TBMSC compared to WJMSC and BMMSC, we suggest this difference may be due to the fact that TBMSC were obtained from aged patients undergoing knee replacement. Coipeau et a. (2009) suggested that MSC were modified inherently because of the burden of the diseases . Information about MSC homing and mobilization may still be conflicting because of the difficulty in isolating and identifying native MSC. Laboratories may base studies on culture-expanded MSC which may lack some cell adhesion molecules or chemokine receptors which contribute to MSC homing. However, therapeutic use of MSC is likley to require such expansion, and cells used here were cultured for from 5 to 7 passages.

Previous studies have looked at adhesion of flowing MSC to endothelial cells or purified endothelial receptors. Indeed, MSC have been proposed to use a multi-step process to cross endothelium similar to leukocytes (Chamberlain et al., 2007) (Henschler et al., 2008). On a P-selectin or E-selectin surface, we noticed almost no adhesion when using WJMSC, BMMSC or TBMSC even when wall shear rate was reduced to 18s-1. However, Ruster et al suggested that there were interactions with P selectin and between MSC integrins and VCAM-1 when human MSC bound to EC that had been treated with tumour necrosis factor-α (TNF) (Ruester et al., 2006). In the same study the authors found that MSC expressed neither P-selectin glycoprotein ligand-1(PSGL-1; CD162) nor the alternative P-selectin ligand–CD24 on their surface (Ruester et al., 2006). In agreement to our studies, they did not find a role for E-selectin for adhesion to EC (Ruester et al., 2006). It should be noted that in this

study, adhesion occurred only at a very low flow (0.01Pa wall shear stress, equivalent to about 14s-1 wall shear rate), and remained when flow was increased. Others perfused rat MSC over TNF-treated EC for 2 hours and observed adhesion at higher shear rates, again attributed to VCAM-1 (Segers et al., 2006). In another study, murine MSC did not bind to murine EC from flow, but did adhere when held stationary for 10min before flow increased (Chamberlain et al., 2011). Subsequently, Aldridge et al. (2012) found that blockade of β1-integrins or of CD44 did reduce adhesion of human MSC to liver sinusoidal endothelial cells (Aldridge et al., 2012). However, again, in this model the authors stopped the flow temporarily for 5 minutes to allow enough time for MSC to bind to HSEC, before re-applying flow. In our laboratory, BMMSC bound to HUVEC under stationary conditions even without stimulation of EC (so that P-selectin or VCAM-1 should not be expressed), and then remained adherent if flow was imposed (Luu et al., 2013). We did find that at very low wall shear rate (14s-1), we could detect some adhesion if the EC were treated with TNF or IL-1, but did not investigate what receptors were used. Other studies in our lab suggested that flowing MSC could not adhere to purified VCAM-1 (G. Nash, unpublished observations). Thus the mechanisms and circumstances under which MSC can bind to intact EC in the circulation remain in doubt (Karp, 2009). The effects of blood cells themselves on MSC adhesion are investigated in Chapter 5.

In other studies, scientists have been purposely manipulating MSC to express selectin surface receptors or ligands to enhance MSC adhesive properties, again implying that 'native' MSC do not capably bind to selectins (Sarkar et al., 2010).Improving MSC homing may be an important step to support the therapeutic

effects of MSC. One of the techniques which has been widely used is cell surface modification which increases sialyl Lewis X (SLeX) because of its role in leukocyte rolling (Teo et al., 2012). Another technique used for cell surface modification is the application of biotinylated lipid vesicles which are fused to MSC to bind streptavidin-linked SLeX (Sarkar et al., 2011). We suggest that manipulation may not be necessary for frankly damaged vessels but may be necessary to increase recruitment to intact EC even if inflamed.

The highly significant reduction in adherent cells noticed with increased wall shear rate, suggests that shear rate and stress play critical roles in EPC and MSCadhesion. This is also the case for leukocytes and platelets, but adhesion occurs at higher shear rates the smaller the cells (upto about $300s^{-1}$ for leukocytes and $>1000s^{-1}$ for platelets) (Watts et al., 2013). At high shear rate conditions, the contact time between cell adhesion molecules and surface substrate is shortened which subsequently decreases likelihood of forming a receptor-ligand bond. In addition, the force caused by shear stress increases, tending to break bonds that do form. Here, cells were barely slowed down by P-selectin, so that a two step adhesion did not appear to occur. On the matrix proteins, all cells appeared to come to an abrupt halt and they did not then move, except to spread gradually.

There has been no research study the role of stem cell diameter in adhesion. Watts et al. reported that cell size, margination, and a cell-free layer influenced the adhesive abilities of platelets and leukocytes (Watts et al., 2013). They showed that platelets were able to adhere at high shear rate and they attributed this to their small size which subsequently minimized the force experienced and their velocity. Also, they suggested that leukocytes were not able to adhere at high shear rate but could

be recruited in venules. As expected, we found cell velocity near the wall increased the bigger the cells, and with increasing shear rate. The velocity measured here at 70s-1 was about 800μm.s-1 which is what is expected for about 10μm radius (see Section 1.3.2.3). The values for leukocytes at a shear rate of 280s-1 where adhesion is just retained on P-selectin was about 1400μm.s-1, which is what is expected for about 5μm radius. Thus the relative velocities of the two cells is what might be expected from MSC being double the size of leukocytes. Interestingly, force goes up with radius squared for a given shear stress (see Section 1.3.1.3) This may explain why the limiting velocity for leukocytes is higher, because at that velocity or shear rate of $280s^{-1}$, the force should be similar to MSC at $70s^{-1}$ (because of their doubled radius compared to leukocytes). It seems that the shear sensitivity of MSC can be largely explained by their size versus leukocytes, which may be surprising as the leukocytes are using a specialised capture receptor, selectins, that act quickly, while the MSC are binding to unspecialised matrix receptors. This subject is considered again in Chapter 6 where mechanisms of attachment were investigated.

Another factor in vivo is margination. During inflammation, fibrinogen production increases, accompanied by decline in venous flow rate and increase in red cell aggregation, which leads to margination enhancement for leukocytes and increased adhesion (Watts et al., 2013). Margination is dependent on cell size, with larger cells tending to move inward in the flow more. MSC are much larger than platelets or leukocytes, and it is not clear what might happen to large MSC in flowing blood. This is considered again in Chapter 5.

3.4 CONCLUSIONS

MSC from several sources showed little adhesion to selectins from flow, but did adhere to collagen or fibronectin. This adhesion was surprisingly effective considering their large size and the expectation that they are not adapted for adhesion from the circulation in the same way as leukocytes are. Our results confirm that the cell adhesion cascade is affected by cell diameter, but also depends on the nature of the receptors and ligands. Our surface-coating system is very useful to study the efficiency of MSC delivery and the results suggest that MSC will bind better to damaged vessels than inflamed vessels. Whether MSC can specifically home remains unclear, although our results might suggest they can e.g., in wound healing. While many could get physically trapped in the microcirculation in vivo, they might adhere in a larger damaged vessels of the arterial or venous circulation.

Chapter 4 : SPREADING AND MIGRATION OF MSC FROM DIFFERENT SOURCES

4.1 INTRODUCTION

If MSC are deposited in a damaged vessel, cell adhesion and spreading on extracellular matrix (ECM) are essential for cell motility, migration into tissue and survival. Cell spreading is the initial kinetic process following adhesion. Cell spreading ability is advantageous to the cell, as demonstrated by cell culture pioneers who established the importance of cell-substrate contact area (Folkman and Moscona, 1978). They suggested that the contact area was a determinant of cell proliferation capabilities (Folkman and Moscona, 1978). Moreover, others showed spreading to determine the fate of the cell, with failure leading either to a dormant state (Stoker and Rubin, 1967) or cell death (Chen et al., 1997). The active mechanisms which control cell spreading behaviour are not fully understood. As cells adhere to the surface, they become flattened and deformed. At the molecular level, the initiation of a signalling cascade is caused by the binding between cellular integrins and the matrix (Hynes, 2002). The signalling events trigger a series of morphological changes of the cell and subsequently produce contractile forces which can support migration (Huttenlocher and Horwitz, 2011).

In the current chapter, we set out to investigate the behaviour of MSC after they had adhered to matrix proteins from flow. Contact area was measured as a function of time for different cell types captured on different surfaces. Subsequently, we measured the ability of the MSC to migrate through porous filters with different coatings. The goal was to compare the behaviour of BMMSC,WJMSC and TBMSC and to assess whether any differences might affect their fate once deposited in damaged tissue.

To investigate cell spreading, we used phase-contrast microscopy during and after perfusion of MSC in the cell adhesion assays described in Chapter 3. This enabled us to monitor quantitatively the dynamics of spreading of individual cells. The cell behaviour was analysed offline using Image-Pro software as described in Section 2.4.2c. Spread cells were defined as large cells with extensive visible filopodia (microspikes) and showing changes from phase-bright to phase-dark appearance (see e.g., Figure 4.1). Non-spread cells were phase-bright, smaller round cells with little or no membrane protrusions. We considered partially phase-bright and phase-dark cells, as spreading cells. Degree of spreading was characterised by measuring the area of the cells. To investigate cell migration, MSC were allowed to settle and adhere on 8μm pore Transwell filters which had been coated either from the bottom or from the top with collagen, fibronectin or albumin. MSC were incubated for 24 hours at 37^{o}C, and cells retrieved from top or bottom of the filter by trypsin and counted using a Coulter counter.

4.2 RESULTS

4.2.1 Rate and degreee of spreading of MSC on collagen or fibronectin

Figure 4.1 shows phase contrast images of the spreading behaviours of three different cell types; (A) WJMSC, (B) BMMSC, and (C) TBMSC at different time points on collagen. The cells were perfused over the collagen for 4 minutes followed by washout of non-adherent cells. They are representative of multiple images taken at different sites in the microslide at each time, and were used to quantify the changes in the proportion of cells spread with time. The images at 5 minutes (i.e., after 1min of washout) show that all types of adherent cells attached to collagen

surface showed no sign of spreading. The cells appear small and round with little membrane protrusion. At time point 15 minutes, the membrane of some BMMSC started to deform and protrude, whereas WJMSC and TBMSC remained round with little change. At 25 minutes, some BMMSC had spread out completely while WJMSC and TBMSC had started showing some changes in their cells membrane. By 35 minutes, most BMSC, TBMSC and WJMSC had spread.

Figure 4.2 shows similar images for cells adhered to fibronectin. Again, at time point 5 minutes, adherent cells showed little sign of spreading. At time point 15 minutes, however, the membrane of WJMSC, BMMSC and TBMSC had started to deform and spread cells were evident. At 25 minutes and at 35 minutes, nearly all BMSC and TBMSC and WJMSC were completely spread.

Quantitative data derived from the microsope images for percentage spread with time are shown in Figure 4.3. Comparison of the percentage of spreading of WJMSC, BMMSC, and TBMSC on collagen is shown in Figure 4.3A. At time point 5 minutes, all cell types showed zero percentage spreading. At time point 15 ,25, 35 minutes, there was steady progressive spreading, with BMMSC spreading percentage being consistently and significantly greater than WJMSC and TBMSC, which were similar to each other. This data shows that BMMSC spread more efficiently on collagen compared to the other cells types.

Figure 4.3B shows similar date for progressive spreading on fibronectin. Taken as a whole, it is evident that MSC spread more rapidly and effectively on fibronectin compared to collagen. At time point 15 minutes, 95% of BMSC had already spread while about 50% of WJMSC and TBMSC had spread. At later time points , BMMSC and TBMSC continued to spread, with TBMSC reaching 100%

spreading and WJMSC reaching a value of about 80% by 35 minutes. Overall, BMSC spreading was faster compared to WJMSC or BMSC. Interestingly, WJMSC and TBMSC behaved similarly in the first 15 minutes, after which TBMSC showed faster spreading behaviour than WJMSC.

To gain further insight into the kinetics of spreading, in separate experiments, individual fields were recorded after washout and the area of the same cells repeatedly measured over time. Figure 4.4 shows phase contrast images of the size of adherent cells for (A) WJMSC, (B) BMMSC, and (C) TBMSC on a collagen surface. Figure 4.5 shows phase contrast images of the size of adherent cells on a fibronectin surface. Figure 4.6 illustrates how the phase contrast images of a single adherent cell at different time points were analysed using Image-pro software.

Figure 4.7 compares the cells area measurements of WJMSC, BMMSC, and TBMSC on collagen and fibronectin at different time points. In Figure 4.7A, on a collagen surface, it can be seen that all cell types showed progressive increase in cell area with time. Overall, the order of areas was BMMSC>TBMSC~WJMSC, with the data showing statistically significant difference in cell size between BMMSC and TBMSC.

In Figure 4.7B, on a fibronectin surface, again, all cell types showed increase in area with time. However, on fibronectin BMMSC and TBMSC had similar large areas, and both were significantly greater in area than WJMSC. Comparing the surfaces, it was evident that the BMMSC and TBMSC spread to larger final sizes on the fibronectin than collagen, while WJMSC reached similar sizes on each surface. On both surfaces, BMMSC spread to greater extent than WJMSC.

In summary, fibronectin tended to support more efficient spreading of MSC than collagen. BMMSC spread more rapidly, in great numbers and to a greater extent than WJMSC. TBMSC had an intermediate behaviour. Because of the clear differences in the more widely studied types of MSC, from bone marrow and Wharton's jelly, and the poor proliferation potential of TBMSC, subsequent studies of cell migration were limited to the first two types of MSC.

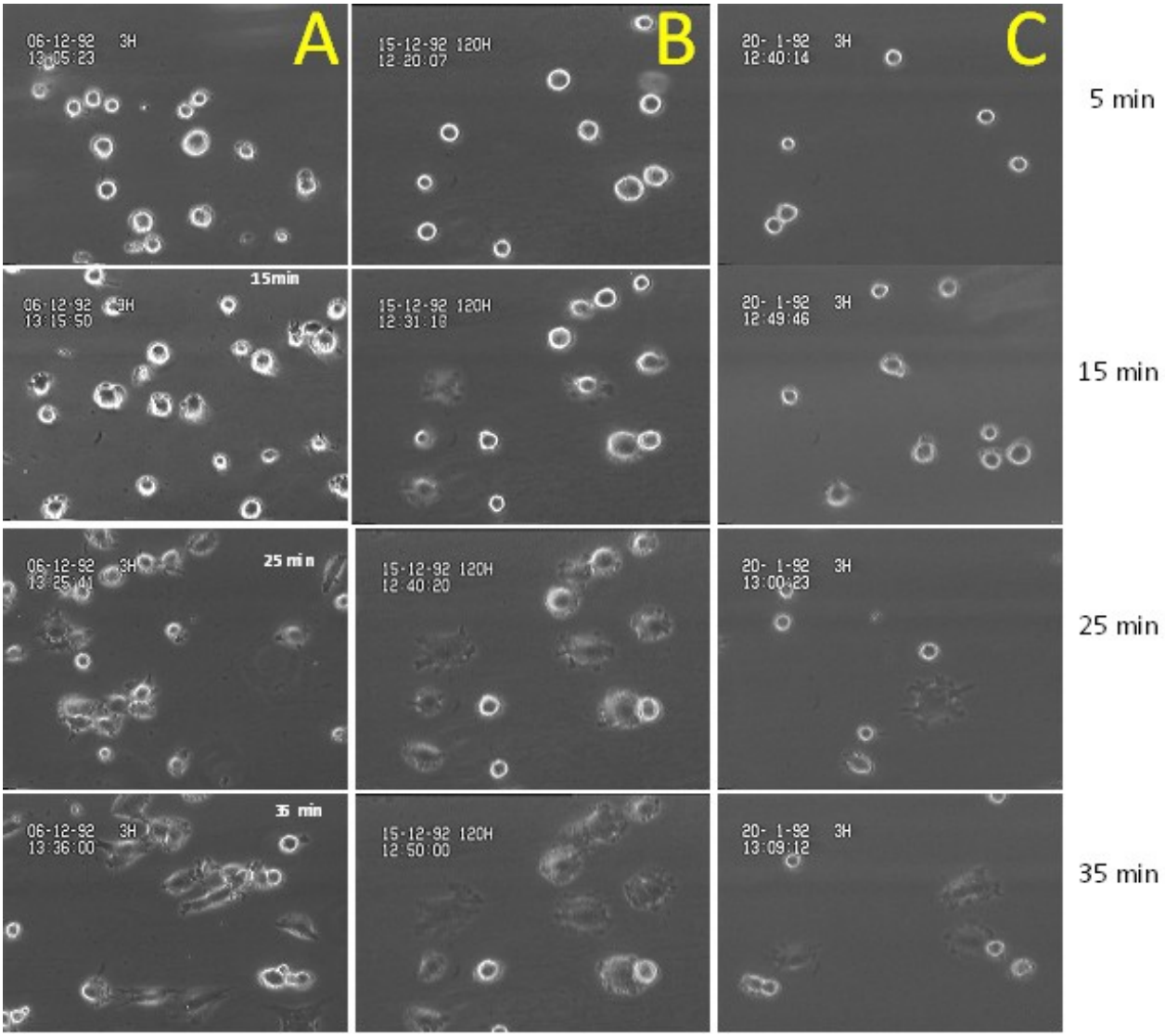


Figure 4-1: Phase contrast images of spreading of (A) WJMSC (B) BMMSC and (C) TBMSC on collagen

Images recorded at 5, 15, 25 and 35 minutes after the start of perfusion for 4 minutes at a wall shear rate of $35s^{-1}$. Images are from separate fields in an experiment representative of 3 others with similar results.

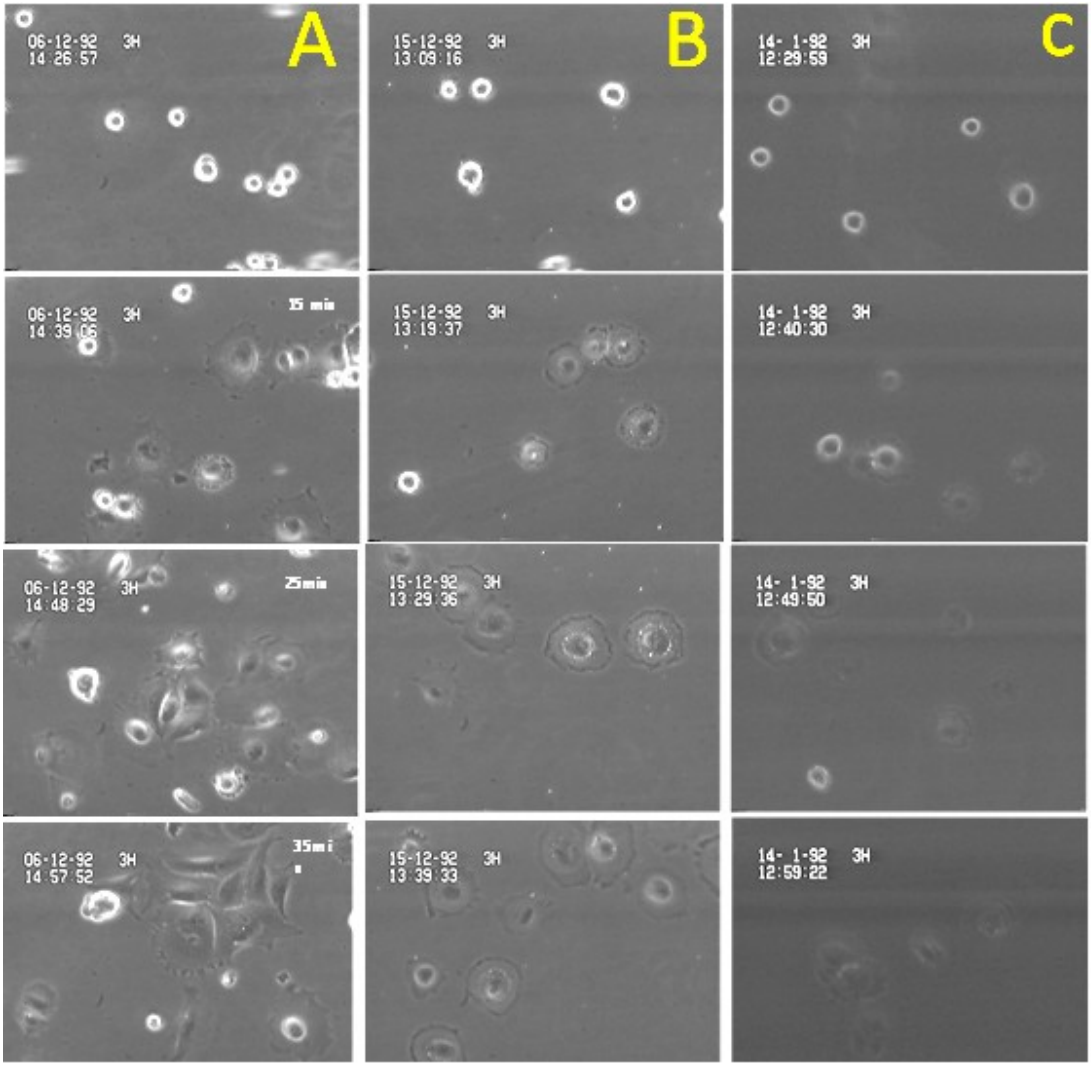


Figure 4-2: Phase contrast images of spreading of (A) WJMSC (B) BMMSC and (C) TBMSC on fibronectin.

Images recorded at 5, 15, 25 and 35 minutes after the start of perfusion for 4 minutes at a wall shear rate of $35s^{-1}$. Images are from separate fields in an experiment representative of 3 others with similar results.

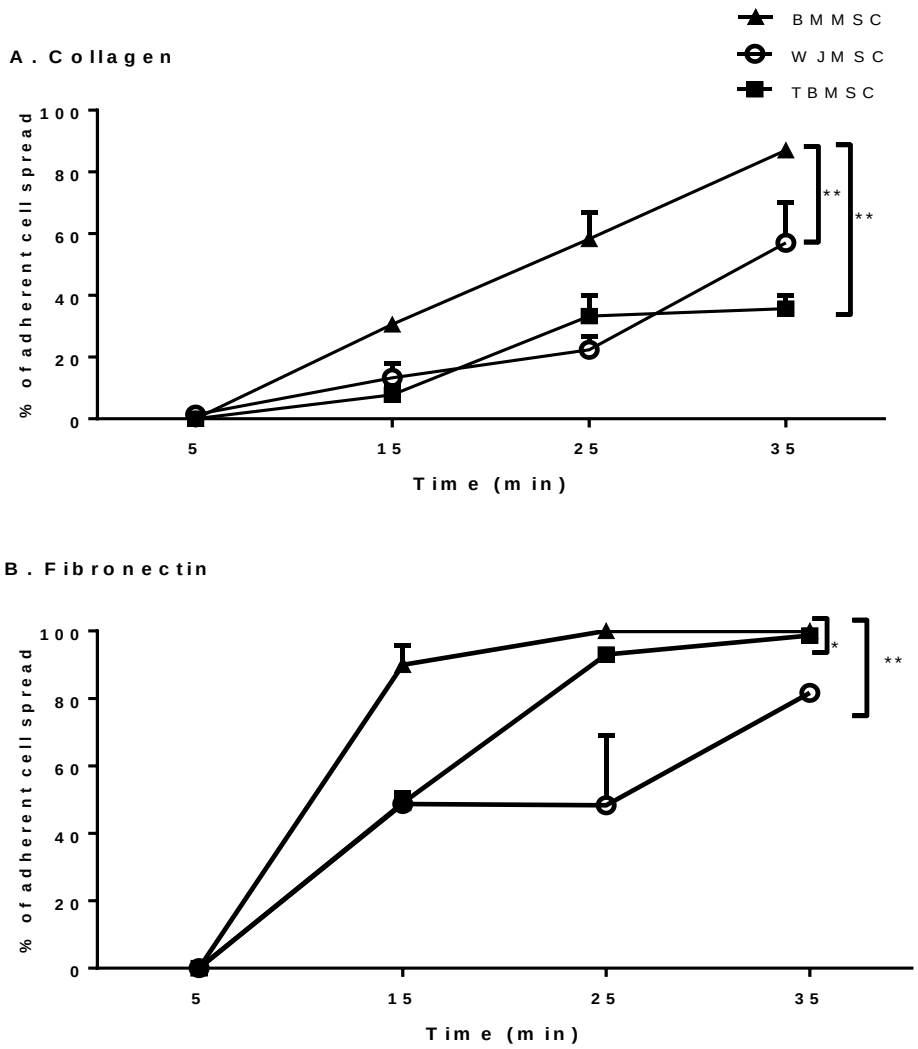


Figure 4-3: Comparison of the percentage of adherent WJMSC, BMMSC and TBMSC spread with time on A. collagen, B. fibronectin. to different surfaces (A) on collagen and (B) on fibronectin surface:

Images were analysed at 5, 15, 25 and 35 minutes after the start of perfusion for 4 minutes at a wall shear rate of $35s^{-1}$. Data are mean ± SEM from 3-4 experiments. For combined data from A and B, ANOVA showed significant effects of cell type and substrate on percentage of cells spreading ($p<0.01$ in all cases). *=$p<0.05$; **=$p<0.01$ for post-hoc comparison of cell types, combining values for all time points, by Bonferroni test

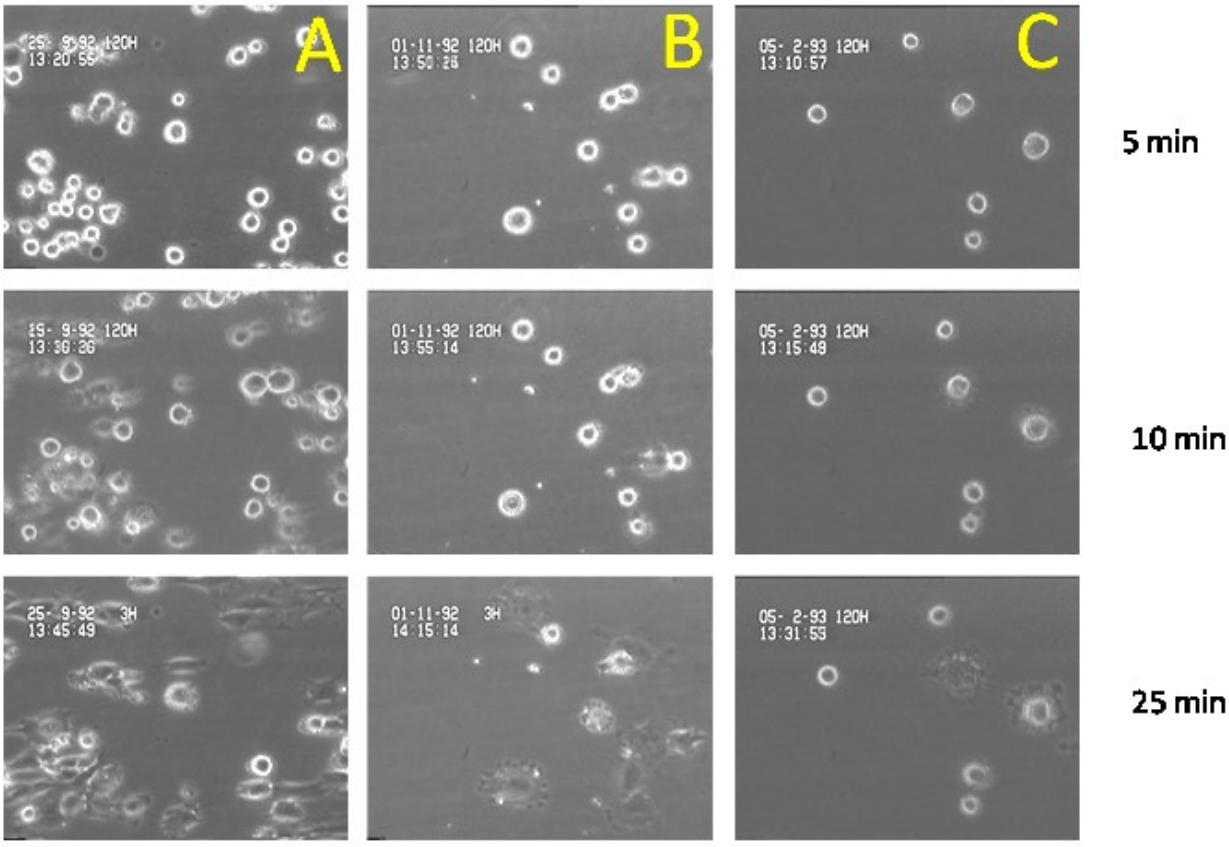


Figure 4-4: Phase contrast images of (A) WJMSC (B) BMMSC and(C)TBMSC adherent to collagen, with the same field followed over time.

Images recorded at 5, 15, 25 and 35 minutes after the start of perfusion for 4 minutes at a wall shear rate of $35s^{-1}$. For each cell type, images are from a single fields in an experiment representative of 3 others with similar results.

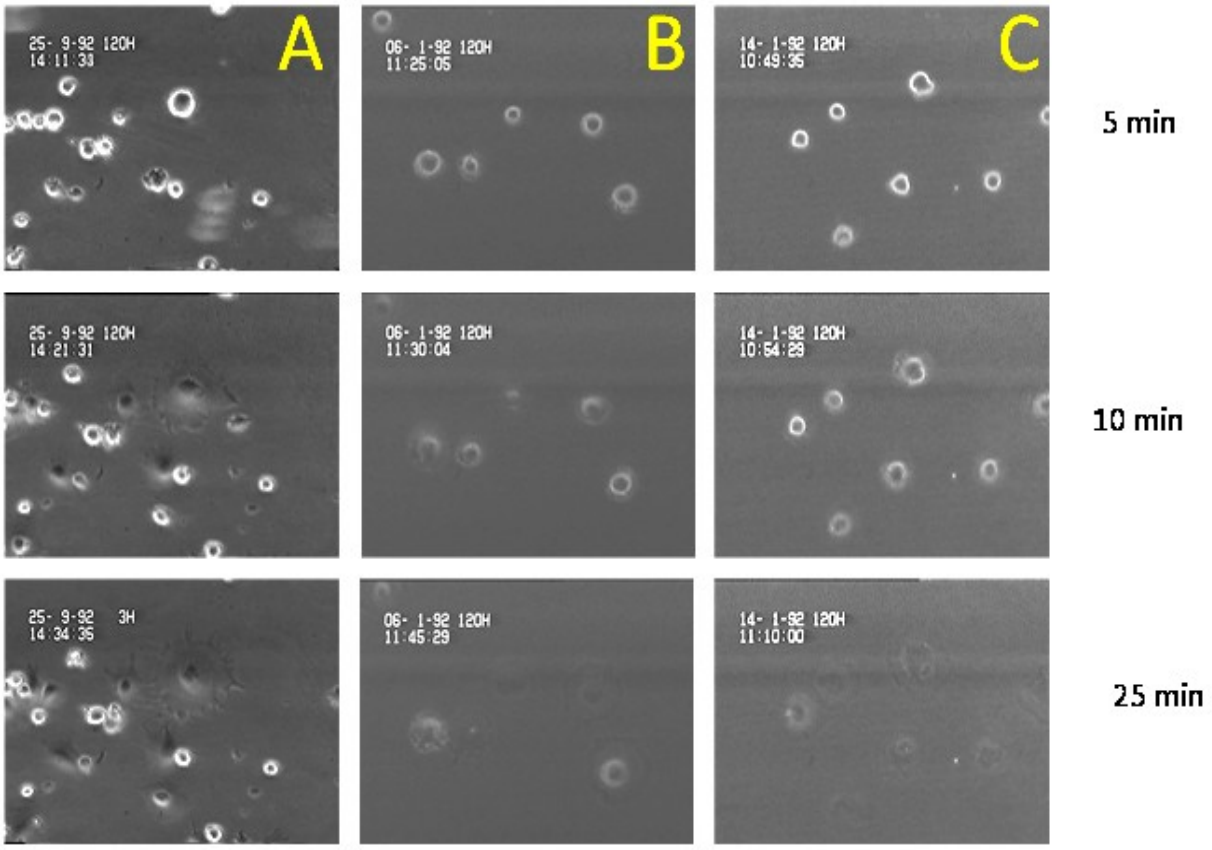


Figure 4-5: Phase contrast images of (A) WJMSC (B) BMMSC and(C)TBMSC adherent to fibronectin, with the same field followed over time.

Images recorded at 5, 15, 25 and 35 minutes after the start of perfusion for 4 minutes at a wall shear rate of $35s^{-1}$. For each cell type, images are from a single fields in an experiment representative of 3 others with similar results.

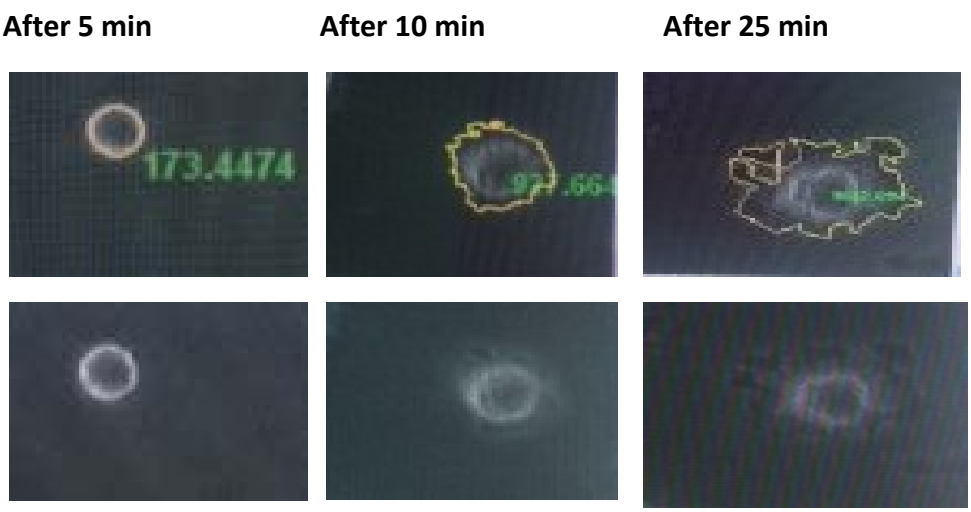


Figure 4-6: Measurement of the area of individualWJMSC on Fibronectin surface.

Images at 5, 10, 25 minutes after the start of perfusion for 4 minutes at a wall shear rate of $35s^{-1}$. Individual frames were digitised and analysed using ImagePro software.

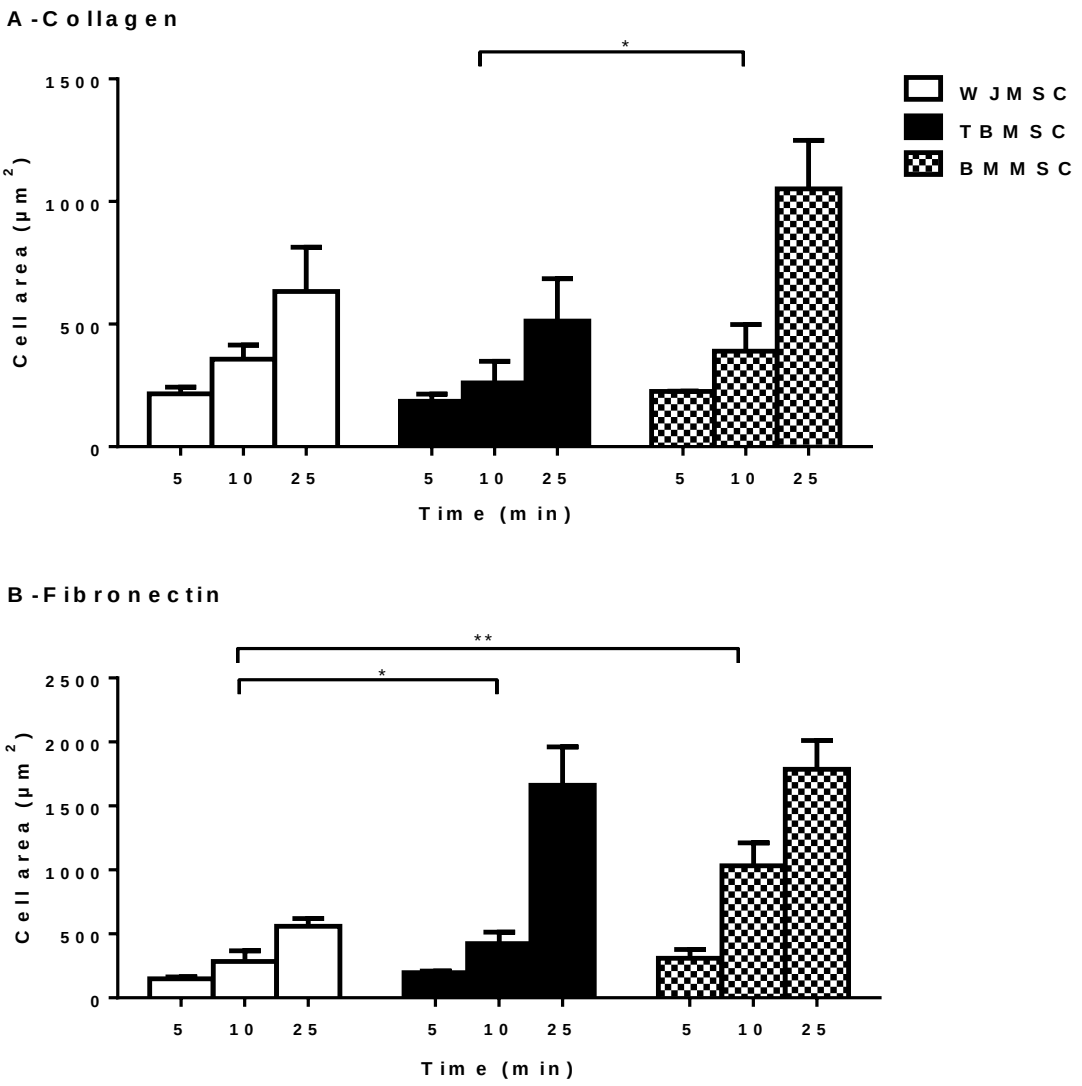


Figure 4-7: Comparison the areas of WJMSC, BMMSC and TBMSC spread on A. collagen or B. fibronectin for different times.

Images were analysed at 5, 10 and 25 minutes after the start of perfusion for 4 minutes at a wall shear rate of $35s^{-1}$. Data are mean ± SEM from 3-4 experiments. For combined data from A and B, ANOVA showed significant effects of cell type, substrate and time on cell area. *=$p<0.05$; **=$p<0.01$ for post-hoc comparison of cell types, combining values for all time by Bonferroni test.

4.2.2 Migration of MSC through porous filters coated with collagen or fibronectin

We compared migration ability for BMMSC and WJMSC using Transwell filters with 8 μm diameter pore. The filters were first coated on the top or the bottom with fibronectin or collagen. BSA was used as a control protein. The filters were subsequently immersed in culture medium which contained FCS, so that all surfaces were effectively 'blocked' with albumin. Comparisons were thus mainly examining the effects of adding collagen or fibronection to BSA on either the upper or lower surface.

In these experiments, the original samples and the cells retrieved from the above and below the filter were counted using a Coulter counter which also measured their diameter. We noticed that the diameter of the retrieved cells was smaller compared to the original sample. (See figure4.11). We thus analysed the migration for the whole population (7-27μm) and for the larger population (12-27μm) separately, to see if there was a link between size and migration.

Figure 4.8 compares Transwell migration for BMMSC and WJMSC after coating filters with collagen, fibronectin or albumin alone. In Figure 4.8A, data are shown for top-coated filters and analysis of all cells (gated from 7-27 μm). In Figure 4.9B, data are shown for top-coated filters and analysis of cells between 12-27 μm in diameter. Figures 4.9C and D show results for the same analyses but where the bottom of the filter was coated. For albumin coating alone, about 20% of MSC migrated across the filter in 24h. Fibronectin coating tended to increase migration compared to albumin. However, collagen coated on top gave a similar migration to albumin, while collagen coated on the bottom gave a higher migration than albumin. Overall, there was a higher level of migration for the WJMSC than for the BMMSC,

with this difference more clearly apparent for the filters with a bottom coating. There was also a tendency to observe a higher degree of migration when the smaller sized cells were included in the analysis.

To make the comparison between coating the top or bottom of filters clearer, the data are re-plotted in Figure 4.9 as Top vs. Bottom, separately for the different cells. It is evident that coating the bottom gave greater migration. This effect was stronger for the WJMSC then the BMMSC. It was again noticeable that the percentage migration was greater when the smaller sized cells were included in the analysis. For example, Figure 4.10 compares directly the results for all cells vs. larger cells only, for MSC migrating on filters with the bottom coated with collagen, fibronectin or BSA.

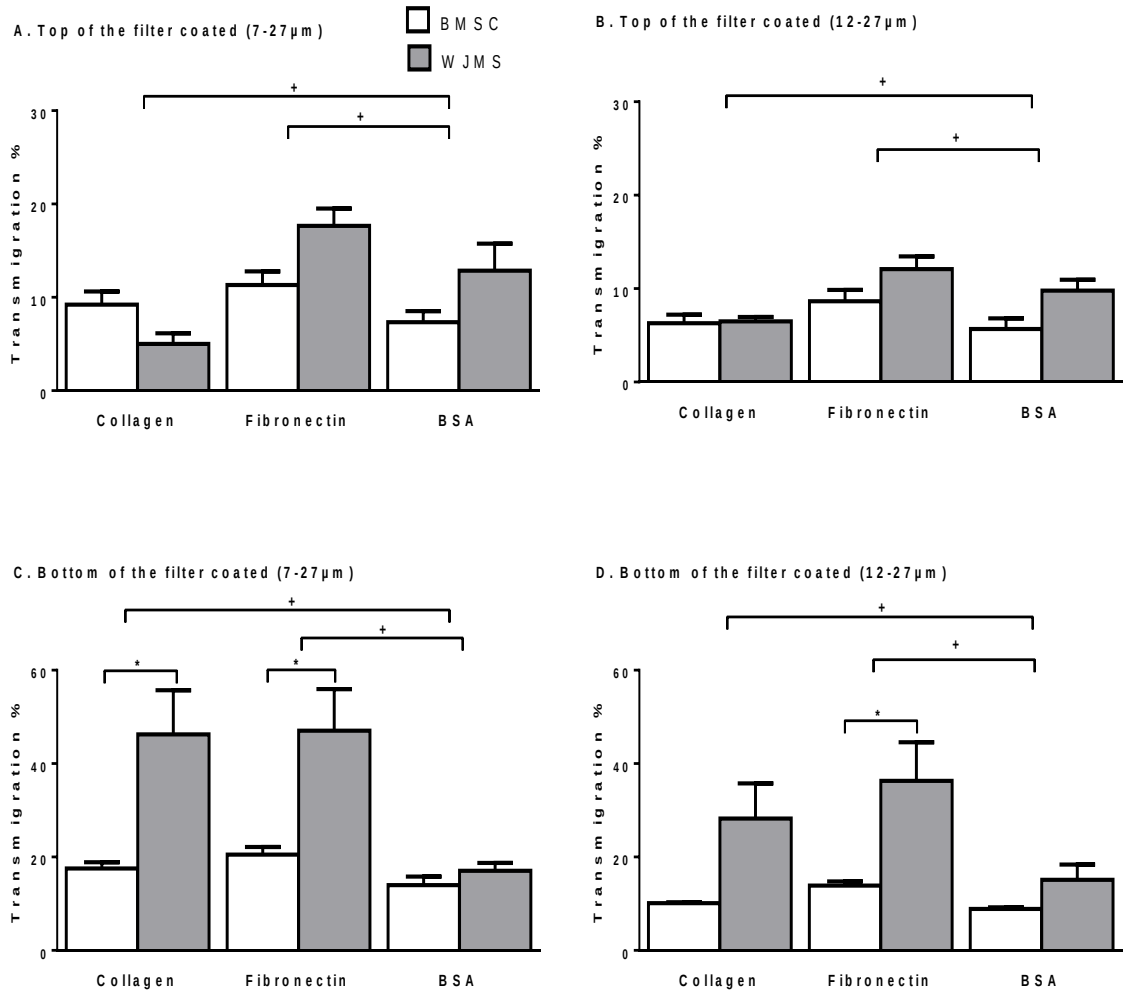


Figure 4-8: Transwell migration: comparison between BMMSC and WJMSC for filters pre-coated on the top (A,B) or bottom (C,D), with collagen, fibronectin or albumin.

Data were analysed for all cells detected by the Coulter counter (A,C), or for only larger cells with diameter above 12µm (B,D). Migration was allowed for 24hrs at 37°C. Data are mean ± SEM from 3 experiments. ANOVA showed that there were significant effects on migration of cell type in C and D ($p<0.01$ in each case) and of coating substrate in A, B and C ($p<0.05$ in all cases). +=$p<0.05$ for post-hoc comparison of adherent substrates including values for both cell types, by Bonferroni test. *=$p<0.05$, ** = $p<0.01$ for comparison of cell types by Bonferroni text.

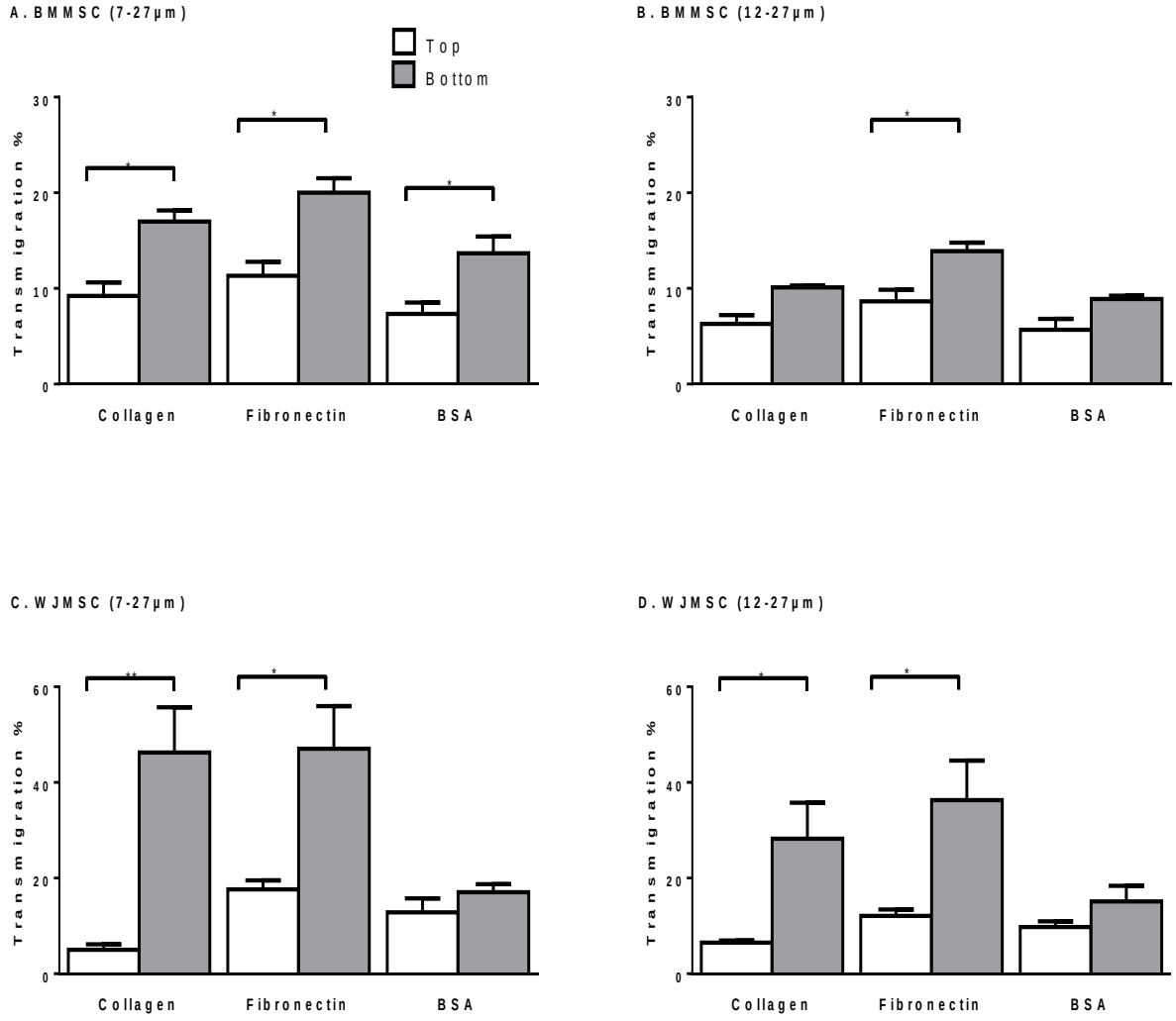


Figure 4-9: Transwell migration: comparison between coating the top or bottom of filters with collagen, fibronectin or albumin for BMMSC (A,B) or WJMSC (C,D).

Data were analysed for all cells detected by the Coulter counter (A,C), or for only larger cells with diameter above 12µm (B,D). Migration was allowed for 24hrs at 37°C. Data are mean ± SEM from 3 experiments. ANOVA showed that there were significant effects of the surface coated (Top vs. Bottom; $p<0.01$ in all cases). *=$p<0.05$, ** = $p<0.01$ for comparison by Bonferroni text.

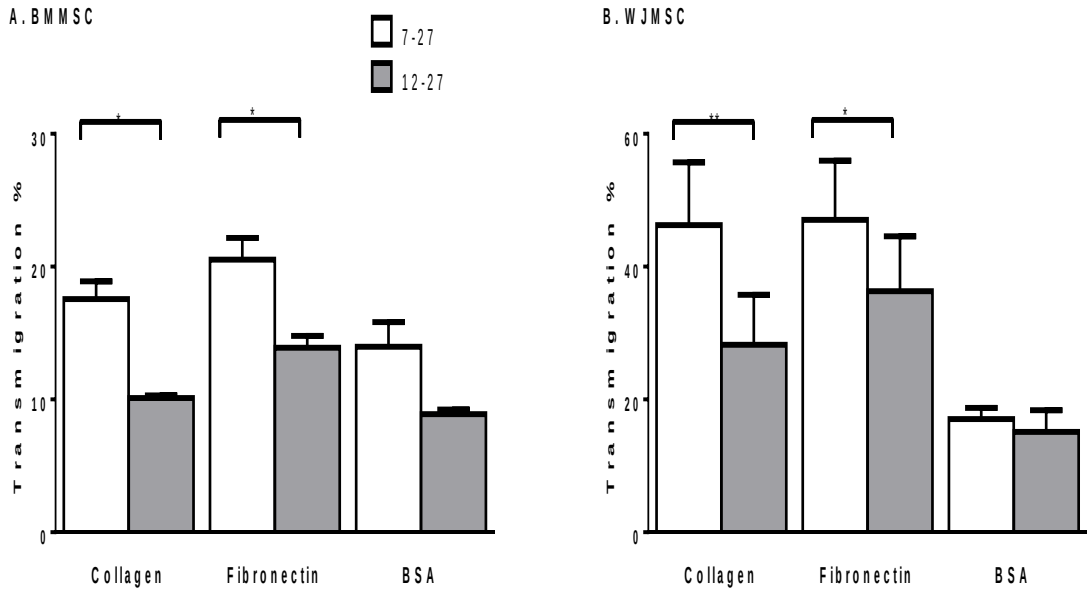


Figure 4-10: Transwell migration: comparison between all cells detected by the Coulter counter (7-27µm diameter) and larger cells (12-27µm diameter), for BMMSC (A) or WJMSC (C).

Migration was allowed for 24hrs at 37°C. Data are mean ± SEM from 3 experiments. ANOVA showed that there were significant effects of cell size on migration ($p<0.01$ for each MSC type). *=$p<0.05$, ** = $p<0.01$ for comparison by Bonferroni text.

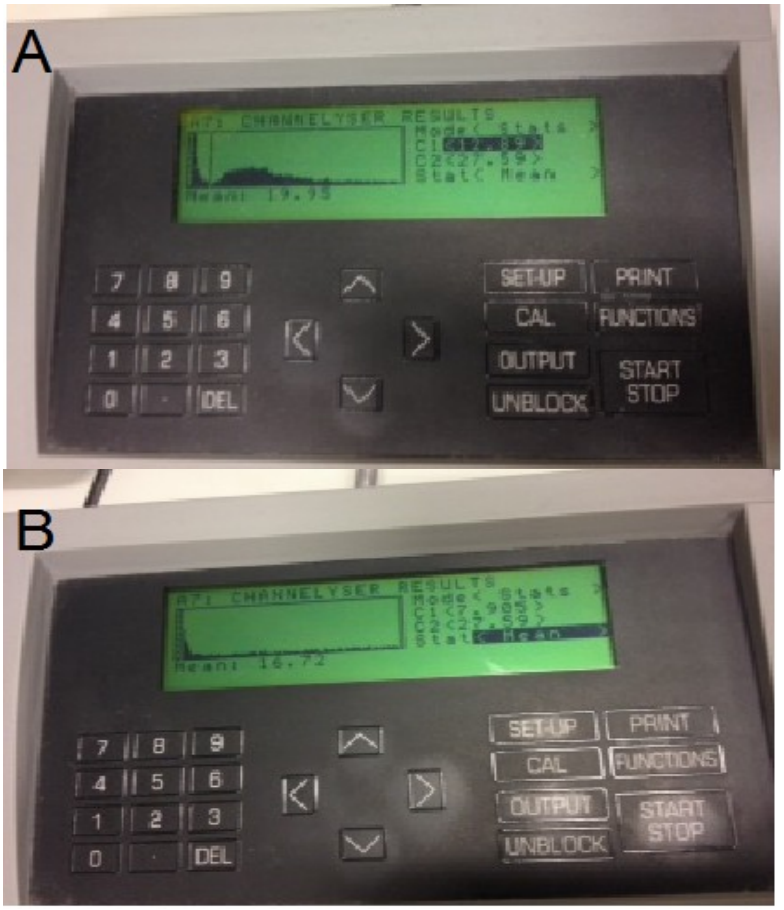


Figure 4-11: : cells size diameter distributionA original sample and B the cells retrived from below the filter after 24hours incubation: the original samples and the cells retrieved from the below the filter were counted using a Coulter counter which also measured their diameter. We noticed that the diameter of the retrieved cells was smaller compared to the original sample.

4.3 DISCUSSION

Having studied adhesion of MSC to matrix proteins, collagen and fibronectin, the next aim of this thesis was to compare the subsequent behaviour of BMMSC, WJMSC, and TBMSC. This would be relevant to their fate when deposited in damaged tissue and might reveal the factors which affect their role there. To do that, we studied their spreading behaviour on collagen and fibronectin, and then their migration through filters coated with these proteins.

Firstly, on collagen, BMMSC spread more efficiently and faster than WJMSC and TBMSC, which behaved similarly. On fibronectin BMSC also spread faster compared to WJMSC or TBMSC, although all cell types approached 100% spread with time. On both surfaces, BMMSC spread to cover a larger area than the TBMSC and WJMSC. We noticed an interesting findings regarding the spreading behaviour of TBMSC on fibronectin, which behaved similarly to WJMSC until about 15 minutes, but then TBMSC spread faster and to a larger area than WJMSC. On collagen the WJMSC and TBMSC behaved nearly identically. The behaviour on fibronectin might relate to differences in signals from the adhesive interaction with this protein (see below). Overall, spreading was more efficient, more rapid and to a greater area on fibronectin than on collagen.

These findings are consistent with those of Ogura N et al. (2004), who found that fibronectin stimulated adhesion, spreading and growth of human BMMSC (Ogura N, 2004). In addition, Veevers et al. (2011) found a receptor, α5β1-integrin, which supported cross talk between growth factor receptor and integrin receptor signals on fibronectin (Veevers-Lowe et al., 2011). This interaction between cell

and fibronectin led to a chain of actions which caused greater phosphorylation of PDGFR-β and subsequently promoted the adhesion and migration of the human BMMSC. They noted that collagen types I or IV had little effect on PDGFR-β activity compared to fibronectin. Neither of these studies evaluated MSC from other sources, and so we have shown for the first time there are cell-specific variations in spreading as well as adhesion, in addition to substrate-specific variations.

The potential therapeutic uses of MSC may depend on their ability to migrate into tissue as well as adhere and spread on the surface. We thus assessed migration across 8μm pore filters coated with different proteins, either on the upper or lower surface. We limited our studies to use of BMMSC and WJMSC, as TBMSC were difficult to obtain and proliferate, and have not been widely studied or used in therapies. We discovered several interesting trends. In contrast to spreading, WJMSC were more effective in transmigration then BMMSC on all surfaces. When the top of the filter was coated, there were was little difference between coating with collagen or albumin, but coating with fibronectin encouraged migration slightly. When the bottom was coated, there was greater migration overall than if the top was coated, and both collagen and fibronectin increased migration compared to albumin. Interestingly, the effect of coating the bottom of the filter appeared to be greater for WJMSC than BMMSC. When the bottom was coated, this showed the greatest difference between the cells. Finally, we unexpectedly observed that there was a greater spread in cell size after migration then in the original cell population, with a greater proportion of small cells (diameter <12μm) observed. These smaller cells showed more efficient migration. Thus, larger cells may migrate less easily through

gaps than smaller ones, although this applied to both types of MSC, which had similar diameters.

To our knowledge, this is the first comparison of the ability to migrate for MSC from different sources on different surfaces. Our findings could be linked to the study noted above by Veevers et al. (Veevers-Lowe et al., 2011), who found that binding to fibronectin promoted signalling through PDGFR-β and trans-filter migration when the bottom side of the filter was coated. They found that the interaction with fibronectin was dependent on α5β1-integrin and not αvβ3-integrin. They also found that binding to collagen did not induce the same level of signalling as fibronectin. However, we found similar migration for collagen or fibronectin coating of the lower side, although coating of the upper side with fibronectin did promote migration more than collagen. The wider potential importance of such observations was illustrated by studies of knockout mice deficient in genes encoding PDGFR-β; these died at late embryonic stages because of failure of mural cell recruitment (Lindahl et al., 1997). Others have shown that migration of 'multi-potent stromal cells' and fibroblasts are also promoted on fibronectin, but through effects on epidermal growth factor-receptor (EGFR) (Wu et al., 2011) (Maheshwari et al., 1999). Cell migration typically consists of protrusion at the front, followed by adhesion there and detachment at the rear (Ridley et al., 2003). Activation of EGFR induces a signal network which promotes the front lamellipod protrusion and dynamic changes in adhesion (Wells A, 2006). The current transfilter assays lack ability to study these dynamic processes in detail, although the assays of adhesion and spreading did follow kinetics of the early events.

The role of migration following systemic infusion of MSC for therapeutic uses is challenging to characterize, and there is limited data on MSC adhesion and migration mechanisms in vivo. There have been a number of studies of migration through endothelial monolayers in vitro. The mechanisms by which leukocytes adhere and go through the endothelium barrier to integrate into the tissue haves been widely studied (Ley et al., 2007) (Woodfin et al., 2010). While it is believed that the process may be similar for MSC, there is limited data to support this. Schmidt et al. (Schmidt et al., 2006) showed slow integration and then migration of MSC through an endothelial cell monolayer over about 2 hours. Steingen et al. (Steingen et al., 2008) reported that MSC migrated through and under endothelial cells stimulated with cytokines in 2-4 hours, assisted by VCAM/α4β1-integrin interaction. They showed that the degrading enzyme MMP-2 was generated during penetration of the endothelium. Chamberlain et al (Chamberlain et al., 2008) showed that murine MSC adherent to murine aortic endothelial cells (MAEC) produced microvillous processes (filopodia) which then were extended to form pseudopodia in multiple directions, and slowly moved into and under the endothelial monolayer, again in hours. In this study, the authors also found that chemokines CXCL9, CXCL16, CCL20 and CCL25 promoted transmigration of MSC through MAEC cultured on filters over 16 hours. Several studies have shown expression of chemokine receptors on human MSC (Chamberlain et al., 2008) (Ringe et al., 2007), and there are common chemokine receptors between leukocytes and MSC (Minguell et al., 2001).

In our studies of transmigration, endothelial cells were not present and we did not add chemokines, but we did find a high proportion of WJMSC could migrate across filters when the bottom was coated. BMMSC did show an effect of coating

the lower surface on migration, but not as strong as for WJMSC. It is possible that migrated cells released chemotactic factors when adherent on the lower surface which attracted those on top, and that WJMSC were more effective in this respect. There were however several differences in adhesion and spreading that may explain differences in migration, which may be the key event in recruitment in vivo. It is also possible that signalling through integrin receptors noted above, is different for the different types of MSC. Investigation of the roles of specific integrins in the adhesion, spreading and migration behaviour seen here might help explain differences between cells and between coating proteins. Studies of the integrins required for adhesion are described in Chapter 6.

Bringing together the results from this Chapter and Chapter 3, we can consider the links between adhesion, spreading and migration for the different MSC. Comparing BMMSC and WJMSC for which we have the most complete data, WJMSC adhered in greater numbers from flow. BMMSC spread more efficiently and quickly, but WJMSC migrated in greater numbers, especially when the lower surface was coated. Thus, spreading does not appear to promote migration. In general, migration speed depends on strength of attachment but also the ability to detach in a regulated manner, with the regulation of formation and loss of integrin bonds being critical factors (Ridley et al., 2003, Huttenlocher and Horwitz, 2011). Too strong an attachment may slow migration. The adhesion we measured here shows ability to support capture from flow, and this may not be the same as the adhesion developed after attachment, and not be closely linked to migration which starts some time after capture. Thus the spread cells may become too strongly attached to move quickly. In vivo it is difficult to predict which of the above

properties will be more important for therapy. WJMSC should be more effectively captured in damaged tissue and migrate faster into the tissue. On the other hand, BMMSC may spread and integrate into the wall faster if e.g., mechanically trapped in small vessels. Further studies in flow models coated with endothelial cells, or by intravital microscopy, might be able to answer these questions about the effectiveness of the different MSC.

4.4 CONCLUSIONS

The cell adhesion, spreading and migration assays described here have provided information on behaviour of MSC that may be relevant to their recruitment to blood vessels and tissue if injected into the blood. We found clear differences in behaviour of MSC that are used in current research and therapy, from bone marrow and Wharton's jelly. Trabecular bone is less frequently used to obtain MSC, and TBMSC behaved similarly to the WJMSC.

The studies described show the most complete comparison of adhesive and migratory behaviour of different MSC to date. However, they also raise questions about the mechanisms supporting adhesion and migration, and whether the adhesive behaviour would be the same if the MSC were added to blood. The latter question is investigated in the next Chapter.

Chapter 5 ADHESION OF MSC TO MATRIX PROTEINS FROM FLOWING WHOLE BLOOD AND INTERACTION WITH PLATELETS

5.1 INTRODUCTION

Studies described in Chapter 3 showed that MSC could bind to matrix proteins, collagen and fibronectin, but not selectins, at wall shear rates up to about $70s^{-1}$, and suggested that the different MSC tested adhered in the order WJMSC>BMMSC>TBMSC. Having studied the spreading behaviour and migration of adherent cells on the different matrix proteins, we decided next to evaluate adhesion from flowing blood, to see if the same order of adhesion occurred or whether presence of other cells altered behaviour.

There are no studies of adhesion of MSC from flowing blood in the literature to our knowledge. Adhesion from blood would be more relevant to the situation when MSC are used for therapy by injection into the circulation. It is known that margination in the blood stream is important for adhesion of leukocytes and platelets (Abbitt and Nash, 2003, Watts, 2015), and it is not clear whether this would happen with large cells like MSC. For instance, for leukocytes, slow flow and red cell aggregation promote margination, but for platelets, these conditions are not so effective (Watts, 2015). Since this thesis was started, some studies have been reported that suggest BMMSC may bind to platelets in blood and be deposited in the microcirculation with them (Langer et al., 2006, Teo et al., 2015)In addition, it has been reported that MSC can bind to platelets stuck to collagen (Jiang L et al., 2012). Thus interaction with platelets might affect adhesion from blood.

In this chapter, we compared adhesion form isolated MSC to adhesion for MSC in whole blood perfused over the coated surfaces at a wall shear rate of $35s^{-1}$. The number of adherent cells of each cell type of MSC was counted for the different surfaces and expressed as a percentage of all those perfused, as described in Section

2.4.3a. Results from these experiments led us to also investigate the ability of flowing MSC to bind to platelets that had been deposited onto fibronectin from blood and binding of MSC to platelets in platelet-rich plasma (PRP). For the latter, as described in Section 2.5, MSC were added to the PRP in a platelet aggregometer and changes in light transmission recorded before the cells were fixed and examined under phase-contrast microscope for morphological changes such as clump formation.

These studies thus aimed to improve our understanding of the behaviour of MSC in blood and the role of blood flow and other blood cells such as platelets in MSC dynamic adhesion properties.

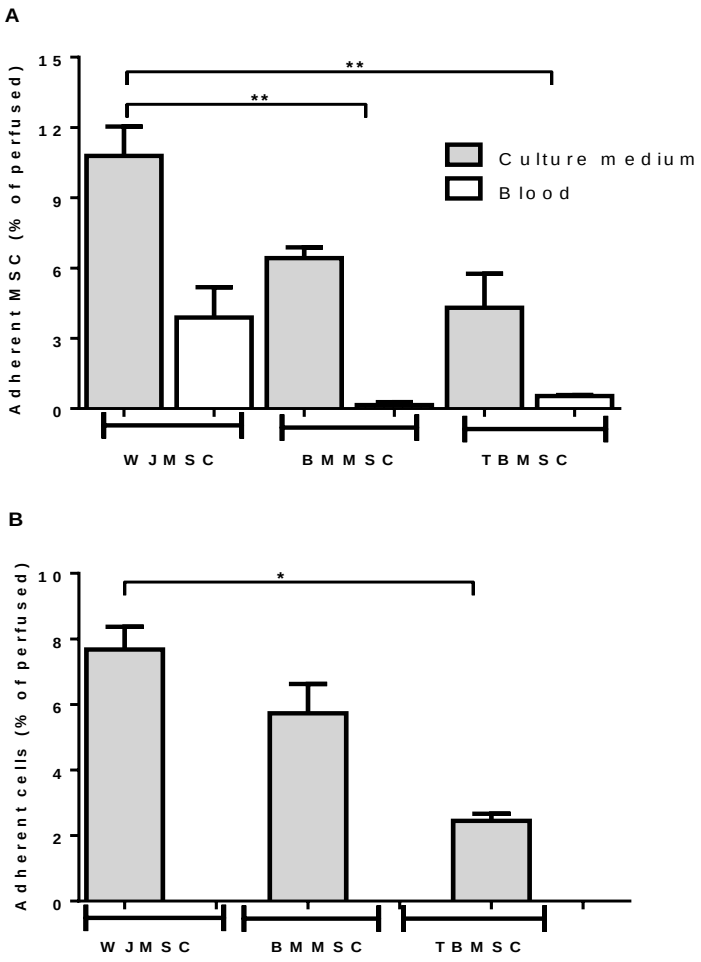
5.2 RESULTS

5.2.1 Comparison of the adhesion of WJMSC, BMMSC and TBMSC added to whole blood

In Figure 5.1, the adhesion of WJMSC, BMMSC and TBMSC are compared when they were added to blood at a fixed count and perfused over collagen or fibronectin. For comparison, data for adhesion of isolated cells at the same shear rate are included. Figure 5.1A shows the adhesion on collagen; we found that after flowing WJMSC, BMSMC, and TBMSC with whole blood, the percentage of adhesion was much decreased compared to adhesion of isolated cells in culture media. Also, by comparing the adhesion of the three MSC, the Figure shows that the adhesion of WJMSC within blood was much higher than that of BMMSC or TBMSC. In fact, we only saw occasional fluorescent BMMSC or TBMSC adhered to collagen. For WJMSC we saw large clumps of cells with several MSC in them (Figure 5.2) and on close examination we could also see fluorescent platelets and

leukocytes which had taken up dye. After washout of blood, we used phase contrast microscopy to examine these clumps and observed small platelet clumps or 'thrombi' (Figure 5.2). Thus, only WJMSC adhered to collagen effectively in blood. They appeared to be associated with platelets, and also bound together by them. Leukocytes were also adherent, probably also binding to the platelets.

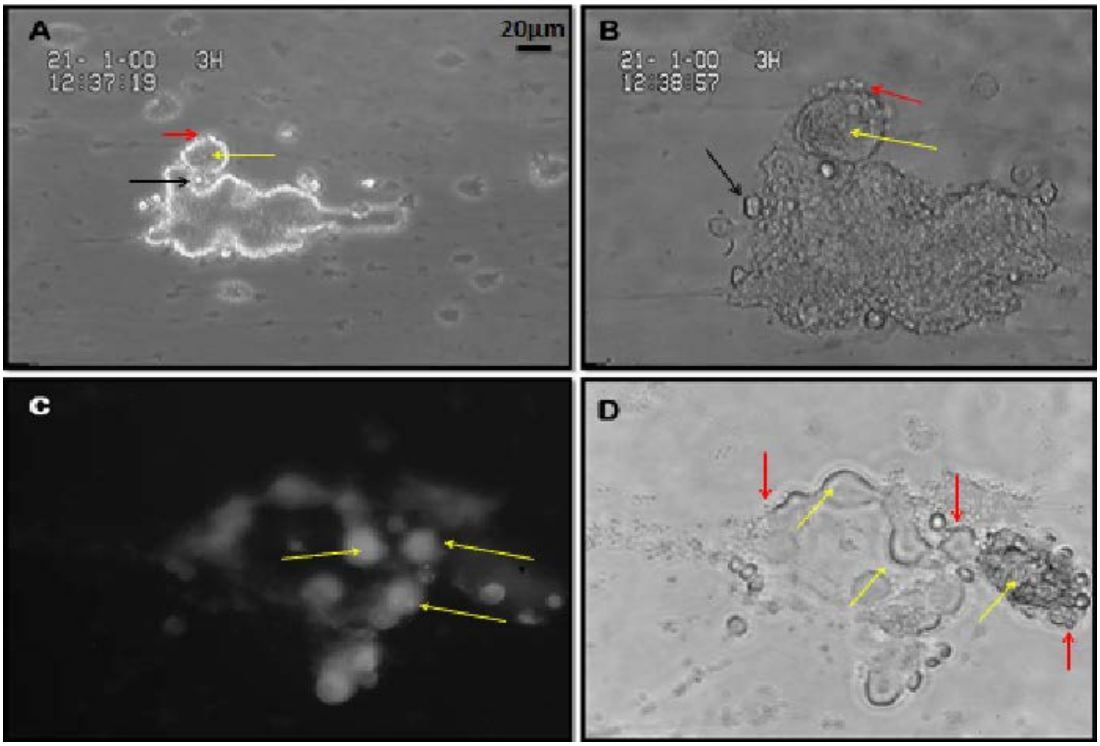
Figure 5.1B shows the data for adhesion on a fibronectin surface; we found that after flowing WJMSC, BMSMC, and TBMSC with whole blood, no adhesion was detectable. When we washed out the blood, however, we noticed that the surface of the fibronectin was covered by spread platelets in a monolayer (see Figure 5.3). We also saw leukocytes rapidly rolling across this surface during washout (see Figure 5.4) These platelets did not form aggregates or build up of 'thrombi' as seen on a collagen surface. It appeared therefore that although MSC, especially WJMSC, might bind to platelets, they did not bind to the platelets on the fibronection. It was possible that the platelet coating also inhibited the MSC binding to the fibronectin itself, and this might be the case for the collagen as well.



5-1:Comparison of the adhesion of WJMSC, BMMSC and TBMSC suspended in culture medium or in whole blood from flow to (A)collagen or (B) fibronectin.

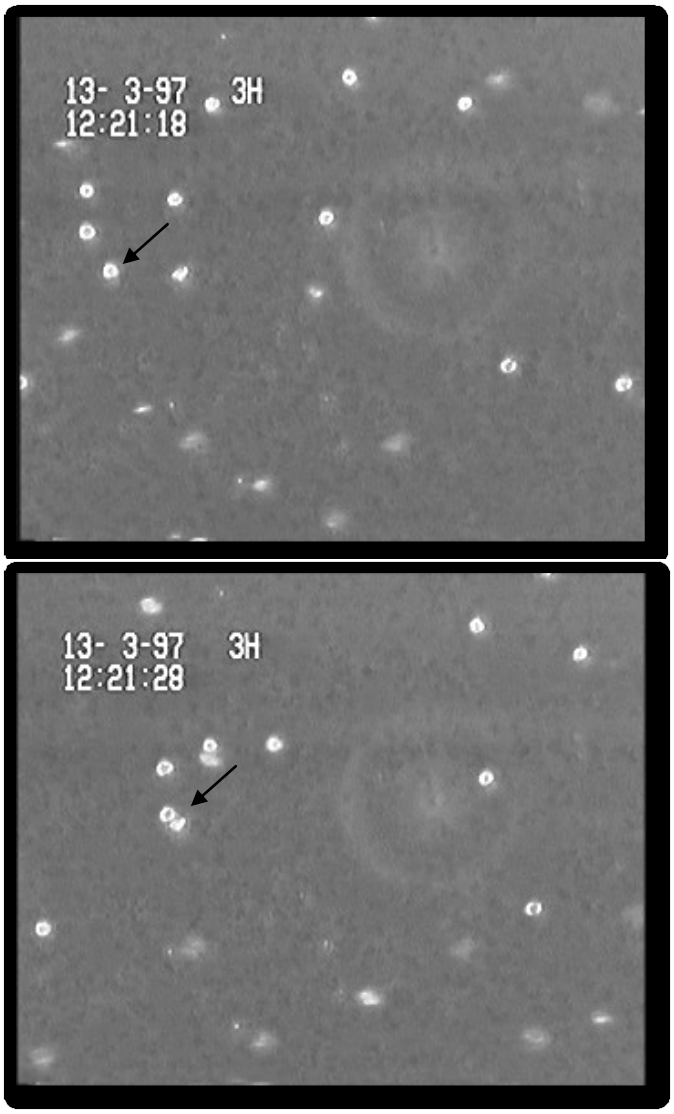
MSC were perfused at a wall shear rate $35s^{-1}$ for 4 minutes before washout and counting of adherent MSC. In both A and B, ANOVA showed that there was a significant effect of cell type and of suspending medium ($p<0.01$ for cell type; $p<0.05$ for suspending medium). Combining data for A and B for blood only, ANOVA showed a significant effects of adherent surface ($p<0.05$). *=$p<0.05$; **=$p<0.01$ for post-hoc comparison of cells type with data for the two media

combined, by Bonferroni test.



5-2: Images of WJMSC and platelets adhered to collagen from whole blood.
A,B. Phase contrast images taken with 20X or 40X objective.
C. Fluorescent and,D phase contrast images of a single WJMSC-platelet aggregate.
Yellow arrow is pointing to WJMSC,Red arrow is pointing to Platelets and Black aroow is pointing to White bloos cells .

5-3: Phase contrast image of platelets adhered to fibronectin from whole blood after WJMSC with whole blood (1.5 x 10^5/ml) **were flown on fibronectin surface at rate 35s^{-1} for 4 minuets**

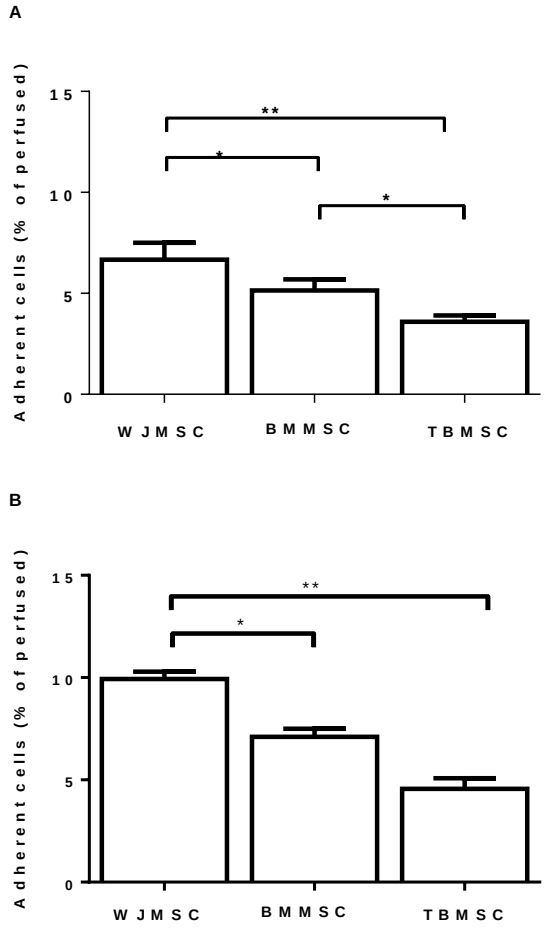


5-4: Leukocytes rolling across platelets monolayerduring washout, picture was taken each 10 second.

5.2.2 Adhesion of flowing isolated MSC to platelets deposited on fibronectin

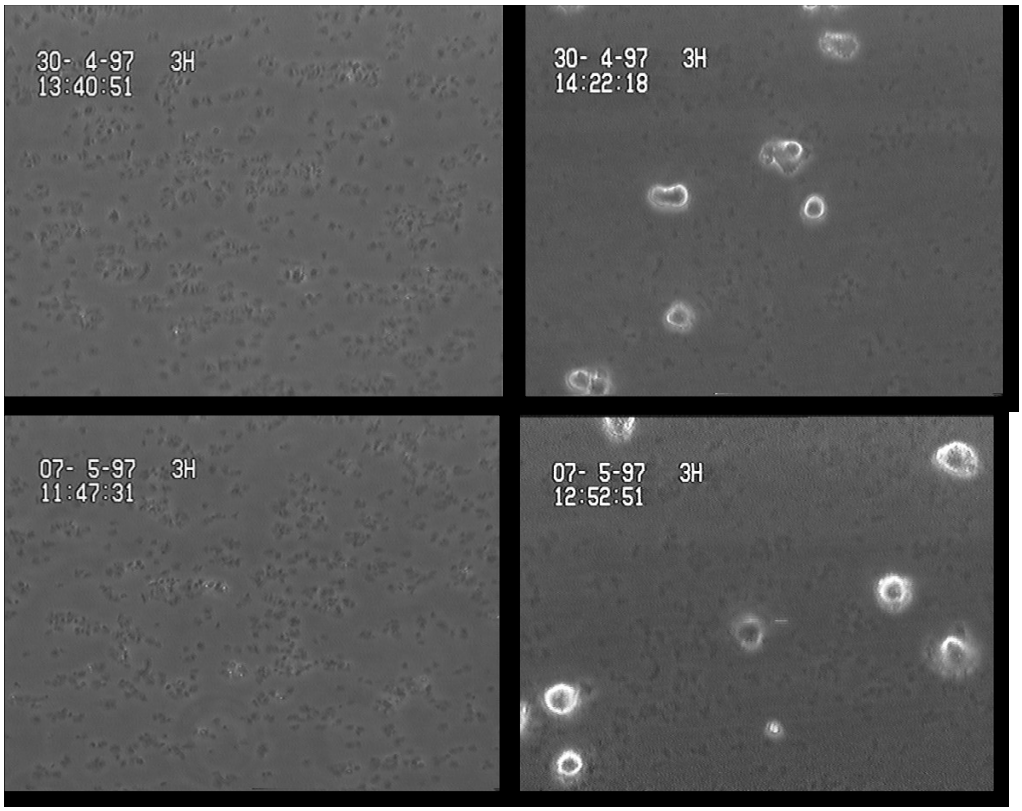
Based on the surprising results described above, we conducted a different experiment where isolated WJMSC, BMMSC, and TBMSC were flowed over platelet monolayer surfaces which had been deposited onto fibronectin from whole blood. Interestingly, adhesion was restored for all the MSC types (Figure 5.5A). The adhesion percentage was again in the order WJMSC>BMMSC>TBMSC. Since these platelets were presumably not fully activated (not forming aggregates from blood), we repeated the test after TRAP had been perfused to activate platelets in the monolayer. Interestingly, we noticed an increase in the adhesion percentage of all MSC compared to 'unactivated' platelet monolayers (Figure 5.5B). Since the platelet monolayers were not perfectly confluent, some adhesion could have been to fibronectin itself (see e.g., Figure 5.6). The effect of platelet activation thus helped show that adhesion was also platelet-specific.

These results raised the question why MSC had not bound to the platelets on the surfaces in the whole blood experiments, when the platelets were deposited on collagen or fibronectin, except the WJMSC for collagen. We considered the hypothesis that if activation was not required to bind platelets to MSC, they might bind in the blood and form a 'barrier' to adhesion to the platelets on the surface. This barrier would not operate if the platelets bound to the MSC were themselves activated (e.g., those bound to WJMSC and not the other MSC), as then cross-bridges would occur.



5-5: Comparison of the adhesion of WJMSC, BMMSC and TBMSC suspended in culture medium to platelets adhered to fibronectin after washout of blood.

Platelets were (A) unstimulated, or (B) TRAP was perfused over them before perfusion of MSC. MSC were perfused at a wall shear rate $35s^{-1}$ for 4 minutes before washout and counting of adherent MSC. In A and B, ANOVA showed that there was a significant effect of cell type on adhesion ($p<0.01$ fin each case). *=$p<0.05$; **=$p<0.01$ for post-hoc comparison of cells type by Bonferroni test.



5-6: Phase contrast image of platelets adhered to fibronectin from whole blood, before and after perfusion of isolated WJMSC at conc (1.5 x 10^5 /ml)for 4 min at shear rate 35s^{-1}.

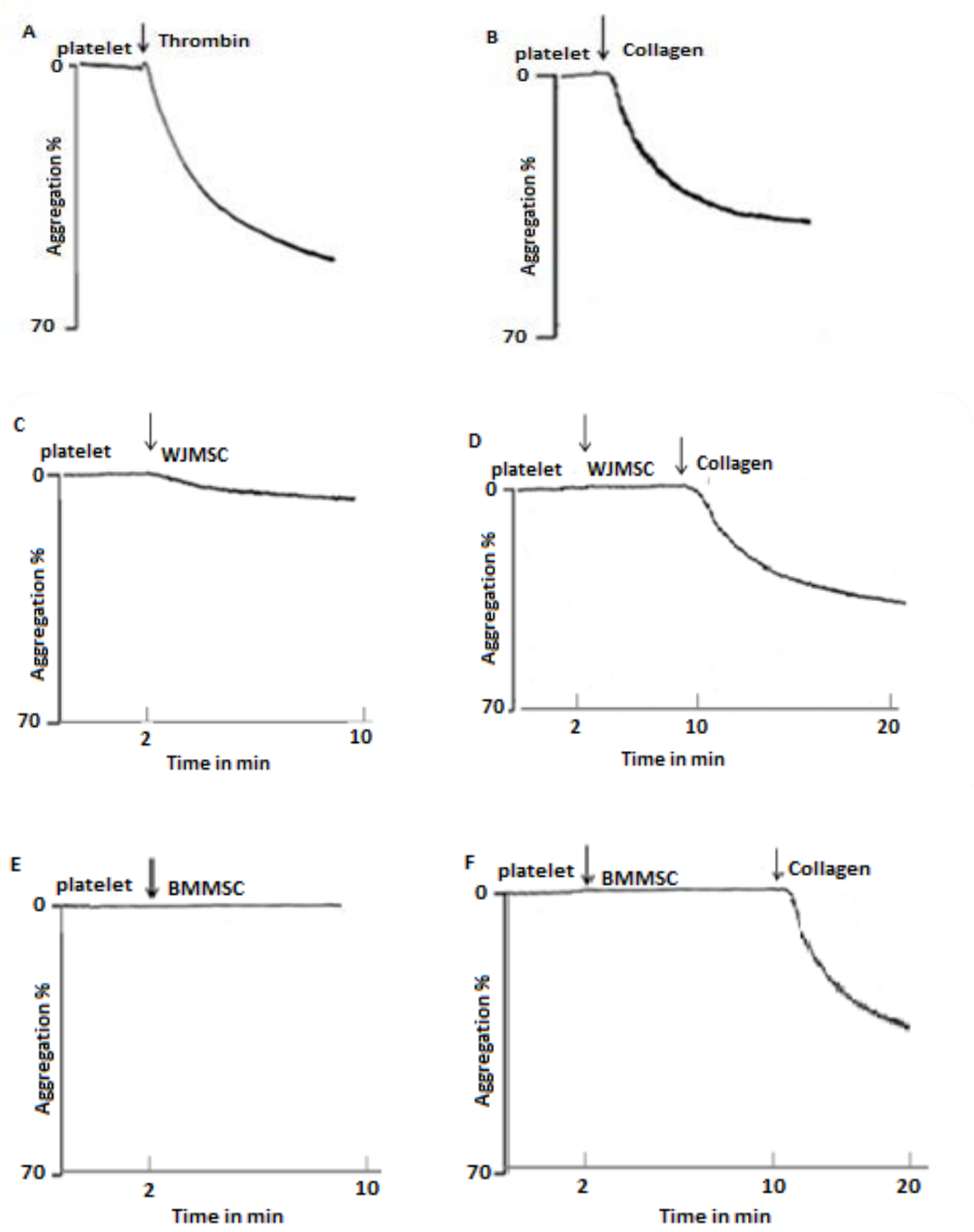
5.2.3 Further studies of direct binding between MSC and platelets

We thus investigated the binding of platelets directly to MSC when they were mixed with PRP in a platelet aggregometer. Figure 5.7A,B shows the aggregation results for PRP alone when thrombin or collagen were added as agonists, as positive controls. In Figure 5.7C,D we show a typical aggregation response between PRP and WJMSC. We noticed a slight gradual increase in light transmission, or slight increase in 'aggregation' after adding WJMSC. After adding WJMSC we also tried adding collagen. We noticed a strong aggregation response. In Figure 5.7E,F we show a typical aggregation response between PRP and BMMSC. BMMSC caused a just detectable change in light transmission. When collagen was added, there was an increase in the aggregation response.

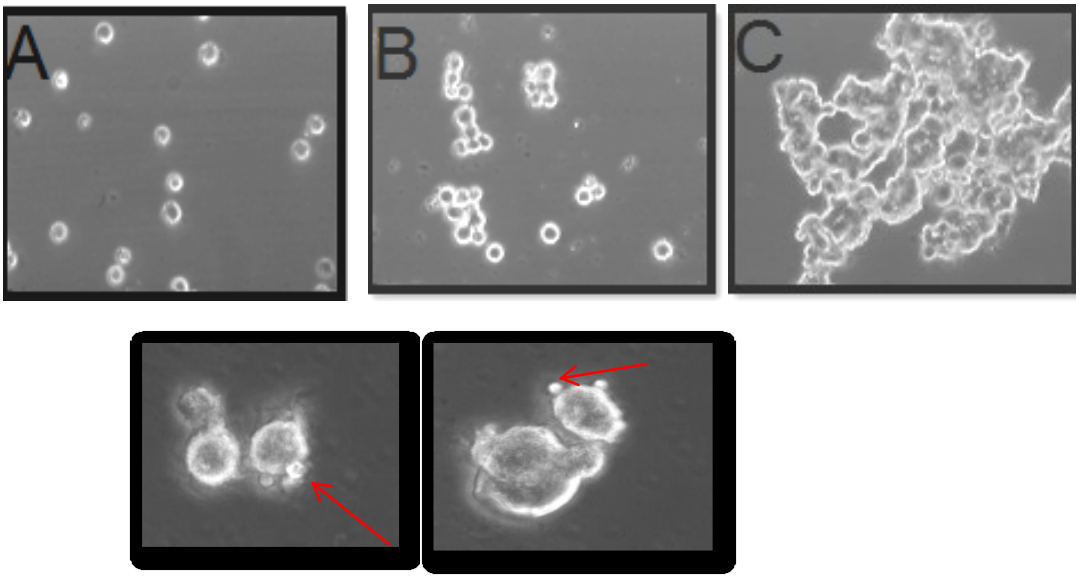
These results suggested an interaction with platelets that was greater for WJMSC than BMMSC, but did not provide clear evidence. When we observed cells retrieved from the aggregometer is was evident that aggregation of WJMSC did occur when they were mixed with PRP, and that platelets could be observed attached to individual cells (Figure 5.8). With collagen added, large multi-MSC aggregates could be observed. We could also see aggregates with BMMSC, especially when collagen was added (Figure 5.9), but overall, aggregates were smaller than for WJMSC.

To obtain further evidence of interaction between MSC and platelets, we tried a 'reverse' experiment. WJMSC were injected into microslides and allowed to settle and adhere for 2 hours. Then blood was perfused over the MSC, followed by washout. Platelets could be observed attach to the sparse MSC (see e.g., Figure 5.10). However, the MSC tended to round up and we could not show consistent

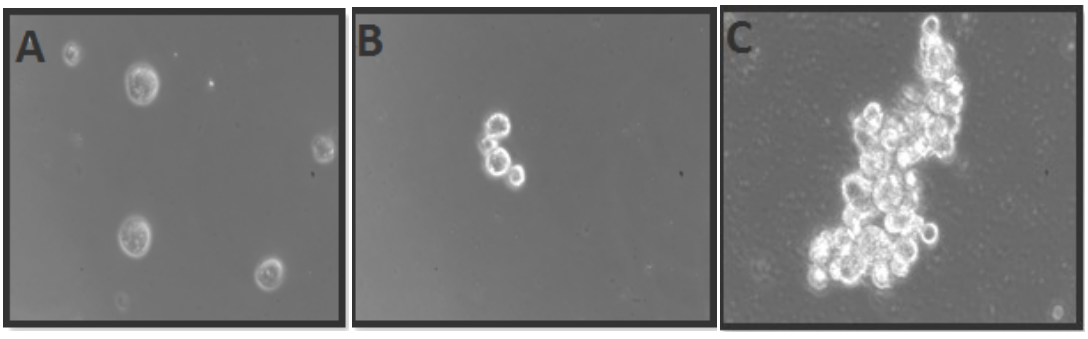
attachment through this method. Since completion of experiments described in this thesis, others in our laboratory made a simpler 'static' assay with MSC deposited on tissue culture dishes to which PRP was added and allowed time to settle. The MSC remained spread and platelets adhered to the WJMSC but far fewer to the BMMSC (see Figure 5.11; data provided by Samera Husain).



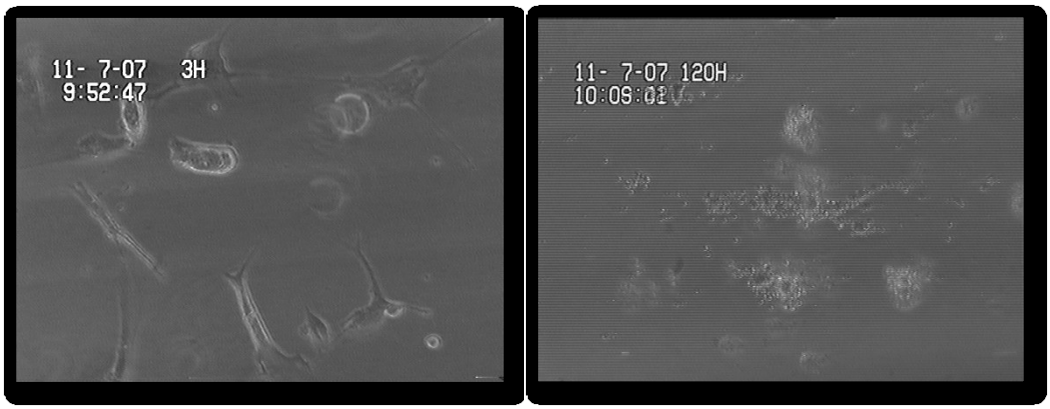
5-7: Printouts from a platelet aggregometer showing platelet aggregation (decrease in absorbance or increase in transmitted intensity of light).
A,B. Aggregation responses to Thrombin (0.1 U/ml) or collagen (2 µg/ml) added to PRP.C,D. Aggregation responses to $2x10^6$/ml WJMSC without or with added collagen (2 µg/mL).E,F. Aggregation responses to $2x10^6$/ml BMMSC without or with added collagen (2 µg/mL). The results are presented as percentage aggregation, with 100% equivalent to transmission through PP . The tracings shown are from an experiment repesentative of 3 others with similar results. Arrowheads mark the moments of addition of cells or agonists.



5-8: Phase contrast image of WJMSC after stirring with A. PPP; B. PRP; C. PRP + collagen.
The smaller pictures show higher magnification of MSC in PRP. red arrow is pointing to platelet.

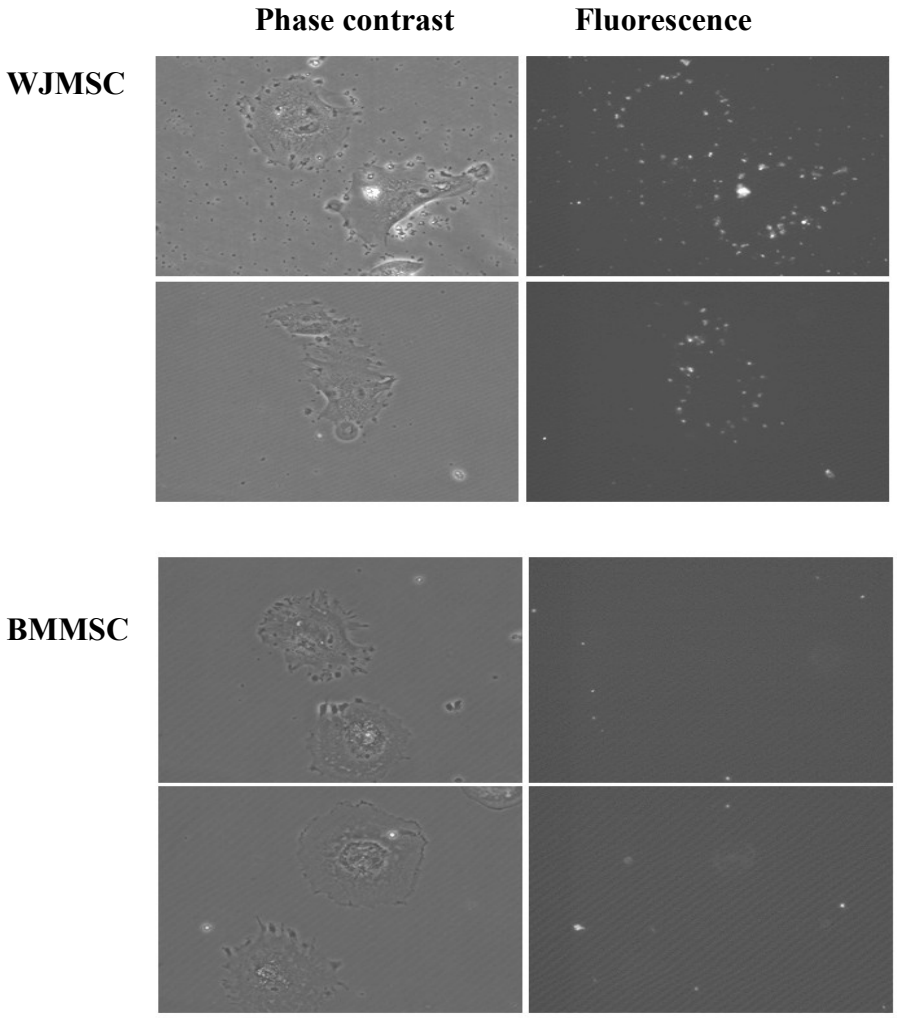


5-9: Phase contrast image of BMMSC after stirring with A. PPP; B. PRP; C. PRP + collagen.



5-10: Phase contrast image of WJMSC adhered to a microslide before and after perfusion of whole blood.

A simpler 'static' assay with MSC deposited on tissue culture dishes to which PRP was added and allowed time to settle. The MSC remained spread and platelets adhered to the WJMSC.



5-11: Phase contrast and fluorescence images of MSC adhered to a culture dish.

Platelets in PRP were stained with rhodamine and added to MSC. The platelets were allowed to settle for 30 minutes and then non-adherent platelets were washed off. (Images provided by Samera Husain)

5.3 DISCUSSION

In this chapter we demonstrated for the first time the adhesive behaviour of flowing MSC when added to whole blood. WJMSC, BMSMC and TBMSC were compared when perfused over surfaces coated with matrix proteins. We demonstrated that MSC within whole blood adhered to collagen or fibronectin much less than isolated MSC. Indeed, there was no detectable adhesion to fibronectin, and on collagen, only occasional adherent BMMSC and TBMSC were seen. In the case of WJMSC, we observed clumps of cells, some large with several MSC, which included platelets and occasional leukocytes. Thus, only WJMSC adhered to collagen effectively in blood, and they were associated with platelets, and also bound together by them. On the protein surfaces themselves, small 'thrombi' of platelets were adhered to collagen, and a nearly confluent layer of spread platelets, without thrombi, were found on the fibronectin.

In subsequent experiments on the fibronectin surface, after whole blood had been perfused and washed out, we found that isolated MSC adhered to the platelet monolayer, in the order WJMSC>BMMSC>TBMSC. Since MSC could bind to platelets, and they could also bind to fibronectin or collagen alone (see Chapter 3) it was puzzling why they were not seen attached to the these surfaces from blood, except for WJMSC and collagen. To investigate further, we added WJMSC or BMMSC to PRP and examined aggregation in a platelet aggregometer which stirs the suspension at high speed. Both MSC aggregated slightly with platelets, and larger aggregates of MSC with platelets were formed if collagen was added; in each case WJMSC formed larger aggregates than BMMSC. Other experiments were done to test adhesion of platelets from blood to MSC on a surface. Although platelets

were observed attached to MSC, behaviour of the MSC was not reproducible. Recently, others in our laboratory have continued investigation based on these results. They showed that unstimulated platelets in PRP could bind to MSC on a surface, again with WJMSC binding more platelets than BMMSC (Husain, Sherif and Nash, unpublished observations). In addition, MSC/platelet aggregation studies have been repeated but with slow mixing speeds. These studies showed strong aggregation of platelets induced by WJMSC (judged by change in light transmission) but not by BMMSC (Sherif and Nash, unpublished observations). Taken together, the above results indicate that all MSC can bind platelets, but that WJMSC bind in greater number and more importantly, activate platelets so leading to formation of aggregates that include the MSC.

Our hypothesis based on the above is illustrated in Figure5.13. MSC bind platelets on their surface when flowing in blood; only for WJMSC, these platelets become activated and adherent for other platelets. For collagen, the surface is also coated with activated platelets. The WJMSC-platelet conjugates bind to these platelets and multicellular aggregates develop. The BMMSC or TBMSC are shielded with non-activated platelets and do not form platelet-platelet attachments onto the collagen. For fibronectin, the surface is coated with unactivated platelets, and these do not bind the MSC whether coated with activated or unactivated platelets. In other words, activation is not required to bind platelets to MSC, and so they form a 'barrier' to adhesion to the platelets on the surface. This barrier does not operate if the platelets bound to the MSC are themselves activated (e.g., those bound to WJMSC), as then cross-bridges can occur with other activated platelets.

The limited literature relevant to these studies supports the existence of MSC-platelet interactions and their relevance in vivo. Teo et al. (Teo et al., 2012) demonstrated that in mice, injection of endotoxin into the ear caused preferential recruitment of infused human BMMSC to microvessels in that ear. However, platelet depletion led to a decrease in this MSC homing, and direct observation showed MSC present with platelets and neutrophils in the inflamed vessels. In another study, Langer et al. (Langer et al., 2009) reported attachment of platelets and infused human BMMSC to the damaged carotid artery of mice, and again, there was a decrease in MSC adhesion when platelets were depleted. Jiang et al. (Jiang L et al., 2012) found that rat BMMSC infused into a rat model of pulmonary arterial hypertension became located in the pulmonary blood vessels and that the number was reduced for animals treated with antibodies against P-selectin or an inhibitor of GpIIbIIIa. They also studied adhesion of MSC to platelets which were adhered to collagen. Under static conditions or flow, MSC bound to the platelets adhered to the collagen, but not to collagen alone. The flow experiments were carried out at a much higher shear rate ($1000s^{-1}$) than in our studies. The deposition of MSC was reduced by treatment with the same antibodies against P-selectin or an inhibitor of GpIIbIIIa.

Each of the above studies used BMMSC, and none directly observed behaviour in blood. Thus they do not clearly test the hypothesis outlined in Figure 5.11. It is not clear in vivo which cells adhered first. The results may suggest that adhered platelets captured perfused BMMSC, but it is also possible that platelets adhered to BMMSC assisted their trapping in microvessels. In vivo, attachment may have been to endothelial cells rather than collagen or fibronectin.

The observation of a monolayer of spread platelets on a fibronectin surface is in agreement with a study by B Savage et al. (B Savage - 1996), who showed that platelets in flowing blood arrested on fibronectin surface through αIIbβ3-integrin (GPIIbIIIa). They also described a monolayer without aggregates. It is well known that platelets adhere to exposed collagen by (vWF) and (GPIb) initially, with firm adhesion and aggregation following through GpIIbIIIa explaining the presence of platelet clumps (Dopheide et al., 2002). We saw that during the wash out step some leukocytes rolled on the platelet monolayer on fibronectin. We also found leukocytes attached to the aggregates on collagen. These findings are expected from previous observations of leukocyte-platelet adhesion in our laboratory (Buttrum et al., 1993, Butler et al., 2007)and may not be linked to the presence of MSC. Rinder et al. (Rinder et al., 1991) reported that platelets and leukocytes may also form aggregates in the blood when platelets are activated, and it is also possible that WJMSC-platelet aggregates could bind leukocytes in the blood as well as on the surface.

These experiments are the first to show that flowing MSC can be marginated in blood. (see figure5.12) The adhesion observed for WJMSC was on the upper surface of the microslide and so it cannot have been due to sedimentation. Even when there was no adhesion, fluorescent MSC added to the blood could be seen flowing near the upper surface. Thus, although MSC are larger than leukocytes, they can still be marginated by aggregated red blood cells at the low shear rates used in these experiments.

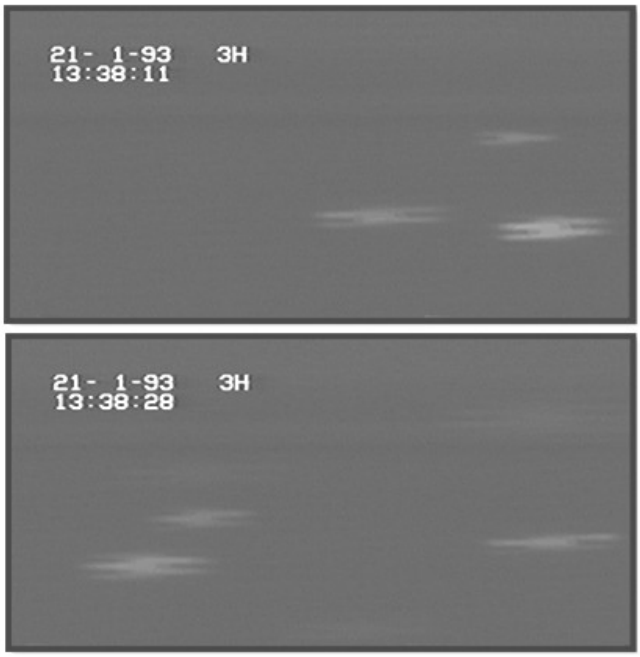
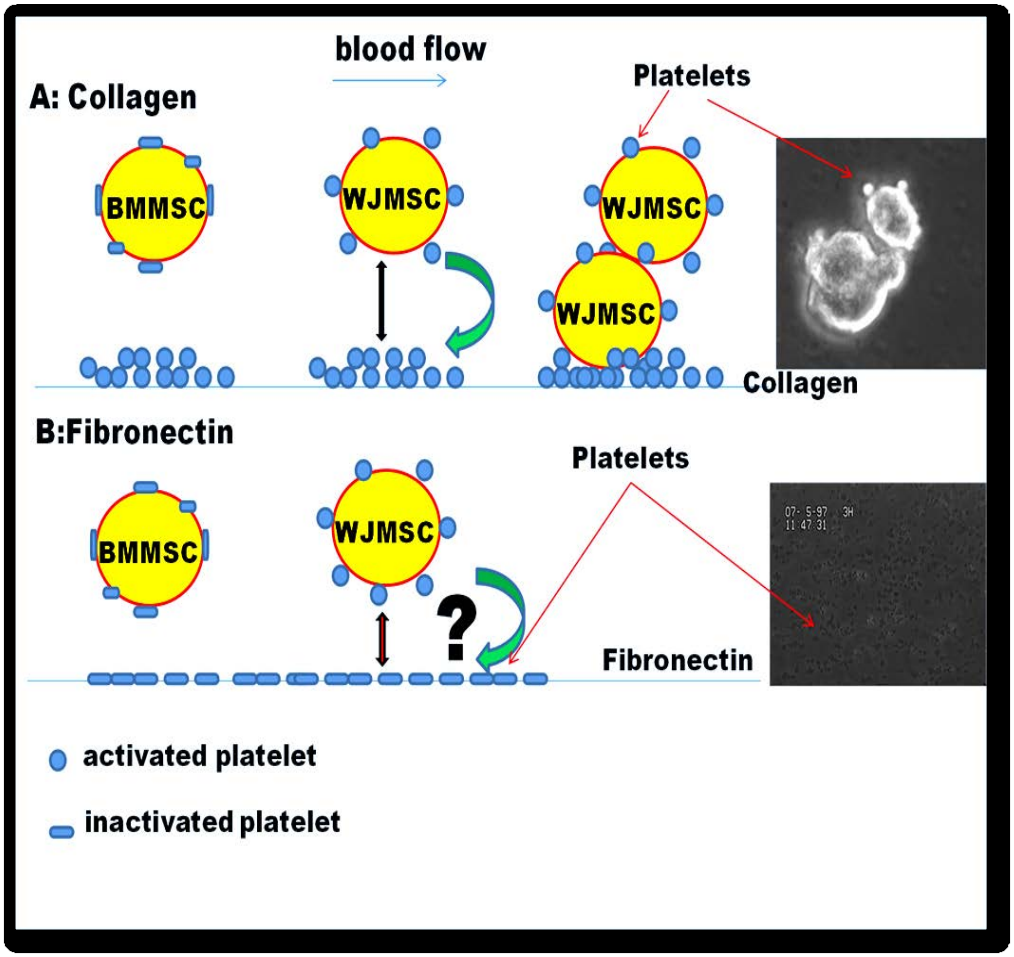


Figure5-12: Fluorescent image ofstained MSC marginated by the red blood cells during blood flow assay at the low wall shear rate used ($35s^{-1}$).

The results described may be relevant to the behaviour of MSC if infused for therapy. Adhesion to the vessel wall may be unlikely for BMMSC or TBMSC for vessels with intact endothelium or even in damaged vessels with exposed matrix. WJMSC might be able to adhere to damaged vessels along with platelets. There is also the possibility that WJMSC could cause thrombus formation in the blood and that this could block microvessels. Infused MSC are cleared from the blood and many become stuck in the lungs (Fischer et al., 2009, Kang et al., 2012) These results suggest that trapping is likely to be mechanical and not adhesive. However, if aggregates formed with platelets, MSC would be trapped more quickly. Others have observed BMMSC stuck in small vessels in inflamed tissue (Teo et al., 2012) with platelets. It is possible the BMMSC carried the platelets there, or that the platelets adhered to the MSC after they were trapped.



5-13: Hypothesis regarding behaviour of MSC and platelets adhering from flow.

For perfusion over collagen (A), the surface coated with activated platelets. BMMSC is shielded with non-activated platelets and do not form platelet-platelet attachments onto the collagen while WJMSC-platelet conjugates bind to these platelets and multicellular aggregates develop. For perfusion over fibronectin (B), the surface is coated with unactivated platelets, and these do not bind theBMMSC and WJMSC whether coated with activated or unactivated platelets

5.4 CONCLUSIONS

Although all MSC could bind to collagen or fibronectin, and also were able to adhere to platelets, under blood flow only WJMSC showed large clumps of cells with several MSC, platelets, and leukocytes on a collagen surface. It appears that WJMSC are able to activate platelets as well as bind them, leading to the build up. Differences in behaviour might arise because of different adhesion molecules on the MSC, such as integrins, or because WJMSC present some other activating agent to platelets. To study the basis of MSC interactions with matrix proteins and with platelets, and mechanisms underlying the behaviour in whole blood, we next studied effects of antibodies able to block possible receptors (Chapter 6). We considered the assay where MSC attached to platelets which had been deposited from blood onto fibronectin the best to study mechanisms of the MSC-platelet adhesion, since the PRP aggregation assay did not have a clear quantitative readout.

Chapter 6 MECHANISMS OF ADHESION BETWEEN MSC, PLATELETS AND PROTEINS

6.1 INTRODUCTION

Platelets play an important role in haemostasis by being the primary agents in forming the initial thrombotic plug (Davi and Patrono, 2007) and in inflammation can assist in leukocyte recruitment (Nash, 1994). The results in Chapter 5 suggest that platelets can also interact with MSC and influence MSC adhesion in blood. However, the mechanism that control that interaction is not well defined. Structurally, the platelet membrane carries a glycocalyx which act as an exterior coat, through which glycoprotein receptors are presented which control platelet adhesion (Cooper et al., 1976). The major adhesive receptors are the glycoprotein (GP) Ib-IX-V complex and GPIIbIIIa (also called αIIβ3-integrin). When collagen is exposed due to vascular injury, there will be immediate platelet binding between GPIb and its ligand vWF which binds from the blood onto collagen. Then, platelets bind to collagen via GPVI and α2β1-integrin, which stabilizes the adhesion and triggers GpIIbIIIa activation which attaches to vWF, fibrinogen and fibronectin to further stabilise adhesion to the matrix and also induced platelet-platelet adhesion, to form the platelet plug (Varga-Szabo et al., 2008). Platelets can also bind from flowing blood onto fibronectin at low shear rates using GpIIbIIIa as the main receptor, but also α5β1-integrin (Beumer et al., 1994).

In addition to platelet adhesion, integrin family members support adhesion of many cells to extracellular matrix, and include receptors classified as collagen- or fibronectin-binding, as shown in Figure6-1 Section 1.3.3.5. Our result in Chapter 5 showed that isolated MSC could bind to collagen and fibronectin in the order WJMSC>BMMSC>TBMSC. In blood, however, we only observed clumps of WJMSC with platelets on collagen, even though we showed all MSC could bind to

deposited platelets. To investigate the adhesive interactions that caused these results, we decided next to determine receptors which supported adhesion between WJMSC or BMMSC, platelets and matrix proteins using selective function-blocking antibodies. Use of TBMSC was limited by our inability to culture reliably beyond passage 3 and by their slow growth rate, and they were excluded from the studies of adhesion mechanisms.

First, we compared adhesion of two isolated cells WJMSC and BMMSC perfused over surfaces coated with collagen and fibronectin at a wall shear rate of 35s-1, with or without antibodies against different integrins. Next, we flowed isolated WJMSC and BMMSC over platelet monolayer surfaces which had been deposited onto fibronectin from whole blood. Again, antibodies against integrins were tested. In whole blood assays we first tried to inhibit platelet adhesion to collagen in whole blood by:

(i) blockade of platelet GPIb-IX-V complex by antibody againt GP1b

(ii) blockade of platelet GPIIbIIIa by using abciximab

Finally, antibodies against integrins with or without anti-platelet antibodies were tested.

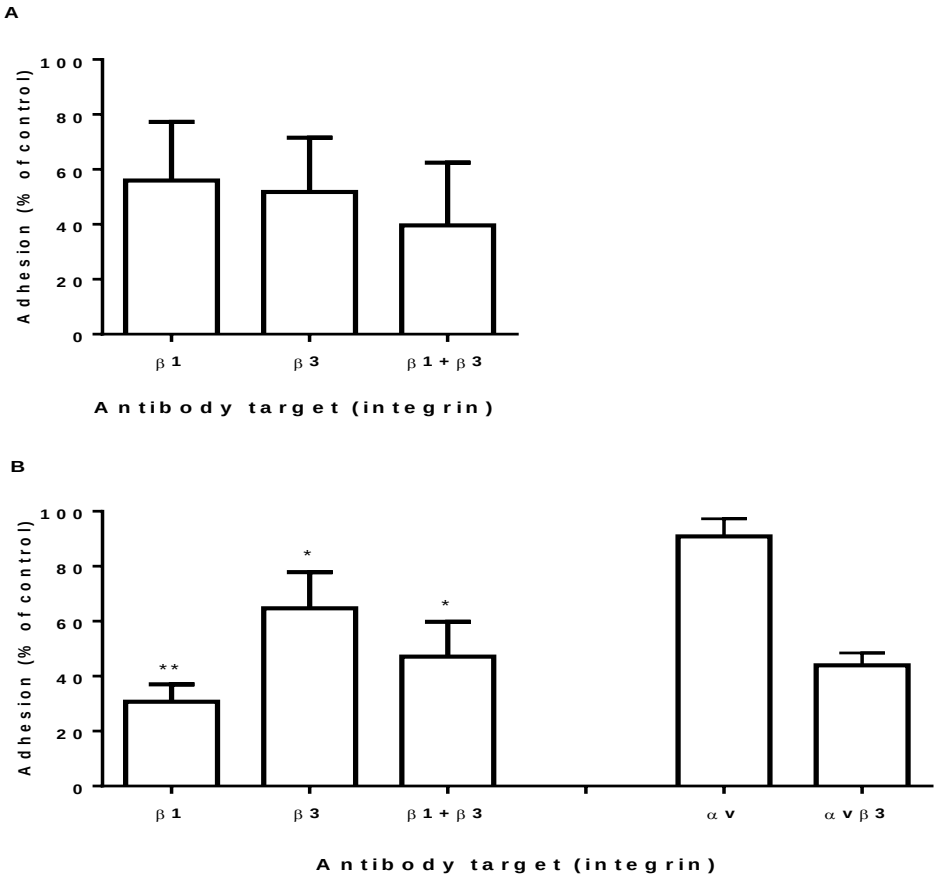
The adhesion assays were as described in Section 2.4, and antibody treatments as in Section 2.8 and Table 2.3.

6.2 RESULTS

We first investigated mechanisms of adhesion of isolated MSC to collagen (Figure 6.1) or fibronection (Figure 6.2). Figure 6.1A shows that when isolated WJMSC were treated with antibody against β1- or β3-integrin, the number of adherent MSC was reduced by nearly 50% compared to untreated controls, and when both antibodies were used, there was a slightly greater reduction. However, there was great variation between experiments and the reduction was not statitistically signficant (e.g., p=0.072 for anti-β3-integrin). In Figure 6.1 B, when isolated BMMSC were treated with antibody against β1- or β3-integrin, or both, the number of adherent cells was significantly decreased compared to control cells. Also, when isolated BMMSC were treated with antibody against β1-integrin, we observed further significant reduction in number of adherent cells compared to non treated cells. Overall, it appears that MSC adhered to collagen using both β1- and β3-integrins. For BMMSC there was evidence that αvβ3-integrin was the particular β3-integrin used, but surprisingly, antibody against αv-integrin was not effective.

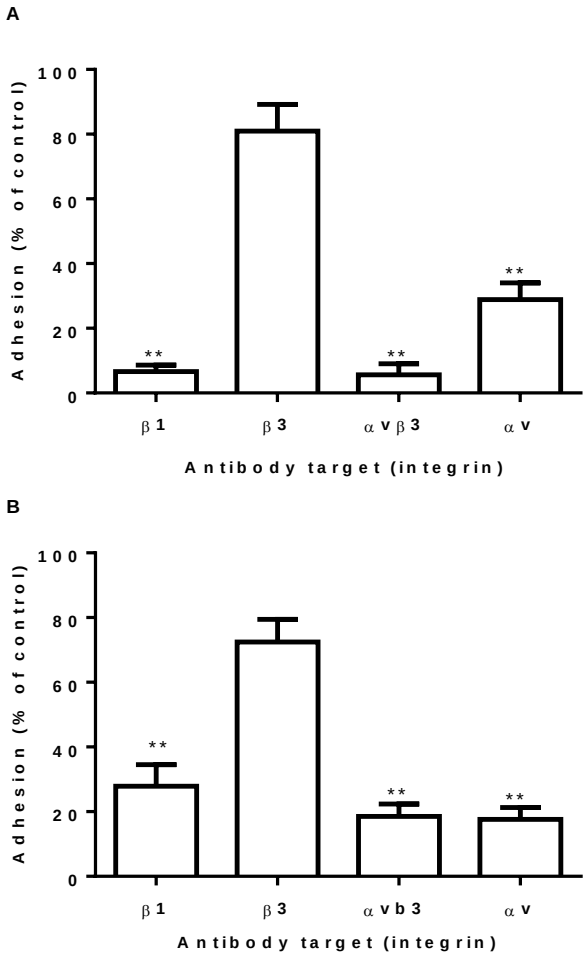
In Figure 6.2A, when isolated WJMSC were treated with antibody against β1- integrin, the number adherent from flow on fibronectin was greatly and significantly decreased compared to control cells. Also, when isolated WJMSC were treated with antibody against β3-integrin, there was a slight but non-significant reduction in number of adherent cells. However, interestingly, we noticed that after treatment with antibody against β3-integrin, adherent MSC lost the ability to spread on the fibronectin surface compared to non-treated cells and remained nearly round (see Figure 6.3). After treatment with anti-αvβ3-integrin, we detected significant decrease in number of adherent cells compared to controls, and this was also the case

when cells were treated with antibody against αv-integrin. Figure 6.2B shows results when isolated BMMSC were treated with the same antibodies. Results were similar to those for WJMSC. Anti β1-integrin reduced adhesion significantly but anti β3-integrin had less effect, with borderline statistical significance (p=0.058). Treatments with anti-αvβ3-integrin or anti-αv-integrin both significantly and markedly reduced adhesion. Overall, it appears that MSC adhered to fibronectin mainly through β1-integrins. Results for β3-integrins were inconsistent, with antibody against αv-integrin or αvβ3-integrin being effective but anti-β3-integrin not. Anti-β3-integrin did inhibit spreading however, showing that the antibody did have a functional effect.



6-1:Effects of antibodies against different integrins on adhesion of (A). WJMSC, (B).BMMSC to collagen.

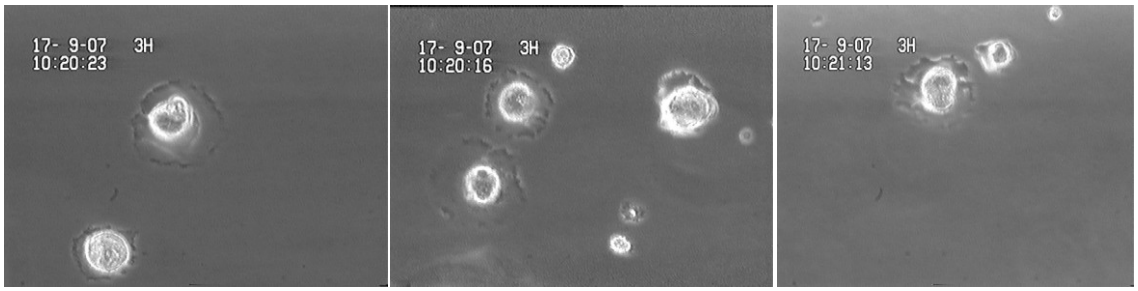
MSC were treated with antibodies and perfused at a wall shear rate of $35s^{-1}$ for 4 min. Data are mean ± SEM from 3-6 experiments (except for antibody against αvβ3 tested on 2 occasions). Not all antibodies were tested in all experiments, but untreated controls were used in every experiment. *=p<0.05; **=p<0.01*for comparison to untreated control by paired t test.
Note: In A, for anti-β3-integrin p=0.072, and for anti-β1+β3-integrin p=0.077.



6-2: Effects of antibodies against different integrins on adhesion of (A). WJMSC, (B).BMMSC to fibronectin.

MSC were treated with antibodies and perfused at a wall shear rate of $35s^{-1}$ for 4 min. Data are mean ± SEM from 3 experiments. **=$p<0.01$ for comparison to untreated control by paired t test.
Note: In B, for anti-β3-integrin $p=0.058$.

Untreated



Antibody against β3-integrin

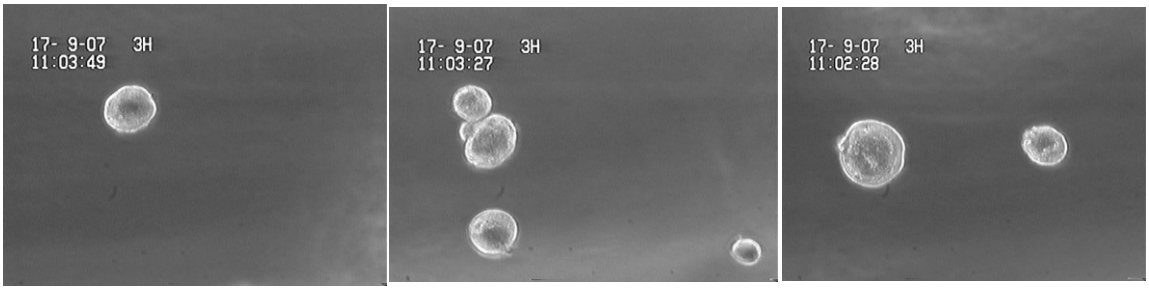


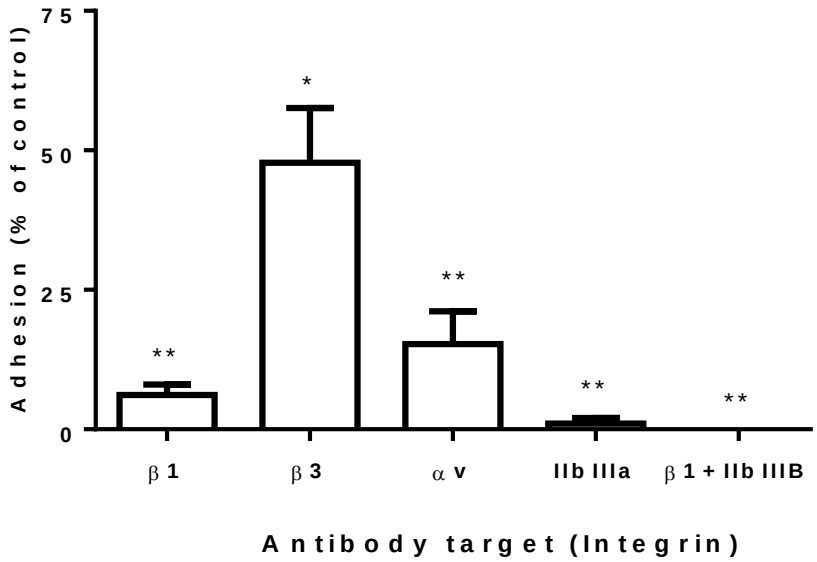
Figure 6-3 Effect of antibody β3-integrin on WJMSC on fibronectin.

Images captured from phase contrast recordings during an adhesion assay. Antibody against β3-integrin caused adherent WJMSC to lose their ability to spread on fibronectin, compare to non treated cells.

Next we investigated the mechanism of adhesion of MSC to platelets deposited from whole blood onto a fibronectin surface. Figure 6.4A shows results when isolated WJMSC were treated with antibodies against β3-,β1- or αv-integrins before being perfused over the platelets. It also shows the effects of adding antibodies against GpIIbIIIa to the blood before perfusion over the fibronectin and washout, with or without anti-β1-integrin added to the MSC. We detected >90% reduction in the number of adherent WJMSC compared to controls when the cells were treated with anti-β1-integrin, and a smaller but significant effect of anti-β3-integrin. Treatment of MSC with anti-αv-integrin also reduced adhesion. When the

blood was treated with anti-GpIIbIIIa, there were adherent platelets but only a few MSC adhered to them. The platelets were not spread but rounded, and we noticed that the MSC rolled across them picking up platelets (see e.g., Figure 6.4B). When the blood was treated with anti-GpIIbIIIa and MSC with anti-β1-integrin, adherent MSC were not seen (Figure 6.4A). In similar experiments for isolated BMMSC treated with anti-β1-integrin, adhesion was 22 ± 10% of the value for untreated controls (mean ± SEM, n=3, $p<0.05$ by paired t test). In 1 or 2 experiments with each, anti-β3-integrin or anti-αv-integrin showed partial inhibition of adhesion of BMMSC, while anti-GpIIbIIIa caused almost complete loss of adhesion. Overall, MSC binding to platelets required β1-integrin, and β3-integrin played a smaller role. When platelet adhesion to fibronectin was inhibited by anti-GpIIbIIIa, there was little adhesion of MSC to the fibronectin, although the MSC could bind to platelets. In that case, adding anti-β1-integrin removed the last adhesion seen.

A



B

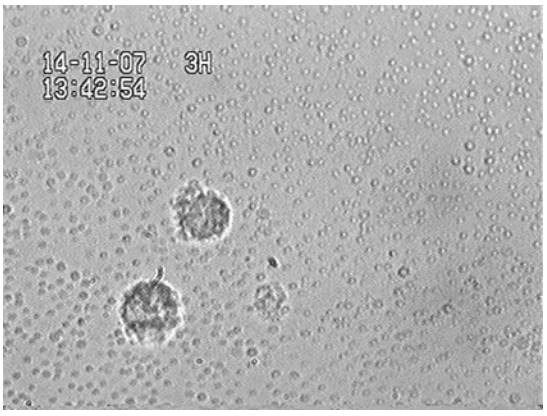


Figure 6-4: Effects of antibodies against different integrins on adhesion of WJMSC to platelets deposited from whole blood onto fibronectin

A. Adhesion when MSC were treated with antibodies and perfused at a wall shear rate of $35s^{-1}$ for 4 min. In experiments with anti-GpIIbIIIa, the antibody was added to the blood to prevent platelet-platelet binding before it was perfused over fibronectin. Data are mean ± SEM from 3 experiments. *=$p<0.05$, **=$p<0.01$ for comparison to untreated control by paired t test.

B. Phase-contrast microscope image of fibronectin surface after perfusion of blood in the presence of antibodies against GpIIbIIIa, during perfusion of WJMSC. Platelets do not spread, and are picked up by 'rolling' WJMSC.

Next, the effects of adding antibodies to whole blood along with MSC and perfusing together over collagen was tested. Similar inhibitory experiments were not done on fibronectin as no adhesion was observed in controls. Figure 6.5A shows the effect on WJMSC binding to collagen in the presence of whole blood, when whole blood was treated with anti-GP1b or anti-GpIIbIIIa. WJMSC again adhered in clumps to collagen. The number was reduced by anti-GpIb and almost totlally inhibited by anti-GpIIbIIIa. Interestingly, the adherent WJMSC after anti-GP1b treatment did not form clumps; instead we noticed single cells surrounded by platelets (see figure 6.6A). Figure 6.5B and 6.5C show results from similar experiments with with BMMSC and TBMSC respectively. As before, very few of these cells showed binding to collagen in whole blood, and when the blood was treated with anti-GP1b or anti-GpIIbIIIa, we saw close to zero adhesion. Examining the surface of the collagen after blood perfusion alone and washout, we saw platelet aggregates as expected on collagen, and smaller numbers of individual platelets with either anti-GpIb or anti-GpIIbIIIa (see Figure 6.6B).

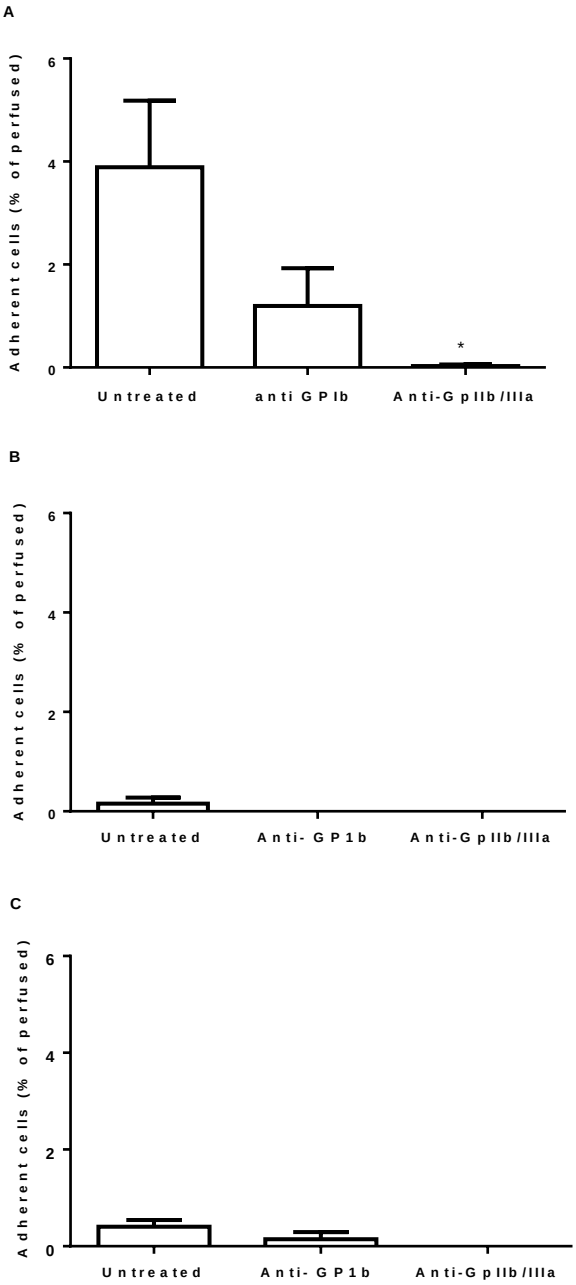


Figure 6-5: Effects of antibodies against different platelet receptors on adhesion of A. WJMSC, B. BMMSC, C. TBMSC to collagen from blood.

Antibodies were added to blood with MSC and perfused at a wall shear rate of $35s^{-1}$ for 4 min. Data are mean ± SEM from 3 (A) or 2 (B,C)) experiments. *=$p<0.05$ for comparison to untreated control by paired t test.

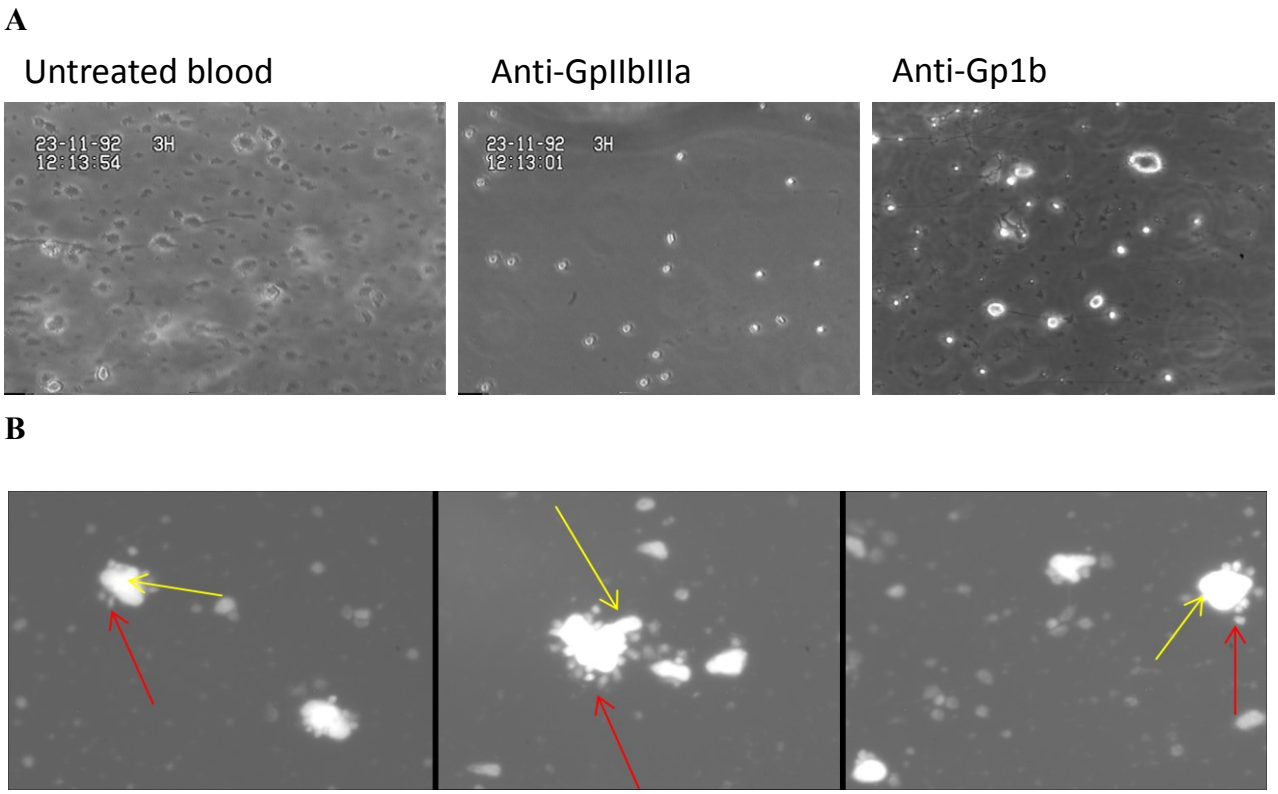


Figure 6-6: Microscope images of collagen surfaces after A. perfusion of blood containing MSC, B. blood alone.

A. Phase-contrast microscope images of collagen surface after perfusion of blood in the absence or presence of antibodies against GpIIbIIIa or GP1b.B. Fluorescence images of WJMSC adherent from blood onto collagen in the presence of antibody against GP1b.Yellow arrow is pointing to WJMSC and Red arrow is pointing to platelet.

Finally, we tried to test the role of different integrins when WJMSC were perfused over collagen. Figure 6.7 shows drastic, significant inhibition of WJMSC adhesion from blood flow compare to control after the MSC were treated with antibody against β1-integrin. In addition, we noticed lesser inhibition of WJMSC adhesion from blood flow after the cells were treated with antibody against β3-integrin or αv-integrin. The reduction was presumably because the MSC did not

adhere to platelets. We decided to test again the effect of inhibition of platelet adhesion to collagen (using anti GpIb and anti-GpIIbIIa), but with or without added anti-β1-integrin treatment of the MSC as well. We considered that if we blocked platelet adhesion to collagen, as well as platelet adhesion to MSC, we might see 'bare' MSC adhere to 'bare' collagen. Inhibiting platelet adhesion to collagen resulted in almost total loss of MSC adhesion (Figure 6.7). The combination of antiGpIb and anti-GpIIbIIIa did leave the collagen surafce with very few platelets on it. Adding anti-β1-integrin to the MSC as well, increased adhesion slightly but not consistently, although the MSC that did adhere were single spherical cells.

Non-specific antibodies were also tested in the above models. In experiments on binding of isolated MSC on collagen or fibronectin, adhesion with non-specific IgG gave adhesion relative to untreated control = 1.01 ± 0.04 (mean ± SEM for 6 independent experiments). In addition, in experiments on fibronectin, adhesion with antibody against α4-integrin gave adhesion relative to untreated control = 1.05 ± 0.22 (mean ± SEM for 4 independent experiments combined). In experiments on binding of isolated MSC to platelets deposited on fibronectin from whole blood, adhesion with non-specific IgG gave adhesion relative to untreated control = 0.98 ± 0.04 (mean ± SEM for 3 independent experiments). In experiments on binding of WJMSC to collagen in whole blood, adhesion with non-specific IgG gave adhesion relative to untreated control = 0.89 ± 0.08 (mean ± SEM for 2 independent experiments).

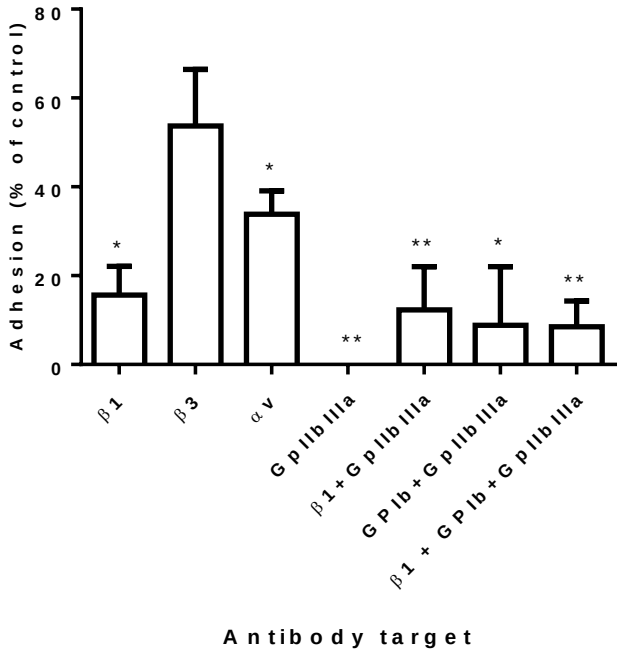


Figure 6-7: Effects of antibodies against different receptors on adhesion of WJMSC to collagen from whole blood

MSC were treated with antibodies against β1-, β3- or αv-integrin and added to blood and perfused at a wall shear rate of $35s^{-1}$ for 4 min. In experiments with anti-GpIb or GpIIbIIIa, antibodies were added to the blood before it was perfused. Not all antibodies were tested in all experiments, but untreated controls were used in every experiment. Data are mean ± SEM from 3 to 9 experiments. *=$p<0.05$, **=$p<0.01$ for comparison to untreated control by paired t test.

Note: For anti-β3-integrin $p=0.068$.

6.3 DISCUSSION

This chapter describes for the first time the adhesion mechanisms when MSC are flowed over matrix proteins, and how these change when MSC are added to blood and also interact with platelets. We followed a stepwise investigation to find out first how MSC can directly bind to collagen or fibronectin or platelets. We then investigated blood to try to find out what interactions were important for adhesion there, and to explain the results that only WJMSC adhered to collagen and no MSC adhered to fibronectin.

When isolated WJMSC or BMMSC were treated with antibody against β1-integrin, we observed marked reduction in number of adherent cells to collagen for both cells types, although the effect was stronger and more consistent for the BMMSC. Both cells types also showed reduced adhesion after treatment with antibody against β3-integrin. A combination of antibodies against both integrins had little extra effect suggesting that some other adhesive mechanism also existed. For BMMSC, we also used anti-αvβ3-integrin and there was a reduction in number of adherent cells similar to anti-β3-integrin. Surprisingly, anti-αv-integrin did not show any effect on number of adherent cells.

On fibronectin, when isolated WJMSC and BMMSC were treated with anti-β1-integrin, we observed huge, significant reduction in number of adherent cells compared to non-treated cells. When isolated WJMSC and BMMSC were treated with anti-β3-integrin, the number of adherent isolated cells only decreased slightly. However, after treatment with antibodies against αvβ3-integrin or αv-integrin, we did detect significant decrease in the number of adherent MSC on the fibronectin surface. Interestingly, adherent WJMSC and BMSC after treatment with anti-β3-

integrin lost the ability to spread on fibronectin surface. It is possible that this antibody affected signaling after adhesion which promoted spreading but not adhesion itself, while the anti-αvβ3-integrin did inhibit adhesion.

Our results are consistent with earlier studies, although none directly tested adhesion of flowing MSC to matrix proteins. Others have reported that β1-integrin is expressed on the surface of undifferentiated MSC (Goessler et al., 2008). Gronthos et al. reported that α1β1- and α2β1-integrins were involved in MSC binding to collagen and that MSC bound to fibronectin through α5β1-integrin (Gronthos et al., 2001). Another study concluded that the interaction between α5β1-integrin and fibronectin was crucial for MSC adhesion and control of cell migration (Veevers-Lowe et al., 2011). MSC were also reported to express αvβ3-integrin which supported MSC adhesion to platelets (Langer et al., 2009).

Our results indicate that both β1- and β3-integrins assist adhesion to collagen and to fibronectin, for WJMSC as well as BMMSC. The earlier adhesion studies (Chapter 3) showed WJMSC adhered at higher levels then BMMSC for both surfaces, but BMMSC spread faster than WJMSC (Chapter 4). Recent studies in our laboratory compared integrin expression for WJMSC and BMMSC by flow cytometry. These studies showed that both cell types expressed both integrins, although the level of β1-integrin appeared much higher. There was a slightly higher level of integrins on the WJMSC (Lewis Clarke, unpublished observations). It is difficult to predict whether these slight differences could explain the different behaviours.

Next we tested how MSC adhered to platelets. We perfused isolated WJMSC and BMMSC over platelet monolayers which had been deposited onto fibronectin from whole blood. We detected major, significant reduction in the number of adherent cells when MSC were treated with anti-β1-integrin, and lesser effect when MSC were treated with anti-β3-integrin. When the blood used to deposit the platelets was treated with anti-GpIIbIIIa, there was little stable adhesion of MSC as the platelets did not spread and they were pulled off the surface by MSC rolling past. This is consistent with the finding that platelets adhere to fibronectin using GpIIbIIIa (Beumer et al., 1994). It also indicates that the platelets do not use this receptor to bind MSC because adhesion still occurred but the platelets came off the surface. When anti-GpIIbIIIa was combined with treatment of MSC with anti-β1-integrin, MSC lost all adhesion ability and rolling was not seen. This shows again that β1-integrin supported adhesion of MSC to the platelets.

Having tested the adhesion mechanism between MSC and collagen and between MSC and platelets, we investigated mechanism of adhesion when MSC were added to whole blood and perfused over collagen. First we investigated the roles of platelet receptors. When whole blood was treated with anti-GPIb, we observed reduction in WJMSC adhesion to collagen. Large aggregates of platelets did not form on the collagen, and the adherent WJMSC after anti-GPIb treatment instead adhered as single cells surrounded by platelets. GpIb reduced platelet adhesion to collagen and build up of aggregates, but clearly did not stop platelets adhering to MSC. Secondly, when whole blood was treated with anti-GPIIbIIIa, we observed a total reduction of WJMSC adhesion to collagen, although a few platelets were adhered on the surface. Without platelet-platelet adhesion it seems WJMSC

could not bind in large numbers or aggregates. However, nor did they bind to the nearly bare collagen, presumably because they still had platelets attached to them and obstructing them.

BMMSC and TBMSC showed very little adhesion on collagen from whole blood, as demonstrated in Chapter 5. After whole blood was treated with anti-Gp1b or anti-GPIIbIIIa, adhesion was barely detectable. Again, it is likely that the MSC did have platelets attached in blood, and could not attach to the exposed collagen directly.

The behaviour of the platelets on collagen is in agreement with the literature which shows that platelets treated with anti-GPIb or anti-GPIIbIIIa have impaired adhesion and aggregation on collagen (Varga-Szabo et al., 2008, Savage et al., 1996). The results are also consistent with the in vivo study by Teo et al. who demonstrated that platelet depletion led to a decrease in MSC homing to the inflammed ear skin (Teo et al., 2012).

Finally, we investigated the role of integrins on MSC, with or without inhibition of platelet adhesion as well. On the collagen surface, we demonstrated that when WJMSC were treated with antibody against β1-integrin, the number of adherent WJMSC from flowing blood was greatly decreased. Antibody against β3- or αv-integrin reduced adhesion but not as much. This is consistent with the finding above that WJMSC bind to platelets via β1-integrin and to a lesser extent β3-integrin. Treatment of platelets with anti-GpIIbIIIa alone or with anti GpIb again reduced adhesion because of the loss of platelet binding to the collagen, but not platelet binding to the MSC in the blood. Treatment of MSC with anti-β1-integrin at the same time as the platelets did not recover adhesion. This should have inhibited

the platelets from binding to the MSC, but they still did not adhere to the collagen except in quite small numbers. It seems that the anti-β1-integrin also stopped that adhesion to collagen.

6.4 CONCLUSIONS

Adhesion of MSC to matrix proteins and to platelets involves both β1- and β3-integrins, with the β1-integrins having greater effect. Previous studies with static assays have shown importance of both families of integrins, but were not in flow. We found β3-integrins affected spreading after initial adhesion. In static systems, it is not possible to separate initial adhesion from spreading and stabilisation afterwards. As expected, platelets used GpIb and GpIIbIIIa to adhere and spread on collagen and GpIIbIIIa on fibronectin. These receptors did not affect interaction between MSC and platelets. We were not able to devise an experiment where we blocked platelet binding to collagen, and platelet binding to MSC, to recover adhesion of flowing 'bare' MSC to 'bare' collagen. This was because the same receptors mediated MSC-collagen and MSC-platelet adhesion, and blockade of adhesion to platelets also blocked adhesion to collagen.

Chapter 7 GENERAL DISCUSSION

7.1 Main findings

In this thesis we aimed to investigate the ability of MSC from different sources to adhere, spread and migrate on different surfaces using dynamic flow assays, and to study the effects of haematological and rheological factors on adhesion when MSC were suspended in blood. The studies were designed to give information on how MSC might behave in vivo if infused for therapy. The main findings are listed below.

7.1.1 Adhesive properties of different progenitor cells under flow

- Progenitor cells could adhere from flow onto collagen or fibronectin and the adhesion was more effective on the matrix proteins than on selectins.
- EPC adhered in greater number to P-selectin than MSC, but still in low numbers, and were slowed in flow slightly by P-selectin.
- MSC and EPC adhesion decreased the higher the wall shear rate, with adhesion not detectable at wall shear rate above $70s^{-1}$.
- The velocity of non-adherent MSC was greater, the bigger the mean diameter of the cell sample perfused, and smaller MSC adhered better than larger.
- WJMSC, BMMSC and TBMSC adhered to collagen significantly more than to fibronectin.
- The different MSC tested adhered in the order WJMSC>BMMSC>TBMSC.

7.1.2 Spreading and migration of MSC from different sources

- On collagen, BMMSC spread more efficiently and faster than WJMSC and TBMSC.

- On fibronectin BMMSC also spread faster compared to WJMSC or TBMSC.
- Spreading was more efficient, more rapid and to a greater area on fibronectin than on collagen.
- WJMSC were more effective in transmigration than BMMSC on collagen, fibronectin or albumin.
- Coating the lower surface with collagen or fibronectin promoted MSC migration compared to coating the top, for both proteins similarly.
- Coating of the upper side of filter with fibronectin promoted migration for fibronectin more than collagen.
- Smaller cells of BMMSC and WJMSC showed more efficient migration.
- Overall, comparing BMMSC and WJMSC for which we have the most complete data, WJMSC adhered in greater numbers from flow but BMMSC spread more efficiently and quickly; WJMSC migrated in greater numbers then BMMSC, especially when the lower surface was coated.

7.1.3 Adhesion of MSC to matrix proteins from flowing whole blood and interaction with platelets

- MSC within whole blood adhered to collagen or fibronectin much less than isolated MSC.
- Only WJMSC adhered to collagen effectively in blood, and they are associated with platelets, and also bound together by them.
- WJMSC formed clumps of cells, some large with several MSC, which included platelets and occasional leukocytes
- On a collagen surface, only occasional adherent BMMSC and TBMSC were seen.

- On collagen, many platelets adhered from blood and formed scattered clumps or 'thrombi' ; on fibronectin platelets formed almost confluent layer of spread platelets but no thrombi.
- Isolated MSC adhered to the platelet monolayer on fibronectin in large numbers, which increased if the platelets were activated with TRAP; adhesion was in the order WJMSC>BMMSC>TBMSC.
- Although MSC are larger than leukocytes, MSC could be seen marginated by the red blood cells in the blood flowing at the low wall shear rate used ($35s^{-1}$).
- Both WJMSC and BMMSC aggregated slightly with platelets, and larger aggregates were formed if collagen was added.
- All MSC could bind to platelets but only WJMSC appeared to activate the bound platelets.

7.1.4 Mechanisms of adhesion between MSC, platelets and matrix proteins

- There was a marked reduction in the number of adherent cells to collagen for isolated WJMSC or BMMSC when treated with antibody against β1-integrin.
- On collagen, MSC adhesion was also reduced after treatment with antibody against β3-integrin.
- A combination of β1-integrin and β3-integrin antibodies treatment, had little extra effect on MSC adhesion.
- There even greater reduction of adhesion to fibronectin for isolated WJMSC or BMMSC when treated with antibody against β1-integrin.
- There was only slight reduction in number of adherent cells to fibronectin for isolated WJMSC or BMMSC when treated with antibody against β3 -integrin antibody, but after treatment with antibodies against αvβ3-integrin or αv-integrin,

there was significant decrease in the number of adherent MSC on the fibronectin surface.

- Antibody against β3-integrin caused adherent WJMSC and BMSC to lose their ability to spread on fibronectin, suggesting that different antibodies against β3-integrin could affect adhesion or spreading separately.
- β1- and β3-integrins assist adhesion to collagen and to fibronectin, for WJMSC as well as BMMSC.
- When isolated WJMSC and BMMSC were perfused over platelet ma marked reduction in the number of adherent cells when MSC were treated with anti-β1-integrin, and lesser effect when MSC were treated with anti-β3-integrin.
- When the blood used to deposit the platelets is treated with anti-GpIIbIIIa, there is little stable adhesion of MSC as the platelets do not spread and they will be pulled off the surface by MSC rolling past.
- Anti-GpIb decreased platelet adhesion to collagen from blood, but did not stop platelets adhering to WJMSC.
- There was almost total loss of WJMSC adhesion to collagen when whole blood was treated with anti-GPIIbIIIa.
- Treating whole blood with anti-Gp1b or anti-GPIIbIIIa, BMMSC and TBMSC showed very little adhesion on collagen from whole blood.
- Overall, even when platelets adhesion was lost from the collagen, MSC were not seen adhering from flowing blood, suggesting they were still 'blocked' by platelets adhered to them.

- Treatment of WJMSC with anti-β1-integrin, greatly reduce the adhesion of WJMSC from flowing blood, while antibodies against β3- or αv-integrin reduced adhesion but not as much.
- Combining blockade of β1-integrins on MSC and platelet adhesion in whole blood did not recover adhesion of individual MSC to the 'bare' collagen. β1-integrins appeared to mediate adhesion of MSC to collagen as well as to platelets in the whole blood assay.

7.2 Relation to previous findings

Adhesion from flow may be the first and critical step for the recruitment of stem cells for vascular protection. For the first time, this thesis reports the comparison in this behaviour for MSC from different tissues: BMMSC,WJMSC and TBMSC. It also assessed their behaviour after adhesion, that might affect their fate once deposited in damaged tissue.

The finding that MSC could bind to matrix proteins, collagen and fibronectin, but not selectins, at wall shear rates up to about $70s^{-1}$ agrees with some but not all literature. One study found adhesion of BMMSC to endothelial cells at shear stress 0.1Pa (equivalent to$140s^{-1}$) (Segers et al., 2006), but others found that shear needed to be reduced to about 0.01Pa ($14s^{-1}$) (Ruester et al., 2006, Luu et al., 2013); Luu et al.,(2013) to detect adhesion, or could not detect adhesion from flow (Chamberlain et al., 2011). Some of these studies showed roles for selectins and VCAM-1 in adhesion (Segers et al., 2006, Thankamony and Sackstein, 2011). We could not reproduce adhesion to selectins here, while others in our laboratory previously examined adhesion to purified VCAM-1 and did not detect adhesion of BMMSC

form flow (Gerard Nash; unpublished observations). Adhesion from flow directly to collagen or fibronectin has not been reported previously. In one study, BMMSC did not adhere to a collagen surface at a wall shear rate of $1000s^{-1}$ (Jiang et al., 2012). In another study, BMMSC were settled onto surfaces and then flow was increased to study the strength of attachment; collagen coating increased the strength (Lan et al., 2003). Differences between results in adhesion studies could depend on the source and culture conditions for the MSC, and possibly also for the endothelial cells. P-selectin used here did support some adhesion of EPC as expected, and could also support rolling of leukocytes.

We found that adhesion was in the order WJMSC>BMMSC>TBMSC. We also found that BMMSC spread more rapidly and efficiently than WJMSC or TBMSC, while migration rate through filters was in the order WJMSC>BMMSC>TBMSC. Most of the studies of adhesion and migration in the literature examine only a single type MSC (BMMSC) using static assays. In this thesis we compared the behaviour of three different types of MSC by using flow adhesion assays to get a better understanding of MSC behaviour and to study the effect of their sources. We expected differences based on the findings of Aldridge et al. (2012) who suggested that the expression of adhesion molecules on MSC were affected by their source and the isolation method (Aldridge et al., 2012). The high WJMSC adhesion compared to BMMSC on collagen and fibronectin may be linked to differences in regeneration ability. WJ MSC have been shown to be more neurogenic and angiogenic than BMMSC, possibly also linked to differences in their secretomes (Hsieh et al., 2013). Regarding the low adhesion and migration of

TBMSC compared to WJMSC and BMMSC, we suggest this difference may be due to the fact that TBMSC were obtained from aged patients undergoing knee replacement. Coipeau et al. (2009) suggested that MSC were modified inherently because of the burden of the diseases.

Cell spreading and migration are the initial kinetic processes following adhesion, which are likely to affect the outcome after MSC adhere or become trapped in vessels. In this thesis -for the first time - we concluded that MSC spread more efficiently, more rapidly and to a greater area on fibronectin than on collagen. We found clear differences in behaviour of MSC that are used in current research and therapy, from bone marrow and Wharton's jelly. Trabecular bone is less frequently used to obtain MSC, and TBMSC behaved similarly to the WJMSC for spreading but not migration. We are not aware of any previous kinetic or comparative similar studies for MSC.

Cell diameter is expected to be one of the main factors which influences shear sensitivity of cell adhesion and thus of MSC recruitment. There has been no previous study of the role of stem cell diameter in adhesion. We showed that small size MSC adhered better than larger cells. Leukocytes are about 10μm in diameter while MSC were about 20μm. Leukocytes use specialised capture receptors, selectins, that act quickly, while the MSC binding we measured was to unspecialised matrix receptors via integrins. This is in agreement with studies assessing the effect of the cell diameter on adhesion behaviours. Watts et al. reported that cell size, margination, and a cell-free layer influenced the adhesive abilities of platelets and

leukocytes (Watts et al., 2013). They showed that platelets were able to adhere at high shear rate and they attributed this to their small size which subsequently minimized the force experienced and their velocity. The wall shear rate thus plays an important role in the adhesion of cells. In this thesis, the highly significant reduction in adherent cells noticed with increased wall shear rate, suggests that shear rate and stress play critical roles in EPC and MSC adhesion. In fact, infused MSC are cleared from the blood and many rapidly become deposited in the lungs (Fischer et al., 2009, Kang et al., 2012). These results suggest that trapping may be mechanical and not adhesive, so that size in important for that reason. If that is the case, the comparisons of data for capture for different MSC may be less important than the comparisons of ability to spread, integrate and migrate. However, the adhesion data for the isolated MSC may be less important than the results for behaviour in the blood.

In addition to the comparison of MSC types, this thesis aimed to improve our understanding of the behaviour of MSC in blood and the role of blood cells such as platelets in MSC adhesion. We demonstrated that MSC within whole blood adhered to collagen or fibronectin much less than isolated MSC. Indeed, there was no detectable adhesion to fibronectin, and on collagen, only occasional adherent BMMSC and TBMSC were seen. Only WJMSC adhered to collagen effectively in blood, and they were associated with platelets, and also bound together by them. There have been no previous studies of MSC adhesion in blood in vitro, but a few studies of flow or of infused MSC in animals that may be relevant. In vitro, Jiang et al. (Jiang et al., 2012) perfused rat BMMSC over collagen with or without platelets

added to the MSC, and found that MSC adhesion only occurred with platelets; these studies were at high wall shear rate ($1000s^{-1}$). In the same study, recruitment of infused MSC to the lung of rats was also dependent on platelets. Teo et al. (GS Teo et al) demonstrated that in mice, injection of endotoxin into the ear caused preferential recruitment of infused human BMMSC to microvessels in that ear. Intravital observations showed that the deposited MSC were associated with platelets and neutrophils. Langer et al. found that platelets attached to endothelial cells in vitro increased adhesion of BMMSC, and that injected BMMSC became attached to damaged arteries via platelets (Langer et al., 2009). These results are consistent with our finding that all MSC could adhere to platelets deposited on a surface or to platelets in suspension, and that WJMSC adhered with platelets to collagen from blood. We revealed that isolated MSC adhered to platelets in the order WJMSC>BMMSC>TBMSC and that the different behaviour of WJMSC was mediated through ability to activate and aggregate platelets. This has not been observed before.

We used function blocking antibodies to obtain a better understating of MSC interactions with matrix proteins and with platelets, in isolation and in blood. We found that BMMSC and WJMSC both used a combination of β1-integrin and β3-integrins to adhere to collagen , fibronectin and platelets, with β1-integrins more important in binding especially on fibronectin and platelets. Our results are consistent with earlier studies. Others have reported that β1-integrin is expressed on the surface of MSC (Goessler et al, 2008). Gronthos et al. reported that α1β1- and α2β1-integrins were involved in MSC binding to collagen and that MSC bound to

fibronectin through α5β1-integrin (Gronthos et al, 2001). Another study showed that α5β1-integrin controlled MSC adhesion and migration on fibronectin (J Veevers-Lowe et al 2011). We observed the interesting result that anti-β3-integrin inhibited adhesion to fibronectin slightly, but strongly inhibited spreading. This suggest that this integrin affected signalling after cells adhered, and so might affect migration as well as α5β1-integrin. MSC were reported to express αvβ3-integrin which supported MSC adhesion to platelets (Langer et al, 2009), but a role for β3-integrins in matrix interactions has not been described previously to our knowledge.

When platelets in blood were treated with anti-GpIb or anti-GpIIbIIIa, there was little stable adhesion of the platelets to fibronectin or to collagen, as expected from previous reports on platelet adhesion to similar surfaces (Beumer at al., 1994)(Varga-Szabo et al, 2008) (B Savage, 1996). These treatments also inhibited any adhesion of MSC in the blood. The results are consistent with the in vivo studies noted above (GS Teo et al)(Jiang et al., 2012)(Langer et al., 2009) who found in different animal models that platelet depletion or antibodies reducing platelet adhesion also reduced the recruitment of MSC. Those studies all used BMMSC, and there is no information to our knowledge on how the increased interaction of WJMSC with platelets would affect adhesion from blood or behaviour in vivo.

7.3 Physiological relevance of this study

MSC have features which make them potential therapies for a number of diseases but their therapeutic uses via infusion may depend on their ability to migrate

into tissue as well as adhere and spread on the surface of vessels. The ability of MSC to differentiate into organ specific cells, makes them useful for regenerative therapy and there have been many clinical trials e.g., to treat spinal cord injury and myocardial infarction (Wei et al., 2013). MSC are also able to suppress immune responses (Teo et al., 2015, Karp and Teol, 2009). However, there is not a large body of data to clearly define the steps by which they would adhere locally (within the vessel) or transmigrate across the endothelium. Our results, and previous studies with endothelial cells, suggest that MSC will bind better from flow to damaged vessels exposing matrix than inflamed vessels with intact endothelium. Thus, whether MSC can specifically home remains unclear, although our results suggest they could adhere, with or without platelets, in wounded tissues. The range of low shear rates where we observed adhesion indicate that recruitment would be more likely in the venous circulation rather than arterial. Alternatively, because of their large size, many could get physically trapped in the microcirculation in vivo.

Shear stress is a critical hemodynamic force because it modulates the function of endothelial cells and also influences the ability of circulating cells to adhere to the vessel wall (Chien et al., 1998, Topper and Gimbrone, 1999). The highly significant reduction in adherent cells noticed with increased wall shear rate in our experiments with isolated cells, suggests that shear rate and stress play critical roles in MSC adhesion. In blood, margination is necessary for effective adhesion, and is dependent on cell size, with larger cells tending to move inward in the flow more. In a low shear rate environment, the velocity of flowing MSC being low will maximize adhesion near to the vessel wall, and margination will also be promoted by

red cell aggregation. Although MSC are larger than leukocytes, they can still be marginated at low shear rates, as we have shown for the first time in this thesis. We observed adhesion for WJMSC on the upper surface of the microslide and so it was not promoted by sedimentation. Even when there was no adhesion, fluorescent MSC added to the blood could be seen flowing near the upper surface. In small vessels in vivo, the efficiency of MSC adhesion may be increased per unit volume of blood perfused because of their occupation of a larger proportion of the lumen and their having a lesser distance to marginate. The results described may be relevant to the behaviour of MSC if infused for therapy for inflamed tissue. During inflammation, fibrinogen production increases, accompanied by decline in venous flow rate and increase in red cell aggregation, which would be expected to assist adhesion of large cells (Watts et al., 2013).

This thesis demonstrated that MSC within whole blood adhered to collagen or fibronectin much less than isolated MSC, showing that their behaviour in vivo will modified by blood components, especially platelets. In the case of WJMSC but not BMSC or TBMSC, we observed clumps of cells which included platelets, indicating that the different cells would behave differently in vivo, in addition to differences in their adhesiveness for matrix proteins and ability to spread and migrate. Adhesion to the vessel wall may be unlikely for BMMSC or TBMSC even in damaged vessels with exposed matrix but WJMSC might adhere to exposed vessels along with platelets. There is also the possibility that WJMSC could cause thrombus formation in the blood and that this could block microvessels and be dangerous in recipients. On the other hand, such thrombi might actually help

recruitment to target tissues, and platelets seem to assist recruitment to damaged sites in vivo (Langer et al., 2009; Jiang et al., 2012; Teo et al., 2015). MSC appear to be rapidly cleared if infused, and it is not certain whether they would be trapped first and then collect platelets, or bind platelets in the blood and then become localised. It does seem to be significant that different MSC interact differently with platelets, and the platelet activation induced by WJMSC should be considered if these cells are used for therapy via infusion.

While MSC will not be' isolated' for long in blood in vivo, the behaviour of the isolated cells is still relevant. The therapeutic uses of MSC may depend on their ability to migrate into tissue as well as adhere and spread on the surface. BMMSC spread more quickly than BMMSC or TBMSC and might become stabilised in the wall of blood vessels more quickly. On the other hand, WJMSC were more effective in transmigration than BMMSC on all surfaces studied here. Veevers-Lowe et al. found that binding to collagen did not induce the same level of signalling in BMMSC as fibronectin (Veevers-Lowe et al., 2011), but in our studies this did not cause a difference in migration. Migration was more efficient when the back of filters was coated, but the migration to collagen or fibronectin were similar. The walls of vessels contain collagen as well as fibronectin, and our results thus suggest that WJMSC would migrate more effectively than other MSC in the vessel wall.

Our results show that the not only is the cell adhesion cascade affected by cell diameter, but also migration. We found that smaller cells in BMMSC and WJMSC samples showed more efficient migration as well as adhesion. Larger cells may migrate less easily through gaps than smaller ones, and this applied to both

types of MSC, which had similar diameters. The size distribution for MSC measured by the Coulter counter was very wide, and there might be some advantage for selecting MSC of smaller size for infusion. Not only could recruitment onto and into the vessel wall be more effective, but their trapping in organs such as the lung might be less, so that more arrive at the target tissue.

7.4 Future work

The in vitro flow assay used in this thesis represents the first attempt to study the rheology of MSC adhesion. It could be used to conduct further studies to investigate the effects of other rheological factors of blood on the margination and adhesion of MSC and their interactions with platelets. Studies could be done with platelets removed from the blood, and for varying levels of haematocrit or red cell aggregation, to study effects of the blood variables with and without platelets present. Using different sources of MSC would be useful to test whether their behaviour differs in the absence of platelets. In this thesis, we used low shear rates in flow adhesion assays; replicating the experiments using high flow shear rate, and possibly disturbed flow, could be used to mimic blood environment in arteries. These studies would provide a more complete understating of the physical factors that have an impact on MSC recruitment.

A question that requires further study is how much WJMSC activate platelets and why they differ from BMMSC. Since completion of experiments described in this thesis, others in our laboratory made a simpler 'static' assay with MSC deposited on tissue culture dishes to which PRP was added. This verified greater adhesion of platelets to WJMSC than to BMMSC. They have also adapted the MSC-platelet aggregation assay to use slower stirring and found that WJMSC cause strong platelet

aggregation judged by changes in light transmission, while BMMSC do not. Future work needs to show the molecular basis for these differences, as they are likely to be important for infused cells.

It is difficult to predict which of the properties such as adhesion, spreading and migration for the different MSC will be more important for in vivo behaviour and therapy. Different types of MSC (WJMSC VS BMMSC) may spread and integrate into the wall faster if e.g., mechanically trapped in small vessels. Further studies in flow models coated with endothelial cells, or by intravital microscopy, might be able to answer these questions about the effectiveness of the different MSC. IN vutri, MSC bound to the platelets adhered to the collagen; it might be possible in vivo to show which cells adhered first. The results may suggest that adhered platelets captured perfused MSC, but it is also possible that platelets adhered toMSC assist their trapping in microvessels.

Comparison of the circulation of different in MSC infused in animal models will provide more insights about the behaviour of different MSC types in the circulation. Recent studies in our laboratory compared the outcome when BMMSC and WJMSC were injected systemically via the tail vein in mice. They showed that WJMSC caused a decrease in blood platelet count but BMMSC did not. Thus MSC show origin-dependent interaction with platelets in vivo that may influence their adhesion to damaged vessels, and potentially cause thrombotic complications. More work needs to be done to investigate the mechanism that control MSC recruitment and interaction with platelets to control these processes.

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