HUMAN BONE TISSUE ENGINEERING USING CORAL AND DIFFERENTIATED OSTEOBLASTS FROM DERIVED-MESENCHYMAL STEM CELLS

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by

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ALCAM	Activated leukocyte cell adhesion molecules
ALP	Alkaline phosphatase
ATP	Adenosine triphosphate
CMM	Cord matrix medium
CPD	Citrate phosphate dextrose
DAB	3,3’ diaminobenzidine tetrahydrochloride
DAB	3,3'-diaminobenzidine
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethylsulfoxide
DNA	Diribonucleic acid
ECM	Extra-cellular matrix
EDTA	Ethylenediamine tetraacetic acid
FBS	Fetal bovine serum
HCl	Hydrochloric acid
hUCM	Human umbilical cord matrix
MNC	Mononuclear cells
MSC	Mesenchymal stem cells
MSCBM	Mesenchymal stem cell basal medium
MSCGM	Mesenchymal stem cell growth media
MSCGS	Mesenchymal stem cell growth supplements
MTT	Dimethylthiazol diphenyl tetrazolium bromide

OC	Osteocalcin
ON	Osteonectin
OPN	Osteopontin
OS	Osteogenic supplement differentiation medium
PBS	Phosphate buffer saline
SEM	Scanning electron microscopy

KEJURUTERAAN TISU TULANG MANUSIA MENGGUNAKAN BATU KARANG DAN OSTEOBLAS TERBEZA DARIPADA TERBITAN SEL STEM MESENKIMA

ABSTRAK

Penggunaan sel-sel stem mesenkima (MSC) di masa hadapan dalam kejuruteraan tisu tulang bergantung kepada keberhasilan pengkulturan sel MSC secara *in vitro*, pembezaan kepada osteoblas dan regenerasi tulang baru di dalam rangka batu karang. Tujuan kajian ini ialah untuk membina satu kaedah pengkulturan untuk sel-sel stem mesenkima dari sum-sum tulang dan darah talipusat manusia kemudian membezakannya kepada fenotip osteoblas diikuti dengan penilaian kebolehan sel-sel ini untuk bercambah secara *in vitro* dan membeza menjadi osteoblas di dalam rangka batu karang. Sel-sel stem mesenkima yang diperolehi dari sum-sum tulang diasingkan, dikultur-kembang dan kemudian dibezakan menjadi osteoblas. Morfologi sel dianalisis menggunakan teknik mikroskop cahaya manakala fenotip keimunannya dengan penanda-penanda permukaan sel pada MSC (CD105, CD166). Sel-sel stem mesenkima dikultur di dalam medium yang mengandungi 10 nM deksametason, 3.5 mM β-gliserofosfat dan 50 μM L-asid askorbik. Pembezaan osteogenik dinilai dengan pewarnaan histokimia [alkalin fosfatase (ALP) dan von Kossa] pada hari ke-7, 14 dan hari ke-21 selepas sel terbeza menjadi osteoblas. Asai biokimia secara kuantitatif dianalisis dengan aktiviti alkalin

fosfatase, kandungan kalsium dan asai protein total. Pewarnaan immunositokimia terhadap osteonektin (ON), osteopontin (OPN) dan osteokalsin (OC) yang bertindak sebagai penanda untuk osteoblas ditentukan setiap penandaannya pada tiga titik masa yang berlainan. Viabiliti sel yang menjalani pembezaan kepada osteoblas di dalam larutan batu karang (1, 10, 25, 50, 100 and 200 mg/ml) dinilai dengan asai dimetiltiazol difiniltetrazolium bromid (MTT). Percambahan dan pembezaan sel-sel di atas rangka batu karang dianalisis dengan menggunakan mikroskop imbasan elektron (SEM). Penanda permukaan sel untuk MSC mengekspresi CD105 dan CD166 yang mengesahkan MSC di dalam sum-sum tulang. Sel-sel dari matriks talipusat mampu bertahan untuk beberapa bilangan pengkulturan sahaja dengan ekspresi yang terhad untuk CD 105. Pewarnaan von Kossa untuk nodul yang bermineral dikesan pada hari ke-14 tetapi aktiviti ALP adalah rendah sepanjang pembezaannya kepada osteoblas. Ekspresi ALP meningkat dengan masa dan asai kalsium berkadaran dengan keputusan histokimia. Osteopontin mencapai tahap maksimum pada hari ke 7 dan beransur-ansur menurun hingga hari ke 21 tetapi rembesan OC meningkat berkadaran dengan masa. Yang menariknya, ON menunjukkan satu corak dwifasa dengan ekspresi yang tinggi pada hari ke-7 dan hari ke-21. Sel-sel kawalan yang dikultur tanpa media osteogenik mengekspresi penandaan yang pudar. Asai MTT dengan signifikannya menunjukkan percambahan sel-sel berbanding dengan sel kawalan apabila di dedahkan kepada kepekatan larutan batu karang yang tinggi. Kajian dengan SEM menunjukkan peliputan sel yang baik pada permukaan batu karang dengan penambunan matriks untuk ketiga-tiga kumpulan iaitu osteoblas terbeza dan MSC tanpa medium osteogenik dan

titisan sel osteoblas. Keputusan yang terhasil mengesyorkan bahawa batu karang ialah satu bahan yang bioserasi yang mampu menyediakan satu rangka sementara yang menarik untuk kejuruteraan tisu tulang.

HUMAN BONE TISSUE ENGINEERING USING CORAL AND DIFFERENTIATED OSTEOBLASTS FROM DERIVED-MESENCHYMAL STEM CELLS

ABSTRACT

The future use of mesenchymal stem cells (MSC) for bone tissue engineering depends on the establishment of *in vitro* MSC cell culture; differentiation into osteoblasts and generation of new bone on coral scaffolds. The purposes of this study were to develop a cultural method for the human bone marrow and cord blood mesenchymal stem cells to differentiate into osteoblasts phenotype followed by assessing their *in vitro* ability to proliferate and differentiate in the coral scaffolds. Mesenchymal stem cells of human were isolated, culture-expanded and then differentiated into osteoblasts. Cellular morphology was analysed by light microscopy technique and the immune phenotype by MSC cell surface markers (CD105, CD166). Mesenchymal stem cells were cultured in MSC media containing 10 nM dexamethasone, 3.5 mM β-glycerophosphate and 50 μM L-ascorbic acid. Osteogenic differentiation was evaluated by means of histochemical stainings [alkaline phosphatase (ALP) and von Kossa] at day 7, 14 and 21 after osteogenic differentiation. Quantitative biochemical assays were analysed by alkaline phosphatase activity, calcium content and total protein assay. Immunocytochemical stainings against osteonectin (ON), osteopontin (OPN) and osteocalcin (OC) served as markers for osteoblasts were each determined at 3 different time-points. Cell

viability for differentiated osteoblasts in eluted corals (1, 10, 25, 50, 100 and 200 mg/ml) were assessed with dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Cell proliferation and differentiation on coral scaffolds were analysed by using scanning electron microscopic (SEM). MSC surface markers expressed CD105 and CD166 which defined MSC in bone marrow. Cells from cord matrix persisted for a limited passage number with limited CD105 expression. Von Kossa stained for mineralised nodules on day 14 but ALP activity was minimal throughout osteogenic differentiation. Alkaline phosphatase expression increased with time and calcium assay correlated with the histochemical findings. Osteopontin reached a maximum on day 7 and gradually decreased until day 21 but the secretion of OC increased time-dependently. Interestingly, ON showed a biphasic pattern with increased expression on day 7 and 21. The control cells cultured in the absence of osteogenic medium produced negligible expression of these markers. MTT assay showed significantly cell proliferation compared to control with administration of high coral concentration. SEM studies exhibited good cell coverage on coral surface with deposition of the matrix for all cell types that is the differentiated osteoblasts, MSC without osteogenic medium and osteoblast cell line. The results suggest that coral is biocompatible material thus provides an attractive scaffold for bone tissue engineering.

CHAPTER ONE

INTRODUCTION

1.1 MESENCHYMAL STEM CELLS

The definition of a stem cell remained contentious for over three decades. “Mesenchyme” designates the developing loose connective tissue of an embryo, mainly derived from the mesoderm and giving rise to a large part of the cells of the connective tissue in the adult (Moore, 1993). Mesenchymal stem cells (MSC) according to Dennis and Caplan (2004), referred to the adult mesenchymal progenitor cells and are defined as cells that have the capacity to self-renew and uncommitted to conduct a specific function as well as the capacity for extensive proliferation and differentiation (Weissman, 2000). To avoid vague terms; Cowen & Melton (2006) concluded a working definition of a stem cell as a clonal, self-renewing entity that is multipotent and thus can generate several differentiated cell types.

Mesenchymal stem cells represent a subset of precursor cells that adhere to the stem cell definition, that is, they are capable of (1) self-renewal or the ability to generate at least one daughter cell with characteristics similar to the initiating cell; (2) differentiation into multiple cells and (3) *in vivo* functional reconstitution of the tissues to which they give rise (Verfaillie *et al*., 2002, Roufosse *et al*., 2004). In 1980, Golde and colleagues investigated the origin of

bone marrow fibroblasts in bone marrow transplant recipients and indicated that the human fibroblast is not derived from a precursor common to haematopoietic cells or lymphocytes. Instead, the cells were mesenchymal cells derived from a different precursor and capable of *in vitro* proliferation even after high doses of total body irradiation. According to Pittenger and co-workers (1999), only a small percentage; estimated at about 0.001 to 0.01% of cells isolated from the light density separation attached and grew as fibroblastic cells that developed into visible colonies.

These cells may be found in cells preparations that have been labelled with various terms include mesenchymal progenitor cells (Minguell *et al*., 2000, Caterson *et al*., 2002), marrow stromal cells (Wilkins & Jones, 1995, Kuznetsov *et al*., 1997, Prockop, 1997), mesenchymal stem cells (Majumdar *et al*., 1998, Tuan *et a*l., 2003); and colony-forming unit-fibroblastic (CFU-f) (Gordon *et al*., 1995, Minguell *et al*., 2000, Minguell *et al*., 2001). Verfaillie and co-workers (2002) described multipotent adult progenitor cells as a subpopulation of cells that co-purified with MSC and have unique properties. Although mesenchymal stem cells were originally isolated from bone marrow (Prockop, 1997, Minguell *et al*., 2001), similar populations have been reported in other tissues such as adipose tissue (Zuk *et al*., 2001), umbilical cord blood (Erices *et al*., 2000, Goodwin *et al.,* 2001, Bieback *et al*., 2004) and trabecular bone (Noth *et al*., 2002).

1.1.1 Sources of mesenchymal stem cells (MSC)

1.1.1(a) Bone marrow stem cells

The most well studied and accessible source of MSC is bone marrow, although in this tissue, the cells are present in a low frequency (Pittenger *et al*., 1999). The cells have attracted interest because the bone marrow stroma was originally thought to function mainly as a feeder layer for the growth of haematopoietic stem cells and progenitor cells (Caplan, 1991, Prockop, 1997). There are three main cellular systems in the bone marrow: haematopoietic, endothelial and stromal (Deans & Moseley, 2000, Tuan *et al*., 2003). Marrow stromal cells provide growth factors, essential cell-to-cell interactions as well as production of matrix proteins essential to maintenance, growth and differentiation of haematopoietic stem cells within the marrow micro-environment (Dennis & Charbord, 2002). Within the bone marrow stromal, there exists a subset of nonhaematopoietic cells referred to as mesenchymal stem or mesenchymal progenitor cells (Minguell *et al*., 2001).

Evidence for the existence of MSC in bone marrow-derived cultures has been provided by the work of Golde and colleagues (1980). They investigated the origin of the bone marrow fibroblasts and indicated that it was not derived from a precursor common to haematopoietic cells but instead, appeared to be mesenchymal cells which were capable of *in vitro* proliferation. Additional evidence for the present of uncommitted MSC has been provided by the work of Simmons and Torok-Storb (1991). By using a murine monoclonal antibody

STRO-1 and plated in long-term bone marrow culture conditions, these STRO-1 positive cells retain the capacity to generate adherent cells layers with identical cellular composition to that of the parent cultures. In addition, these cells were able to support the generation of haematopoietic cells. Recent efforts demonstrated that given the right stimuli and local environment, they develop into various cell types *in vitro* (Prockop, 1997, Caterson *et al*., 2002, Yuasa *et al.,* 2004).

1.1.1(b) Cord blood stem cells

Though bone marrow has been represented as the main available source of MSC, the use of bone marrow-derived cells is not always acceptable owing to high degree of viral exposure and the significant decrease in the cell number and the proliferation or differentiation capacity along with age (Campisi, 2001, Fehrer & Lepperdinger, 2005). In addition, it requires a painful invasive procedure to obtain a bone marrow sample. Thus the need to find an alternative MSC source has emerged especially if MSC are given repeatedly. It was found that the frequency of progenitors in umbilical cord blood (UCB) equals or exceeds that of marrow, and greatly surpasses that of adult blood (Broxmeyer *et al*., 1989). Umbilical cord blood (UCB) was well known to be a rich source of haematopoietic stem cells (Rubinstein *et al*., 1995) with practical and ethical advantages but the issue about the existence of MSC in UCB has not clearly resolved. It was reported that fresh UCB-derived mononuclear cells seeded on flasks without supportive stromal layers did not form stroma in long term culture conditions (Hows *et al*., 1992). Wexler and co-researchers (2003) concluded

that neither fresh UCB nor peripheral blood with stem cell mobilisation contained MSC. Numerous attempts to isolate MSC from UCB have either failed (Mareschi *et al*., 2001, Wexler *et al*., 2003) or have demonstrated a low frequency of mesenchymal progenitors in UCB of full term deliveries (Erices *et al*., 2000, Goodwin *et al*., 2001).

Optimal combination of different media as well as different growth factor cocktail has been evaluated continuously (Kohler *et al*., 1999, Balducci *et al*., 2003). Those experiments resulted in expansion of progenitors but long-term culture initiating cells have not been successfully amplified so far. In addition, Chivu and colleagues (2004) have tested several culture protocols for *in vitro* maintenance of UCB stem cells and evaluated the influences of different growth factors supplement and conditioned medium on UCB cultures. They concluded that the *in vitro* expansion of stem cells from UCB was dependent upon controlled experimental conditions of culture.

1.1.1(c) Cord matrix stem cells

In order for the potential of stem cells to be realised, they must be available in high numbers and they should be easy to isolate, purify and expand in number without induction of spontaneous differentiation and to differentiate into the cell type of choice. This requires an easily accessible, plentiful source of stem cells. Other than umbilical cord blood, adult stem cells have been isolated and propagated from the umbilical cord matrix (Mitchell *et al*., 2003, Romanov *et al*., 2003, Mizoguchi *et al*., 2004). The umbilical cord represents the link

between mother and fetus and important in the transfer of nutrients and oxygen during pregnancy. Mesenchymal connective tissue fills the space between the vessels and the simple squamous epithelium lining the surface of the umbilical cord. It contains a very low number of cells and high amounts of extra-cellular matrix (ECM) components, mainly collagen, hyaluronic acid and several sulphated proteoglycans (Nanaev *et al*., 1997, Gogiel *et al*., 2003). Cells in the umbilical cord matrix (UCM) or tissue are also known as Wharton's jelly or mucous connective tissue (Ross *et al.,* 2002). Within the umbilical cord are three large vessels and their walls. Studies by Kobayashi and co-researchers (1998) revealed that the stromal cells of Wharton's jelly at term pregnancy possessed the morphological characteristics of myofibroblasts which have a contractile function. This function provides the elasticity of Wharton's jelly and protects the umbilical vessels from compression by, for examples, fetal movements and uterine contractions (Nanaev *et al*., 1997, Kobayashi *et al*., 1998). The Wharton's jelly has high amounts of peptide growth factors such as insulin-like growth factor I (IGF-I), fibroblast growth factor (FGF), transforming growth factor β (TGF-β), platelet-derived growth factor (PDGF) (Sobolewski *et al*., 2005). The high amount of peptide growth factors strongly stimulated the cells to produce large amounts of collagen and glycosaminoglycans.

Recent studies showed that only a few researchers have isolated stem cells from human umbilical cord (Mitchell *et al*., 2003, Wang *et al*., 2004a) and in animals from porcine (Mitchell *et al.,* 2003, Medicetty *et al*., 2004). These UCM cells have been reported to be able to differentiate into neuron and glial cells, epidermal phenotype, osteogenic and adipogenic lineages (Mitchell *et al*., 2003, Weiss *et al*., 2003, Wang *et al*., 2004a). Studies have shown that this new

cell source may be another versatile, replacement material for cardiovascular tissue engineering (Kadner *et al*., 2002, Schmidt *et al*., 2005).

1.1.2 Isolation and maintenance of mesenchymal stem cells

Mesenchymal stem cells are generally isolated from aspirated of bone marrow harvested from the anterior iliac crest of the pelvis in humans (DiGirolamo *et al*., 1999, Pittenger *et al*., 1999). Many studies have defined conditions for isolation of the MSC from adult bone marrow and subsequent expansion of these cell populations (Majumdar *et al*., 1998, Pittenger *et al*., 1999). Different protocols exist for isolation and expansion of MSC from bone marrow aspirates (Caplan, 1991, Prockop, 1997). These isolation techniques generally rely upon density gradient fractionation to isolate the MSC from a bone marrow aspirate although density gradient fractionation does not completely preclude haematopoietic cell contamination and does not yield a pure population of cells. On the other hand, Caterson and co-workers (2002) tried to compare the isolation of MSC from bone marrow using both a direct plating method and by density gradient fractionation but did not find any discernable difference between the MSC populations isolated by both methods. Moreover, direct plating is less labour-intensive and allowed greater yield of MSC. However, the haematopoietic cell population in contrast to the MSC does not adhere to tissue culture plastic. For this reason, subsequent medium changes remove the haematopoietic cellular contaminants, thereby leaving a relatively pure cell population of proliferating MSC (Caterson *et al*., 2002).

Optimal growth characteristics and maintenance of the multiple differentiation potential of the MSC is also dependent upon the appropriate selection of a serum lot for medium supplementation (Jaiswal *et al*., 2000, Bieback *et al*., 2004). According to Dennis & Charbord (2002), stroma formation is observed by culturing in a medium containing a high concentration of screened horse and fetal bovine serum (FBS) in where they are cultured in 10% or 20% FBS. Therefore, serum provides many factors that are important for the transport, presentation and utilisation of essential molecules and nutrient molecules to support cell growth. Accordingly, the further expansion of MSC after isolation from bone marrow is necessary for their application in regenerative medicine because the number of MSC in bone marrow aspirate is very small (Pittenger *et al*., 1999).

1.1.3 Characteristics of mesenchymal stem cells

At present, the characterisation of human MSC lags significantly behind that of bone marrow haematopoietic cells. Mesenchymal cells isolate by adherence to plastic culture surfaces have characteristics properties that have been well-defined by a number of investigators (Colter *et al.,* 2000, Weissman, 2000, Caterson *et al*., 2002, Cowen & Melton, 2006). Both Weissman (2000) and Cowen and Melton (2006) described the stem cells as having the capacity for self-renewal and extensive proliferation and differentiation. Another studies by Colter and co-workers (2000) noted the MSC to have high cell heterogeneity with different expansive capacity depending on the presence of recycling uncommitted MSC. When cultures of bone marrow-derived MSC are examined

on the basis of cell proliferation status, they appear to be non homogenous (Minguell *et al.,* 2001). The work formed by Colter and co-researchers (2000) has shown that in stationary cultures of bone marrow, MSC subsist a minor population of small and agranular cell (labelled as RS-1 cells) with a low capacity to generate colonies. These quiescent RS-1 cells express an antigenic profile that is different from that displayed by the most abundant, fast-growing and committed precursors found in expanded cultures of MSC. By studying a precursor-product relationship between RS-1 cells and committed precursors, the authors came to the conclusion that the high expansive capacity of MSC depend on the presence of RS-1 cells. In turn, RS-1 cells may cycle under stimulation of factors secreted by the most mature mesenchymal progenitor cells. Thus, it seems that RS-1 cells may represent an *in vitro* subset of recycling uncommitted MSC (Minguell *et al*., 2001).

Mesenchymal stem cells are multipotent adult progenitor cells which have the ability to develop into a number of different cell types and contribute to the regeneration of mesenchymal tissue such as bone, cartilage, adipose, tendon, ligament and muscle (Minguell *et al*., 2000, Caterson *et al*., 2002). These adult mesenchymal stem cells have been isolated from bone marrow that is taken from the iliac crest and expanded. Studies have characterised these cells by their ability to proliferate in culture and they resemble a fibroblast-like morphology (Digirolamo *et al*., 1999, Colter *et al*., 2001). Upon isolation, these cells uniformly test positive for adhesion molecules (CD105, CD166) (Bowen *et al*., 1995, Barry *et al.,* 1999, Alsalameh *et al.,* 2004), extra-cellular matrix (CD44, CD90) (Oswald *et al*., 2004, Campioni *et al*., 2006) and other surface

proteins. In addition, these cells are able to maintain normal karyotypes and telomerase activity even at high passages (Pittenger *et al*., 1999).

Another feature of the mesenchymal stem cells (MSC) is that it changes dramatically with the age of the individual. Caplan (2006) suggested there were a few possibilities of how MSC decrease with age, for examples (1) the decrease in cell-to-matrix ratios during the differentiation events in embryogenesis and (2) many of the progenitor cells have been converted to differentiated cells during formation of tissues in embryo. Furthermore, from birth to teens, the increase in body-part sizes is many fold, thereby involve the direct conversion of MSC into differentiated phenotypes decreasing further the level of MSC. Based on the knowledge that MSC have large expansive potential, Digirolamo and colleagues (1999) proposed that MSC apparently lose their multipotentiality and approach and/or express apoptotic features with increasing age.

1.1.4 Differentiation of mesenchymal stem cells

Mesenchymal stem cells, other than been defined by their plastic adherent growth and subsequent expansion under specific culture conditions; their MSC characteristics can also be thought of as a committed lineage with limited potential for *in vitro* and *in vivo* differentiation (Javazon *et al.,* 2001). The differentiation of a MSC into an osteoblast was proposed to have four main stages: (1) mesenchymal stem cells, (2) osteoprogenitor cells, (3) pre-osteoblasts, (4) osteoblasts and (5) mature osteocytes (Long, 2001, Heng *et al*.,

2004). Induction of differentiation into osteoblasts, adipocytes and chondrocytes under appropriate culture conditions have been demonstrated extensively (Jaiswal *et al*., 1997, Mackay *et al*., 1998, Pittenger *et al*., 1999, Kotobuki *et al.,* 2005). What drives the differentiation of human MSC is not entirely known. Induction of MSC with osteogenic medium (OS) which consisted of β-glycerophosphate, ascorbic-2-phosphate, dexamethasone and fetal bovine serum (Leboy *et al*., 1991, Cheng *et al*., 1994, Jaiswal *et al*., 1997, Coelho & Fernandes, 2000b, Hoshi *et al*., 2001) triggers a series of molecular events including activation of signal transduction pathways (Jaiswal *et al*., 2000, Karsenty, 2001) and expression of osteogenic marker genes including alkaline phosphatase, osteopontin, osteonectin, osteocalcin and Cbfa1 (Weaver *et al*., 1997, de Oliveira *et al*., 2003, Thorwarth *et al*., 2005). When cell cultured in monolayer in the presence of these supplements, the cells acquire an osteoblastic morphology with up regulation of alkaline phosphatase activity and deposition of a calcium-rich mineralised extra-cellular matrix (Calvo *et al*., 1996, Seibel, 2005) with a heterogeneous populations of cells and mixed differential potentials (Candeliere *et al*., 2001).

In one study it has been reported that human bone marrow mesenchymal stem cells could be passage in medium containing fetal calf serum up to the 38^{th} population doubling level while still maintaining their osteogenic potential (Bruder *et al*., 1997). In support of these findings, a study by Stenderup and colleagues (2001) showed that the number and proliferative capacity of osteoprogenitor cells *in vitro* are maintained during aging. Conversely, a study by D'Ippolito and colleagues (1999) demonstrated that the

number of MSC with osteogenic potential decreased early during aging in humans and may be responsible for the age-related reduction in osteoblast number (Sekiya *et al*., 2002, Suguira *et al*., 2004). Addition evidence for the decreased osteogenic potential of MSC with age is shown by Huang and co-researchers (2001). Their study showed decreased osteoblastic function of mouse pre-osteoblastic cells (MC3T3-E1) after serial passage as was detected by the changes in the expression pattern of selected genes by micro-array analysis.

Thus, the exact environment needed for MSC to differentiate into each different cell type is not well understood, but includes varying combinations of nutrients (Jager *et al*., 2003, Stute *et al.,* 2004), growth factors (Stringa *et al*., 1995, Kanatani *et al.,* 2002, Weismann *et al.,* 2003, Salasznyk *et al*., 2004) and mechanical stimulus (Mueller *et al.,* 1999, Gerber & ap Gwyn, 2002). These researchers rigorously analysed bone marrow MSC in order to prove their adherence to the "stem cell" definition. They studied the progeny of colonies expanded from single adherent marrow cells, proving self-renewal and multilineage differentiation. This showed that at least some marrow cells represent true pluripotent stem cells rather than a mixture of committed progenitor cells. To date, the self-renewal capacity of MSC remains in question. Nonetheless, these *in vitro* studies (Kotobuki *et al.,* 2005, Mauney *et al*., 2005, Ohgushi *et al*., 2005) and other *in vivo* studies (Bruder *et al*., 1998a, Chen *et al.,* 2002, Mauney *et al.,* 2005, Gravel *et al*., 2006) showed that MSC can commit to the bone cell lineage and develop to the state of matrix mineralisation.

1.1.5 Human MSC cell surface markers

Mesenchymal stem cells isolated from the bone marrow by culture of adherent had shown considerable heterogeneity in terms of morphology and immunotype (Colter *et al.,* 2001) and thus currently, there is still the lack of a definitive marker for identification of MSC. Surface markers are useful in characterising the stem cell as isolated or cultured, and as a means to begin to understand its potential interactions with neighbouring cells and the cell environment (Pittenger & Martin, 2004). Antigenic phenotype of MSC is not unique but represents features of mesenchymal, endothelial and epithelial cells (Minguell *et al*., 2001).

Recently, a pattern of surface molecule expression typical of MSC has been identified; based on evidence of an extensive panel of monoclonal antibodies, including lineage specific markers (Gronthos & Simmons, 1995, Barry *et al.,* 1999), adhesion molecules (Pittenger *et al.,* 1999, Pittenger & Martin, 2004), extra-cellular matrix (Prockop, 1997) and growth factor receptors (Pittenger *et al.,* 1999, Minguell *et al*., 2001). For examples, the development of a series of monoclonal antibodies raised towards surface MSC antigens (Bruder *et al*., 1997) along with other antibodies developed to characterise bone marrow stromal cells (Simmons & Torok-Storb, 1991, Gronthos & Simmons, 1995) have been identified for MSC. Haynesworth and colleagues (1992) proposed that the presence of specific, distinct antigens that were identified by the monoclonal antibodies on the cell surface of marrow-derived MSC, while these antigens were negative for osteoblasts suggested that these recognised epitopes were

regulated during development. Currently, many investigators have explored CD105 (endoglin) as an important antigenic determinant in the identification of mesenchymal stem cells (Barry *et al*., 1999, Alsalameh *et al*., 2004, Stute *et al*., 2004) and also for CD166 (activated leukocyte cell adhesion molecule/ALCAM) (Bruder *et al*., 1998b, Arai *et al*., 2002, Alsalameh *et al*., 2004). Both are monoclonal antibodies directed against cell adhesion molecules that mediate static cell-cell interactions.

1.1.5(a) CD105 (Endoglin)

Currently, there is still the lack of a definitive marker for MSC although many attempts have been made to use cell-surface antigens as reagents in the isolation and identification of MSC. In general, these antibodies have been raised against intact human MSC (Haynesworth *et al*., 1992, Bruder *et al*., 1997, Pittenger *et al*., 1999). Examples of antibodies that have been described against human MSC include SH-2, SH-3 and SH-4 (Haynesworth *et al*., 1992). The SH-2 antigen present on the surface of human MSC has been further characterised and has been shown to react with CD105 or endoglin (Barry *et al*., 1999), a member of the transformation growth factor-beta family. Previous studies demonstrated that this cell surface antigen is highly expressed on human endothelial cells (Cheifetz *et al.,* 1992) and expressed in bone marrow of patients with haematologic malignancies (Campioni *et al*., 2006). In addition, Cheifetz and colleagues (1992) found out that these antibodies recognised epitopes present on the surface of MSC and not on haematopoietic cells and the antigens disappeared upon osteogenic differentiation. In another study,

Pittenger and co-workers (1999) suggested the most accepted profile for immunotyping is the co-expression of CD105 and CD73 although CD44, CD90 and CD29 are also important antigenic determinants. However, none of these antigens are exclusive to MSC. Similarly in other studies, the co-expression of CD105 and CD166 has been suggested to differentiate MSC from other mature mesenchymal cells (Alsalameh *et al*., 2004, Oswald *et al.,* 2004). Endoglin was also expressed in other source of stem cells such as human umbilical cord blood-derived mononuclear cells (Erices *et al*., 2000) and the Wharton's jelly of umbilical cord matrix (Wang *et al*., 2004a).

1.1.5(b) Activated leukocyte cell adhesion molecule (ALCAM/CD166)

ALCAM was first identified on thymic epithelial cells (Patel *et al*., 1995) and activated leukocytes (Bowen *et al*., 1995). The expression of ALCAM on MSC was initially reported by Bruder and colleagues (1998b) where SB-10 antibody identified reacted with MSC and osteoprogenitor cells and later known as activated leukocyte cell adhesion molecule (ALCAM or CD166). This expression disappears once the MSC embark on an osteogenic pathway and begin to express alkaline phosphatase (ALP). The expression of ALCAM on MSC was also reported by Pittenger and co-workers (1999). These observations indicate that ALCAM may play a role in the progress of osteogenic differentiation although the precise mechanism of that activity remains to be elucidated (Bruder *et al.,* 1998b). Alternatively, it means that the use of antibodies can, not only be used to confirm the presence of committed osteoprogenitors but also be used to identify MSC which may potentially

differentiate into osteoblasts (Arai *et al*., 2002); suggests the ALCAM positive MSC to have a role in early bone development. Other than MSC, ALCAM is also expressed on human blastocysts (Fujiwara *et al*., 2003), perichondrium (Arai *et al*., 2002) and endothelial cells (Ikeda & Quertermous, 2004). ALCAM was thought to play a role as an adhesion molecule involved in the development of haematopoietic stem cells and endothelial progenitors (Ohneda *et al*., 2001).

1.2 BONE

1.2.1 Bone structure and function

Bone is a dynamic tissue, which remodels and repairs itself throughout life. Bone tissue has three major functions in the body: it offers mechanical support to the body, it protects major vital organs and serves as a mineral reservoir and maintains calcium and phosphate homeostasis (Cullinane & Einhorn, 2002). The feature that distinguishes bone from other connective tissue is the mineralisation of its matrix, which produces an extremely hard tissue capable of providing support and protection. Bone also provides the environment for haematopoiesis, which takes place in the marrow of long bones in adults (Ross *et al.,* 2002, Knothe Tate *et al*., 2004). The diversity of functional requirements of bone tissue is reflected by its complex architecture. In the adult skeleton bone tissue is either arranged in a trabecular pattern (cancellous bone) or in a compact pattern (cortical bone) (Ross *et al*., 2002). Cortical bone is almost solid with less than 10% porosity and ubiquitously present in long, short and flat bones. In contrast, cancellous bone is organized in a porous sponge-

like pattern (Christenson, 1997, Kneser *et al*., 2006). This type of bone harbours a large part of the bone marrow and is essentially present in the metaphysis of long bones, the iliac crests and the vertebral bodies (Moore & Dalley, 1999).

1.2.2 Embryology of bone development

In embryology, the skeletal system develops from the mesoderm which will later divide into paraxial and lateral plate mesoderm (Moore & Persaud, 1993). From the differentiating mesoderm, there arise two lineages with osteogenic potential. The paraxial mesoderm differentiates into somites which give rise to the axial skeleton; whereas the lateral plate mesoderm gives rise to the appendicular skeleton (Sadler, 1990, Moore & Persaud, 1993). The process of bone development involves four distinct phases: (1) migration of cells with osteogenic potential (mesenchymal cells) to the site of future skeletogenesis; this is followed by (2) mesenchymal–epithelial interactions that lead to (3) condensation (or aggregation) of mesenchymal cells and (4) subsequent differentiation into either the chondrogenic or osteogenic lineage (Heng *et al*., 2004).

1.2.3 Bone cell development

Bone cell development can be classified into two phases: (1) osteopoiesis and (2) osteogenesis/bone ossification (Long, 2001). The cellular hierarchy of bone precursor cells development is artificially divided into a number of developmental stages: (1) mesenchymal stem cells, (2)

osteoprogenitor cells, (3) pre-osteoblasts, (4) osteoblasts, and (5) mature osteocytes (Long, 2001, Heng *et al*., 2004). Once the pluripotent progenitor cells have committed to the osteoblastic lineage, they progress through three developmental stages of differentiation: proliferation, matrix maturation and mineralisation (Dworetzky *et al*., 1990).

1.2.3(a) Osteoprogenitor cells

Mesenchymal stem cells are a pluripotent population capable of generating multiple stromal cell lineages (Deans & Moseley, 2000, Herzog *et al*., 2003). It is unclear whether these are derived from more primitive progenitors but one of the primitive progenitors that is the osteoprogenitor cells; which are committed to the bone cell lineage are responsible for the expansion of osteoblast numbers and can be detected by antibody specific for osteoprogenitor cells (Joyner *et al*., 1997). Human osteoprogenitor cells have been cultured from both adherent (D'Ippolito *et al.,* 1999, Nishida *et al*., 1999) and non-adherent (Long *et al*., 1995, Oyajobi *et al*., 1999) populations of human bone marrow, although the relationship between the two phenotypes is unknown. Autoradiography using tritiated thymidine shows that osteoprogenitor cells are undifferentiated mesenchymal cells that are found in the stroma surrounding bone marrow (Qidwai, 2004). Since these mesenchymal cells have the ability to develop into a number of different cell types and contribute to the regeneration of mesenchymal tissue such as bone, cartilage, adipose, tendon, ligament and muscle; they are multipotent adult progenitor cells (Minguell *et al*., 2000, Caterson *et al.,* 2002).

When cultured on biodegradable scaffolds under mineralising conditions, human osteoprogenitor cells were observed to grow and differentiate into osteogenic phenotype (Yang *et al*., 2001, Mauney *et al.,* 2005) as demonstrated by the formation of mineralised foci of bone cell development. Osteoprogenitor cells were first used to delineate the process of osteogenesis and to define the developmental hierarchy of murine bone lineage cells (McCulloch *et al*., 1991) and the number of osteoprogenitor cells in bone marrow act as an index of skeletal growth and maturation (Nishida *et al*., 1999).

1.2.3(b) Pre-osteoblasts

The pre-osteoblasts are transitional in nature, bridging the progenitor cells with the mature osteoblasts (Long, 2001). Pre-osteoblasts lack the well-developed protein-synthesising capability of the mature osteoblasts and do not have the characteristically localised, mature rough endoplasmic reticulum or Golgi apparatus of the mature cell (Sims & Baron, 2000). Also, these cells usually stain less intensely for alkaline phosphatase; which implied that they have not yet acquired all the characteristics of mature osteoblasts (Puzas, 2000). By ultrastructural observation of the Haversian canal, it is identified by its location between the lining of the blood vessels and the mineralised zone of the osteoid seam; a layer which has a significant role in bone modelling and osteogenesis (Hirohata *et al.,* 1981).

1.2.3(c) Osteoblasts

Osteoblasts are the bone forming cells arise from osteoprogenitor cells, which are found in endosteum and periosteum and responsible for formation and remodelling of bone matrix in particular at sites of active bone production (Laros, 1976, Olsen *et al.,* 2000). They range from 20 to 30 micron in maximum diameter, and are best seen along the osteoid seam where new bone is being formed (Laros, 1976, Hirohata *et al.,* 1981). The plasma membrane of the osteoblast is characteristically rich in alkaline phosphatase enzyme; whose concentration in the serum is used as an index of bone formation (Jaiswal *et al*., 1997, Sims & Baron, 2000). Mature osteoblastic cells are highly polarised with a well-developed Golgi apparatus typical of highly secretory cells, intensely basophilic cytoplasm and abundant rough-surfaced endoplasmic reticulum (Hirohata *et al.,* 1981; Sims & Baron, 2000). The main secretory product of osteoblasts is type 1 collagen (90% of the total matrix protein) (Fedarko & Robey, 2000). The predominant non-collagenous protein is osteocalcin (Irie *et al*., 1998) but osteoblasts secrete other non-collagenous proteins including osteopontin (Irie *et al.,* 1998, Gerstenfeld, 1999, Perrien *et al*., 2002), osteonectin (Mundlos *et al.,*1992, Yan & Sage, 1999), bone growth factors (Weir *et al*., 2000), cytokines (Alsina & Roodman, 2000) and certain hormone receptors (Martin *et al*., 2000).

1.2.3(d) Osteocytes

After the completion of bone formation, a small portion (10-20%) of osteoblasts embedded within the newly formed extra-cellular matrix that they produced called osteocytes (Junqueira *et al*., 1998, Ross *et al*., 2002). Osteocytes are concerned more with bone maintenance than with new bone formation (Laros, 1976). Osteocytes are smaller than osteoblasts and they have lost many of their cytoplasmic organelles. Osteocyte cell bodies are found in lacunae inside the bone matrix and have numerous processes which extend out from the lacunae into canaliculi and contact processes from other osteocytes (Knothe Tate *et al*., 2004). The canalicular and lacunar surfaces add tremendously to the surface area of mineral available for exchange with tissue fluids and adjacent cells (Laros, 1976, Junqueira *et al.,* 1998). Even though the metabolic activity of the osteoblast decreases dramatically once it is fully encased, these cells still produce matrix proteins. Before becoming trapped in the matrix, the osteocytes test positive for the presence of alkaline phosphatase activity as functional mature osteoblasts. Osteocytes express a number of osteoblast markers in low levels including osteopontin, osteonectin, osteocalcin and bone sialoprotein (Knothe Tate *et al.,* 2004).

1.2.3(e) Osteoclasts

The osteoclast is a large multinucleated cell which is responsible for bone resorption (Ross *et al*., 2002). It is usually found in contact with a calcified bone surface and within a lacuna which is the result of its own resorptive

activity. They are generally located in the part of the cell away from the bone surface. The most prominent features of the osteoclast are the zone of contact with the bone characterised by the presence of a ruffled border due to multiple infoldings of plasma membrane, producing the brush border characteristics of an active osteoclast (Hirohata *et al.,* 1981, Gay, 2005) and the surrounding zone of attachment (sealing zone). The ruffled border provides an extensive surface area of specialised plasma membrane through which acid is secreted and hydrolytic enzymes are released (Gay, 2005). The sealing zone is a ring of contractile proteins that attach the osteoclast to the bone surface thus appears to function as a seal around the bone-resorbing compartment (Hirohata *et al.,* 1981, Sims & Baron, 2000). Mitochondria are abundant, indicating that osteoclasts have a high level of aerobic metabolism and osteoclasts contained numerous granules rich in calcium phosphate (Gay, 2005). The osteoclasts appears to undergo apoptosis after a cycle of resorption (Kameda *et al.,* 1995), characterised by loss of the ruffled border, detachment from the bone surface and condensation of the nuclear chromatin (Hirohata *et al.,* 1981).

1.2.4 Osteogenesis (bone formation)

Bone formation *in situ* can take place in two different ways (Olsen *et al*., 2000). First, endochondral ossification, in which mineralised bone tissue forms on a cartilage scaffold, and second, intramembranous ossification, in which condensed mesenchymal cells differentiate into osteoblasts at ossification centres in the absence of a cartilaginous scaffold (Olsen *et al*., 2000, Marks & Odgren, 2002).

1.2.4(a) Intramembranous ossification

During intramembranous ossification, mesenchymal cells proliferate within a highly vascularised area of the embryonic connective tissue forming cell condensations (Junqueira *et al*., 1998, Ross *et al*., 2002). These cells will synthesise a woven bone matrix while at the periphery, mesenchymal cells continue to differentiate into osteoblasts. Once the blood vessels are incorporated between the woven bone trabeculae, they will form the haematopoietic bone marrow. Later, this woven bone will be remodelled and progressively replaced by mature lamellar bone (Sims & Baron, 2000).

1.2.4(b) Endochondral ossification

Endochondral ossification is the process by which the skeletal cartilage scaffolds are replaced by bone (Junquiera *et al*., 1998). The scaffolds elongate and expand in width by proliferation of chondrocytes as well as by deposition of cartilage matrix. Shortly after their formation, chondrocytes in the central region of the cartilage undergo further maturation to hypertrophic chondrocytes (Junqueira *et al*., 1998, Ross *et al*., 2002). Angiogenic factors secreted by hypertrophic chondrocytes induce sprouting angiogenesis from the perichondrium. With the vessels come the osteoblasts, osteoclasts and the haematopoietic cells. These result in the formation of primary ossification centres (Olsen *et al*., 2000) where within these centres, the hypertrophic cartilage matrix is degraded, the hypertrophic chondrocytes undergo apoptosis, osteoblasts replace the disappearing cartilage with trabecular bone followed by

the formation of the bone marrow (Ross *et al*., 2002). At the same time, osteoblasts in the perichondrium form a collar of compact bone around the middle portion (diaphysis) of the cartilage, so that the primary ossification centre ends up being located inside a tube of bone. At one or both ends (epiphyses) of the cartilage, secondary ossification centres are formed, leaving a plate of cartilage (growth plate) between epiphysis and diaphysis. In the growth plate, a coordinated sequence of chondrocyte proliferation, hypertrophy and apoptosis results in longitudinal growth of long bones (Junqueira *et al*., 1998, Ross *et al*., 2002). At the same time, these processes are coordinated with growth of the epiphysis and radial growth of the diaphysis (Sims & Baron, 2000).

1.2.5 Mechanism of bone formation

Once the pluripotent progenitor cells have committed to the osteoblastic lineage, bone formation occurs by three coordinated processes of proliferation with matrix secretion, matrix maturation and mineralisation of the osteoid matrix (Dworetzky *et al*., 1990). According to them, in normal adult bone these processes occur at the same rate so that the balance between matrix production and mineralisation is equal. Proliferation phase consists of cell multiplication, growth, maturation and the development of extra-cellular matrix. Type I collagen, one of the first products formed during bone maturation and on which future mineralisation will take place is initially deposited rapidly without mineralisation, producing a thickening osteoid seam (Laros, 1976). The second phase, matrix maturation is characterised by early osteogenic markers such as alkaline phosphatase, osteopontin and osteonectin (Dragoo *et al*., 2003). This is