Chapter 1

Introduction

Introduction

Autogenous bone grafting is a gold standard procedure aiming to repair bony defects and enhance new bone formation. An adequate bone formation and acceleration of bone regeneration in reconstruction of skeletal defects are not overcome by this reconstructive procedure. Allografts and alloplasts are introduced as alternative grafting materials to alleviate disadvantages of autogenous bone grafting, which are inherent donor site limitations including inadequate bone volume and donor site morbidity and discomfort (Lew D. et al., 1992). These materials are not superior to autogenous bone. However, they have variable degradation rates and poor physical properties and can cause tissue reaction and possibly transmit contagious diseases (Mellonig et al., 1992). These shortcomings have inspired the development of tissue engineering in which the properties of synthetic compounds are manipulated to enable delivery of an aggregation of dissociated cells and growth factors into the defect sites in a manner that will result in the formation of new tissue (Langer and Vacanti, 1993).

One of the common approaches for the engineering of new tissues is related to biomaterials and cell culture. Biomaterials are fabricated as scaffolds and functioning as carriers of cells and growth factors and matrixes for attachment and growth of cells. Scaffolds should be osteoconductive, biocompatible and biodegradable (Langer and Vacanti, 1993) and have three-dimensional (3-D) porous structures. The 3-D structures of scaffolds provide surface contact and temporary mechanical support for functional cells and maintain space for tissue development. Scaffolds should be gradually degraded and eventually replaced by the regenerated tissue (Ma and Choi, 2001).

Scaffolds currently use in cell culture are natural and synthetic polymers such as collagen, ceramics, hydroxyapatite (HA), tricalcium phosphate (TCP), poly(L-lactic acid) (PLLA), poly(glycolic acid) (PGA), and poly(lactic acid-co-glycolic acid) (PLGA) (Thompson et al., 1995). Limitations of natural polymers such as collagen is fast degradation rate (Friess, 1998); ceramics are their fragility and slow resorption rate (Pilliar et al., 2001) and synthetic polymers are acidicity of their degradation products

(von Recum et al., 1995). Acidity of the degradation product accelerates the degradation rate of the polymers and induces an inflammatory tissue reaction (Vert et al., 1994).

Many current researches have been using natural biodegradable polymers such as collagen, gelatin, fibrin, and alginates as tissue scaffolds because they facilitate cell attachment and maintenance of differentiation function. There are many forms of these materials such as hydrogels, porous structures, sheet and fibrous matrices. Porous matrices provide a suitable microenvironment that promotes osteoblast adhesion and proliferation, maintains differentiated function without hindering proliferation, and helps in the formation of an extracellular matrix (ECM) (van Tienen et al., 2002;Wake et al., 1994).

Chitosan is a partially deacetylated derivative of chitin which is the prominent structural polysaccharide in the exoskeleton of insects, crustaceans and invertebrates. The chemical structure of chitosan cossresponds to glycosaminoglycans in the ECM of human tissue. Recently, much attention has been given to utilization of chitosan in biomedical applications, such as a wound healing agent, bandage material, hemodialysis membrane and drug delivery vehicle. Chitosan has been applied to conduct the ECM formation in tissue regenerative therapy. It is biocompatible, biodegradable in an acidic environment at pH lower than 6 (Francis Suh and Matthew, 2000) and osteoconductive (Muzzarelli et al., 1993a;Muzzarelli et al., 1993b). Many studies have combined chitosan with other biomaterials such as inorganic ceramics and bioactive polymers to improve physical properties and further enhance the tissue regenerative efficacy of chitosan. Collagen is a major component of ECM and enhances proliferation, migration and differentiation of osteoblast-like cells (Lynch et al., 1995).

Advantageous properties of chitosan and collagen are combined to improve properties of the scaffolds. Many reports have fabricated chitosan-collagen composites into scaffolds to be applied in tissue engineering but the properties of the scaffolds in supporting growth and differentiation of osteoblasts in a long term culture and the effects of the amount of collagen in chitosan-collagen composites to the physical structure of the scaffolds have not yet been reported.

Review of Literatures

Tissue engineering of bones

Tissue engineering utilizes both the engineering and life science disciplines to either maintain existing tissue structure or to enable tissue growth (Langer and Vacanti, 1993). The term tissue engineering has implied to some combination of cells, the 3-D scaffold materials and bioactive peptides that can modulate cellular activities, be delivered into the desired site in a patient's body, and direct new tissue formation into scaffolds (Lee and Mooney, 2001).

Consequently, delivery systems for protein therapeutics that promote tissue regeneration are of great interest (Centrella et al., 1994;Wozney, 1992). Recently, with the advancement of material science in a drug controlled release, it is possible to increase the efficacy of these drugs, which generally have very short half life (Langer, 1990). The suitable scaffolds can deliver the bone morphogenetic protein (BMP) in a controlled manner, as well as provide a matrix that can guide the cells to proliferate and differentiate into the large tissue masses (Saito and Takaoka, 2003).

The development of biomaterials for application in tissue engineering has recently focused on the design of biomimetic materials that are able to interact with surrounding tissues by biomolecular recognition (Hubbell, 1999;Healy, 1999). When biomaterials are exposed to biological environments, ECM proteins are non-specifically adsorbed on the surface of nearly all the biomaterials, then cells indirectly interact with the biomaterial surface through the absorbed ECM proteins. The design of biomimetic materials is an attempt to make the materials such that they are capable of eliciting specific cellular responses and directing new tissue formation mediated by specific interactions, which can be manipulated by altering design parameters instead of by non-specifically adsorbed ECM proteins (Shin et al., 2003).

An important characteristic of an artificial matrix is its micro-architecture (Brauker et al., 1995), which entails having sufficient porosity and interconnections between the pores for cell proliferation, diffusion of nutrients, clearance of wastes, and infiltration of progenitor cells. The micro-architecture must also have sufficient specific surface area to allow for cell attachment and the spreading, and appropriate shape of the pores for cells to attach and differentiate (Brauker et al., 1995;Nade et al., 1983).

The principles of tissue engineering have been applied to virtually every organ system in the body. Considerable attention has been focused on orthopaedic and maxillofacial applications, including the engineering of bone (Nakahara et al., 1992), cartilage (Cima et al., 1991), periodontal tissue repair (Anderegg et al., 1995) and dentin regeneration (Nakashima M., 1994). The engineering of artificial bone is a recent approach to treat patients with bone defects. Production of a bone-like tissue in vitro is based on the ability of osteoblasts to migrate, proliferate, and differentiate in culture. The ultimate step of extracorporeal bone tissue production should be mineralization of a bone-like extracellular scaffold. Thus, engineering of artificial bone should consider not only differentiation but also the mineralization phenomena (Wiesmann et al., 2003). Two basic approaches can be performed to implant cell-containing materials. Firstly, cells can be grown in petri dishes and detached then seeded onto matrices (collagen, polymers, calcium phosphate materials). Secondly, cells are grown on scaffolds under cell culture conditions to develop a hybrid construct *in vitro* for implantation *in vivo* (Meyer et al., 1993).

Bone regeneration requires a morphogenetic signal, responsive host cells that will respond to the signal, a suitable carrier of this signal delivering it to specific sites and serve as a scaffold for the growth of the responsive host cells, and a viable, well vascularized host bed (Croteau et al., 1999;Burg et al., 2000). To succeed, techniques are required to optimize the differentiation of precursor cells and the development of a functional ECM on the 3-D scaffolds (Ignatius et al., 2005). Various scaffolds were developed to allow for bone regeneration by maintaining a space that facilitates the migration and differentiation process. These scaffolds were mainly engineered on the basis of natural or synthetic polymers.

The Cells

The cellular component of tissue-engineered construct is ultimately responsible for performing the function of the tissue that it is designed to replace. The process of assembling a tissue-engineered construct begins with the identification of the relevant cell type and the process of isolation of these cells from the native tissue. To obtain sufficient numbers of cells from the available tissues, cell population from native tissue

can be expanded *in vitro* cell culture systems. It is essential to ensure that the expanded cells retain their phenotypic functions.

Recent advances in stem cell biology showed that mesenchymal stem cells (MSCs) could differentiate into cells of mesenchymal tissues such as bone, cartilage, muscle, tendon, ligament and fat. Stem cells were expected to play an important role in the repair of skeletal defects (Caplan, 1991;Pittenger et al., 1999). Culturing of undifferentiated (stem/progenitor) cells having a higher proliferative capacity is a promising technology (Cancedda et al., 2003). Differentiation of such cells *in vitro* can be directed by controlling or changing the culture conditions after their expansion. Direction of cellular differentiation *in vivo* is a consequence of the new 'physiological' microenvironment in the transplanted area.

Mesenchymal stem cells

Stem cells are cells from an embryo, fetus, or adult that under certain conditions can reproduce for long periods. They can also give rise to specialized cells of body tissues and organs. Recent evidences suggested that stem cells derived from the brain (Clarke et al., 2000) and bone marrow (Reyes et al., 2001;Jiang et al., 2002) that could be expanded to a very significant number of cell doublings without any signs of cell senescence. By manipulating cell culture conditions, embryonic stem cells can differentiate into cells of different lineages displaying pluripotential differentiation capacity of embryonic stem cells (Cancedda et al., 2003). An adult stem cell is undifferentiated (unspecialised) cell presenting in a differentiated tissue, which renews itself and becomes specialized to yield all of the cell types of the tissue from which it was originated. Their progeny includes both new stem cells and committed progenitors with a more restricted differentiation potential. These progenitors in turn give rise to differentiated cell types (van der Kooy and Weiss, 2000).

Apart from the higher proliferative capacity at least two additional considerations favor the use of stem/progenitor cells instead of differentiated cells for tissue engineering. Firstly, a perfectly engineered tissue with differentiated cells will give unpredictable results and eventually fail in an improper microenvironment. The engineered tissues are avascular when implanted and transplanted cells are exposed to an initial phase of hypoxia and insufficient nutrition until blood vessels invade the engineered construct. Thus, it would seem wiser to implant stem/progenitor cells with scaffolds specifically designed to allow for a rapid and correct vascularization. In this

way, new tissue formation, host vessel invasion and in some cases of neo-angiogenesis would occur at the same time as in a physiological repair process. Secondly, it is of critical importance that transplanted cells not only have the capacity to make specialized cells for the immediate repair or replacement of a tissue, but they also retain a high regenerative potential in order to guarantee correct function and cell turnover over time, possibly for a lifetime (Cancedda et al., 2003).

Bone marrow stromal cells (BMSCs) are responsible for the maintenance of bone turnover throughout life and can be regarded as a mesenchymal progenitor/ precursor cell population derived from adult stem cells. Cultured BMSCs can be stimulated to differentiate into bone, cartilage, muscle, marrow stroma, tendon, fat and a variety of other connective tissues (Makino et al., 1999;Bianco and Robey, 2000) and, under certain conditions also into cell types derived from different germ layers (Sanchez-Ramos et al., 2000;Woodbury et al., 2000).

The harvest of a limited bone marrow sample is an easy and relatively safe procedure. Large numbers of BMSCs can be obtained in cultures, making it possible to engineer transplantable constructs composed of these cells in appropriate scaffolds. The properties of BMSCs are deeply influenced by the microenvironmental conditions. Culture conditions that allow expansion without loss of differentiation potential are difficult to establish for most adult stem cells. Culture conditions for BMSCs remained essentially the same as the ones originally described by Friedenstein (Friedenstein et al., 1966). However, to obtain a large number of osteoprogenitor cells, the effects of several growth factors on proliferation and differentiation of osteoblasts were investigated (Bonewald et al., 1990;Erlebacher et al., 1998;Rosen et al., 1988;Linkhart et al., 1996;Hollinger et al., 1998;Uludag et al., 2000;Wozney, 1998;Boyne et al., 1997;Yoshida et al., 1999).

Expressions of osteoblastic phenotypes and osteoblastic differentiation markers

The proliferation and differentiation of the osteoblasts are important events during bone turnover and are controlled by local growth factors and systemic hormones (Heino et al., 2004). In the earlier phase of differentiation, multipotential stem cells differentiate into osteoprogenitor cells, preosteoblasts and osteoblast respectively. In a

terminal phase of differentiation, osteoblasts are embedded deeply within the mineralized bone matrix and become osteocytes (Palumbo et al., 1990).

Cells in the osteoblast lineage exhibit several specific phenotypes that can be used to characterize the osteoblastic differentiation of cells. Alkaline phosphatase (ALP) activity is one of the phenotypic markers of osteoblasts. ALP activity is expressed when cells are in differentiation stages of preosteoblasts but it is absent from mature osteoblasts (Doty and Schofield, 1976). An increase in specific activity of ALP in a population of bone cells indicates a progress of osteoblastic differentiation into terminal or mature stages. The expression of ALP in differentiating osteoblastic cells is found prior to the expression of osteocalcin, a non-collagenous protein in ECM (Aronow et al., 1990;Owen et al., 1990). The ALP plays a crucial role in the initiation of mineralization of ECM and the expression of this enzyme is down-regulated after the starting of the mineralization stage or expression of osteocalcin (Beck et al., 1998;Aubin et al., 1995;Malaval et al., 1999).

Among bone matrices, type I collagen is the most abundant protein of the organic matrix of bone synthesized by active osteoblasts and conductive to mineral deposition (Franceschi and Iyer, 1992;Genovese et al., 1984;Cowles et al., 1998). It also binds to non-collagenous matrix proteins that initiate and regulate mineralization (Ohsawa et al., 2000).

Osteocalcin, also called bone Gla-protein, is a small noncollagenous protein of bone matrix that is specifically found in fully developed mineralized matrices of bone, teeth and mineralized cartilage (Desbois and Karsenty, 1995;Cowles et al., 1998;Sato et al., 1998). It is predominantly synthesized by mature osteoblasts during mineralization and incorporated into the ECM of bone (Desbois and Karsenty, 1995). The osteoblast cells in culture do not express osteocalcin until mineralization of ECM is about to occur (Gerstenfeld et al., 1987;Stein and Lian, 1993).

Osteopontin, also termed bone phosphoprotein, bone sialoprotein I, secreted phosphoprotein 1 (SPP), has been isolated from bone, and is an Arg-Gly-Asp-Ser (RGDS)-containing, phosphorylated, sialic acid-rich, calcium-binding protein. It presents in bone matrices, the proximal convoluted tubules of kidney, neurons, and sensory and secretory cells in the internal ear. It is also expressed by hypertrophic chondrocytes and chondrocytes in mineralizing cartilage. Osteopontin may mediate the attachment and spreading of osteoblasts. Its phosphorylation and sulfation could have roles in

biomineralization. Grzesik and Robey (1994) proposed that osteopontin might be an early marker of cell adhesion and had various effects on mineral formation and growth (Boskey et al., 1993;Singh et al., 1990;Hunter et al., 1994). Osteopontin is localized within the developing bone cells in the embryonic femur during an early stage of osteogenesis and appeared before starting of mineralization in the earlier stage of osteogenesis. Its expression is found before the expression of osteocalcin. Therefore, osteopontin was thought to be an earlier marker of osteoblastic differentiation than osteocalcin, possibly identifying osteoblast precursor cells (Aubin and Liu, 1996).

Owen and coworkers (Owen et al., 1990) showed that the induction of both high osteocalcin and osteopontin mRNA levels depended on the ability of osteoblasts to form a mineralized ECM. The expression of osteopontin and osteocalcin by osteoblasts cultured on the surface of the material may provide a surface of material that is favorable to the formation of a mineralized ECM (Attawia et al., 1999).

Bone sialoprotein (BSP) has higher content of sialic acid and glutamic acid and is more highly sulfated than osteopontine. It binds to cells via an integrin, the vitronectin receptor. It presents in chondrocytes in the mineralized zone of hypertrophic cartilage, osteoblasts, and osteocytes. BSP mRNA appears to be restricted to active osteoblasts and especially high at sites of new bone formation, new mineralized tissue formation and developing sutures (Chen et al., 1992) with less expression in mature bone (Chen et al., 1991).

MC3T3-E1 cell line

The MC3T3-E1 pre-osteoblastic cell line is a spontaneously immortalized cell line of newborn mouse calvaria derived cells. It is a well-accepted model of osteogenesis *in vitro* (Sudo et al., 1983). MC3T3-E1 cells behave as immature, committed osteoblast cells, which continue to differentiate in a response to intracellular and extracellular signals. Upon reaching confluence, this clonal cell line differentiates along osteoblastic lineage, sequentially expressing characteristic phenotypes of osteoblasts in a manner closely mimicking that of primary cultures of fractions 3-5 calvarial osteoblasts (Franceschi and Iyer, 1992). The phenotype of this cell line is stable as long as stocks are rigorously maintained by passaging before confluency. When cultured in the presence of ascorbic acid and β-glycerophosphate, MC3T3-E1

cells in a postconfluent cultures will mineralize ECM accumulating hydroxyapatite (Sudo et al., 1983). Ascobic acid supplemented in a culture medium is an essential factor controlling growth and differentiation of this cell line. It stimulates these cells to undergo a developmental sequence that includes the proliferation of undifferentiated precursors of osteoblasts, which subsequently differentiate into postmitotic osteoblasts being capable to express the osteogenic phenotypes (Franceschi and Iyer, 1992).

MC3T3-E1 growth and differentaition

During the proliferative phase, these cells undergo DNA synthesis and cell division resulting in a rapid increase in cell numbers and confluency. At confluence, proliferation is downregulated and expression of the osteogenic phenotype is increased indicating the presence of mature osteoblasts. Osteoblasts produce ALP, type I collagen, and deposit an ECM containing non-collagenous proteins (e.g. osteopontin, bone sialoprotein and osteocalcin) on the substrate, which is subsequently mineralized (Kanno et al., 2004).

The MC3T3-E1 cell line has been widely used in the studies of proliferation and differentiation. Sudo and coworkers (Sudo et al., 1983) investigated the capacity of a clonal osteogenic MC3T3-E1 cell line. MC3T3-E1 cell line had high ALP activity in the confluent state and was able to differentiate into osteoblasts and mineralize bone matrix *in vitro*. The cells in the growing state showed a fibroblastic morphology and grow to form multiple layers. On days 21, clusters of cells exhibiting typical osteoblastic morphology were found in osmophilic nodular regions. Such nodules increased in numbers and size with incubation time and became easily identifiable with the naked eye by days 40-50. In the central part of well-developed nodules, osteocytes were embedded in a heavily mineralized bone matrix. Osteoblasts were arranged at the periphery of the bone spicules. Numerous matrix vesicles were scattered around the osteoblasts and young osteocytes.

Matrix vesicles and plasma membranes of osteoblasts, young osteocytes, and lysosome-rich cells showed strong reaction to cytochemical staining for ALP activity and calcium ions. Minerals were initially localized in the matrix vesicles and then deposited on well-banded collagen fibrils. Deposited minerals consisted exclusively of calcium and phosphorus, and some of the crystals had matured into hydroxyapatite crystals.

The results indicated that MC3T3-E1 cells had the capacity to differentiate into osteoblasts and osteocytes and to form calcified bone tissue *in vitro* (Sudo et al., 1983).

Franceschi and Iyer (1992) studied the role of collagen synthesis in osteoblastic differentiation of MC3T3-E1. MC3T3-E1 cells expressed osteoblast markers and mineralized ECM only after exposure to ascorbic acid. Mineralization was further stimulated by β-glycerophosphate. The expression of osteoblast markers followed a clear temporal sequence. The earliest effects of ascorbic acid were to stimulate type I procollagen mRNA and collagen synthesis (24 hours after ascorbate addition), followed by induction of ALP (48-72 hours) and osteocalcin mRNAs (96-144 hours). Actions of ascorbic acid on osteoblast marker gene expression were mediated by an increase in collagen synthesis and/or accumulation because parallel dose-response relationships were obtained for ascorbic acid stimulation of collagen accumulation and ALP activity.

Wada and coworkers (Wada et al., 1998) investigated the temporal changes in osteoblastic phenotype in isolated rat calvaria cells including formation of mineralized bone nodules up to day 20 in culture. These cells formed unmineralized nodules by day 5. Mineralization was observed at the center of nodules by day 10, and nodules became larger on day 15. The nodules were surrounded by numerous ALP-positive cells. ALP activity was gradually increased by day 20. Parathyroid hormone responsiveness increased with time in culture. Osteoblasts produced no osteocalcin by day 10, but its synthesis was detected from day 15. The bone porphogenetic protein (BMP-2 and BMP-4) mRNAs appeared in the cells forming nodules. These observations suggested that BMPs played an important role in the formation of mineralized bone nodules. It was confirmed that osteoblasts enzymatically isolated from newborn rat calvaria were a useful tool for studying the differentiation process of osteoblasts.

Wang and coworkers (Wang et al., 1999) isolated a series of subclonal MC3T3-E1 cell lines with high or low differentiation/mineralization potential after growth in the presence of ascorbic acid. Subclones were characterized in terms of their ability to mineralize a collagenous ECM *in vitro* and *in vivo* and expressed osteoblast-related genes. When compared with non-mineralizing cells, mineralizing subclones selectively expressed mRNAs for the markers of mature osteoblasts which were bone sialoprotein, osteocalcin, and the parathyroid hormone/parathyroid hormone-related protein receptor. In contrast, ALP mRNA, a marker of pre-osteoblasts, presented in certain non-

mineralizing subclones as well as mineralizing subclones. After implantation of the MC3T3-E1 into immunodeficient mice, highly differentiating subclones formed bone-like ossicles resembling woven bone, while poorly differentiating cells only produced fibrous tissue. These subclones were very useful for studying critical events in osteoblastic differentiation and mineralization.

There are many studies using MC3T3-E1 cell line to assess biocompatibility of materials. Norman and coworkers (Norman et al., 1994) seeded MC3T3-E1 osteoblast-like cells onto sintered and non-sintered porous coralline hydroxyapatite (HA), and onto non-porous HA discs. This *in vitro* study demonstrated that coralline HA supported the growth of osteoblast-like cells and porous discs supported growth of higher numbers of cells than non-porous discs. Sintering encouraged cell growth, with higher numbers of cells adhereed to sintered porous HA discs by day 7. The results suggested that HA could provide a support for osteoblast cells as part of a matrix.

Simon and coworkers (Simon et al., 2002) assessed the biocompatibility of a new composite bone graft consisting of calcium phosphate cement (CPC) and poly(lactide-co-glycolide) (PLGA) microspheres (approximate diameter of 0.18–0.36 mm) using cell culture techniques. In this study, MC3T3-E1 osteoblast-like cells were seeded onto graft specimens and evaluated with fluorescence microscopy, scanning electron microscopy and the WST-1 assay (an enzymatic assay for mitochondrial dehydrogenase activity). The cells were able to adhere, attain a normal morphology, proliferate and remain viable when cultured on the new composite graft (CPC–PLGA) or on a control graft (CPC alone). These results suggested that the new cement consisting of CPC and PLGA microspheres was biocompatible.

Isama and Tsuchiya (2003) clarified the effects of low molecular weight (MW) poly(L-lactide) (PLLA) on the proliferation and differentiation of mouse MC3T3-E1 osteoblast-like cells. The results indicated that low MW PLLA enhanced the differentiation of MC3T3-E1 osteoblast-like cells with no effect on the proliferation. PLLAs increased the ALP activity and calcium content of MC3T3-E1 cells up to the similar level to the calcification. It was suggested that the increase of the ALP activity was a key step stimulating the calcification of MC3T3-E1 osteoblast-like cells.

Ehara and coworkers (Ehara et al., 2003) studied a using of alpha-tricalcium phosphate (α-TCP) and tetracalcium phosphate (TetCP) as bone substitution materials. MC3T3-E1 cells were cultured and monitored for up to 7 days after confluence to

investigate the impact of α-TCP and TetCP on cell differentiation. They found that these materials did not influence the proliferation rate of MC3T3-E1 cells, but increased ECM formation and promoted a shift to a higher differentiation state and mineralization of ECM. These properties may produce an environment, which stimulate osteogenesis.

O'Brein and coworkers (O'Brien et al., 2005) studied the relationship between cell attachment and viability in collagen-GAG (CG) scaffolds by seeding MC3T3-E1 mouse clonal osteogenic cells onto the scaffolds. They found that the attachment of the numbers of viable cells on the CG scaffold was decreased with increasing mean pore sizes of CG. Cell attachment and viability of MC3T3-E1 cells were primarily influenced by scaffold specific surface area with a pore size ranging from 95.9-150.5 μm.

Xu and Simon (In press) developed strong and macroporous calcium phosphate-chitosan scaffolds (CPC-chitosan). Biocompatibility of the material was tested. MC3T3-E1 cells were seeded on the materials and found that MC3T3-E1 cells were able to attach, spread and proliferate on CPC–chitosan scaffolds. The cells with sizes of 20–50 μm including the cytoplasmic extensions infiltrated into the 165–271 μm macropores of the scaffolds.

The scaffold

A porous 3-D scaffold has been used extensively as carriers of cells and growth factors in the field of tissue engineering. The 3-D scaffold is function as a substrate and an analog of the ECM in providing physical support and regulating behaviors of cells on biomaterials such as cell migration, contraction, and division. A bioactive scaffold should promote adhesion and differentiation of cells by not hindering cell proliferation. A scaffold regulates the generation of new tissue and maintains function of the ECM as a template directing the growth and organization of cells (Thomson et al., 1997).

The scaffold may be applied as a carrier of growth factors or osteogenic proteins and cells. It is generally agreed that a porous structure with interconnected pores and a large surface area promotes tissue ingrowth and attachment (Hu et al., 2001). The optimal pore sizes allowing tissue ingrowth are in the range of about 100-150 μm (Zeltinger et al., 2001). Internal pores of the scaffolds should be well-interconnected pore structure, in which the porous structure will support high density

cell seeding and provides efficient nutrients and oxygen supply to the seeded cells as well as facilitating the excretion of waste product from the internal structures of the scaffolds (Ranucci and Moghe, 1999).

Pore structure significantly affects cell binding and migration *in vitro* and influences the rate and depth of cellular ingrowth *in vitro* and *in vivo* (van Tienen et al., 2002;Wake et al., 1994). Cell adhesion and activity vary considerably with cell types, scaffold compositions, and pore sizes (Chvapil, 1977;Doillon et al., 1986). The pore size of scaffolds significantly influences the morphology and phenotypic expression of cells (LiVecchi et al., 1994;Nehrer et al., 1997;Kuberka et al., 2002). A number of cell types exhibit a preference for binding to scaffolds with pore sizes significantly larger than the characteristic cell sizes. Implanted cells frequently utilize a characteristic bridging mechanism which adjacent cells act as support structures to assist bridging large pores (Borden et al., 2003).

The bridging mechanism of cells can be seen in fibrovascular tissue ingrowth into PLLA scaffolds, osteoblast adhesion to PLGA scaffolds, and rat marrow cells binding to porous copolymer scaffolds (Wake et al., 1994;Claase et al., 2003;Borden et al., 2003). Based on the principles of the scaffold design and a series of experiments, it was postulated that there was an optimal pore size for each specific tissue engineering application. The effect of pore size on cell adhesion could be seen on the adhesion of cells on collagen-glycosaminoglycan (CG) scaffolds. The attachment of viable MC3T3-E1 cells on the CG scaffold was decreased with increasing mean pore size of the scaffolds. It was found that the pore sizes range of 95.9–150.5 μm supported attachment and viability of MC3T3-E1 cells (O'Brien et al., 2005). Hu and coworkers (Hu et al., 2001) suggested that pore sizes between 100 and 350 μm and porosities of more than 90% were preferred in bone regeneration.

In addition, the scaffold structure must bear loads to provide stability for new formed tissue and to fulfill its volume maintenance function. On the microscopic level, evidence suggested that cell growth, differentiation and ultimate tissue formation were dependent on mechanical input to the cells (Butler et al., 2000;Sikavitsas et al., 2001). Desirable aspects of scaffold chemistry may include specific interaction with, or mimicry of ECM components, growth factors, or cell surface receptors (Hubbell, 2003).

Materials for fabrication of the scaffold should be degradable. The degradation rate may be fast or slow in which a completion of scaffold degradation can be ranged

from a very short (days) or a long (several months) duration depending on the type of replaced tissue and regeneration rate. Additionally, the biological property of degradation products is another important factor determining the growth and differentiation of cells. Therefore, a good knowledge of the degradation products is essential for a selection of biomaterials for a fabrication of scaffolds (Gross and Rodriguez-Lorenzo, 2004).

The scaffold can be developed using either natural or synthetic polymers. Synthetic polymers (polylactic acid (PLA), polyglycolic acid (PGA), coplolymers of PLA and PGA (PLGA), polyanhydrides, polycarpolactones, polycarbonates, and polyfumarates) have advantages over natural polymers in that they can be easily mass-produced and their properties can be designed specifically for each application. The mechanical properties, degradation rate, and pattern of synthetic polymers can be manipulated to create scaffolds with optimal bio-mechanical properties. However, tissue reaction to polymers and their degradation products and slow degradation rate are major hindrances for an application of synthetic polymers in tissue engineering (Suh et al., 2001). Natural polymers have been widely applied in organ regeneration. This is because the polymers are bioactive matrices being able to promote cell attachment and adhesion and maintain differentiation capacity of cells. Synthetic and natural polymers applied in tissue engineering are commonly produced in different forms of fibrous meshes, porous sponges or foams (Hubbell, 2003). Collagen, chitosan and hyaluronic acid are examples of natural polymers commonly used in tissue engineering.

Chitosan

Chitosan, a bio-copolymer comprising glucosamine and *N*-acetylglucosamine, is the alkaline deacetylated products of chitin, derived from the exoskeletons of insects and shells of crustaceans (Felt et al., 1998) and can also be found in the cell walls of some fungi such as *Mucor rouxii* (Synowiecki and Al Khateeb, 2003). Chitosan containing more than 50% free amine in the structure.

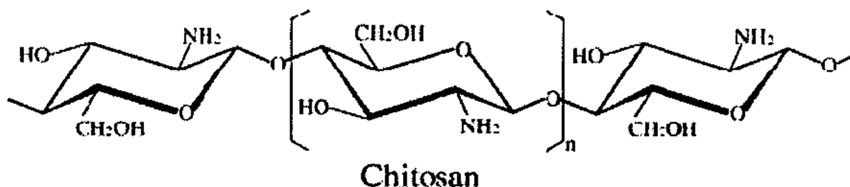

Figure 1 Structure of chitosan (Kumar et al., 2000)

Chitosan is a crystalline polysaccharide. Its molecular weight is ranging from 300-1500 kD. Structure of chitosan is similar to structure of naturally occurring glycosaminoglycans (GAGs) which are components of ECM. The *N*-acetylglucosamine moiety, which is a structural feature in chitosan, is also found in the GAGs. As GAGs influence specific interactions of ECM with growth factors, receptors and adhesion proteins, it is postulated that the analogous structure in chitosan may also have related bioactivities similarly to function of GAGs in ECM. It was reported that chitosan oligosaccharides had a stimulatory effect on macrophages and the effect was linked to the acetylated residues (Peluso et al., 1994). Both chitosan and chitin exert chemoattractive effects on neutrophils *in vitro* and *in vivo* (Usami et al., 1998;Usami et al., 1994).

Chitosan is a positively charged polymer and has high charge density in solution. The charge density allows chitosan to form insoluble ionic complexes with a wide variety of water-soluble anionic polymers (Francis Suh and Matthew, 2000). It is insoluble in water but soluble in organic acids at pH under 6.5. In diluted acids (pH < 6), free amino groups are protonated and the molecule becomes fully soluble below ~ pH 5. The pH-dependent solubility of chitosan provides a convenient mechanism for processing under mild conditions (Chenite et al., 2000;Francis Suh and Matthew, 2000).

Deacetylation levels of chitosan regulate attachment of cells on surface of chitosan. A high percentage of deacetylated chitosan supports attachment of cells, while the low percentage of deacetylated chitosan does not. Commercially available preparations have degree of deacetylation ranging from 50-90% (Francis Suh and Matthew, 2000).

Chitosan is biodegradable (Onishi and Machida, 1999). It is degraded by lysozyme-mediated hydrolysis process targeting acetylated residues. Biodegrading process of chitosan in aqueous medium is slow, even if lysozyme is present. There are evidences that some proteolytic enzymes show low levels of activity with chitosan. The degradation products are chitosan oligosaccharides of variable length. The degradation rate is inversely related to the degree of crystallinity (Lee et al., 1995;Tomihata and Ikada, 1997) which is controlled mainly by the degree of deacetylation. Highly deacetylated chitosan (e.g. >85%) shows higher levels of crystallinity and exhibits lower degradation rates. Chitosan may last several months *in vivo*. The minimum crystallinity

of chitosan exhibits intermediate levels of deacetylation and higher degradation rates. In fact, the low degradation rate of highly deacetylated chitosan implants is believed to be due to the inability of hydrolytic enzymes to penetrate the crystalline. So chitosan sponge is expected to remain stable in shape and size during cell culture (Madihally and Matthew, 1999).

Chitosan has been reported to be safe, non-toxic, non-immunogenic, and biocompatible. It is hemostasis, chemotaxis, bacteriostatic, fungistatic, wound healing accerelator and osteoconductive. It can be made into many forms including gels, films, membranes, fibers, beads, powders, solutions, and sponges. It has many commercial and biomedical applications in dentistry and medicine (Muzzarelli et al., 1993b;Muzzarelli et al., 1993a;Fakhry et al., 2004).

Biomedical application of chitosan

Chitin and chitosan have been used in a wide variety of biomedical applications, including surgical thread, bone healing promoting materials (Muzzarelli et al., 1988b), wound dressing (Cho et al., 1999), sustained release drug carrier systems (Chandy and Sharma, 1993;Oungbho and Muller, 1997) and tissue engineering (Ma et al., 2001;Madihally and Matthew, 1999).

Chitosan and wound healing

The effect of chitosan on wound healing has been evaluated by several investigators in animal study models. Chitin and chitosan improved wound healing in animals (Minami et al., 1993;Okamoto et al., 1993), accelerated increasing of tensile strength of wounds and promoted growth of granulation tissue with abundant neovasculature (Muzzarelli et al., 1988a). Chitosan scaffold enhanced sythesis of collagen in the first few days of wound healing (Chung et al., 1994) and induced the production of type III and IV collagen in tendon (Minami et al., 1996;Kojima et al., 1998). The application of *N*-carboxybutyl chitosan in wound management lead to formation of well organized cutaneous tissue and reduction of anomalous healing (Biagini et al., 1991).

Chitin and chitosan inhibited contraction of fibroblast-populated collagen lattice (FPLC). An inhibition effect was dependent on the level of deacetylation of the polymer. The higher the level of deacetylatation of the polymer was the lower of the level of

contraction. High decetylated chitosans were more biologically active than chitin and low deacetylated chitosan. It was useful as a potential anti-scarring agent in wound healing therapy (Howling et al., 2002).

Chitosan and hemostasis

Chitosan is able to induce coagulation and can be applied to open wounds in surgery. Chitosan formed a coagulum in contact with defibrinated blood, heparinized blood and washed red blood cells (Malette et al., 1983). Several studies have shown safety and efficacy of chitosan as a topical hemostatic and wound healing agent in various animal study models. Chitosan achieved hemostasis via cellular aggregation independently of normal clotting or platelet aggregation mechanisms (Klokkevold et al., 1992;Klokkevold et al., 1991;Klokkevold et al., 1999). Thus, chitosan could be used as an effective hemostatic agent in system with altered platelet activity or when normal coagulation pathway was hindered (Subar and Klokkevold, 1992). Additionally, chitosan allowed the promotion of normal tissue regeneration (Biagini et al., 1991). The study of Ishihara (2002) reported that chitosan hydrogel in a mouse study model effectively stopped bleeding from the artery and significantly induced wound contraction and accelerated wound closure and healing.

Chitosan and biocompatibility

It is essential to improve biocompatibility and physical properties of chitin and chitosan in the tissues because they are not structural component in human tissues. Mori and coworkers (Mori et al., 1997) examined the effects of chitin and its derivatives on the proliferation of fibroblasts. The results showed that chitosan had no effect on proliferation and did not directly accelerate ECM production of fibroblast *in vitro*. The ECM production could be increased by an addition of transforming growth factor-β1 (TGF-β1) and platelet derived growth factor (PDGF) into the culture system (Ueno et al., 2001). VandeVord and coworkers (VandeVord et al., 2002) studied biocompatibility of porous chitosan scaffolds implanted in mice. A minimal immune response and little implant encapsulation were noted. Tissue ingrowth was limited to the edges of the scaffold.

Chitosan and three dimensional scaffolds

Chitosan has a high potential as a biomaterial in tissue engineering. It has both reactive amino and hydroxyl groups that can be chemically modified and is relatively

easy to manipulate for different pore structures (Yagi et al., 1997;Madihally and Matthew, 1999). Chitin gel underwent lyophilization process produced porous matrices with pore sizes ranging from 100 to 500 μm. Varying of pore size and porous structure of matrix were dependent on the various pre-treatment procedures of chitin gels prior to lyophilization and also a varying of freezing temperatures and cooling rates. Mouse and human fibroblast cell cultures exposed to these chitin matrices were found to be growing and proliferating indicating the feasibility of using these porous chitin matrices for cell transplantation applications to regenerate tissues (Chow et al., 2000).

Ma and coworkers (Ma et al., 2001) investigated the utility of bilayer chitosan scaffolds to support growth and differentiation of human neonatal dermal fibroblasts. They found that chitosan scaffold remained stable in shape and size during the cell culture period. Fakhry and coworkers (Fakhry et al., 2004) studied the effect of chitosan on MC3T3-E1 osteoblast and 3T3 fibroblast cell attachment and suggested that chitosan supported the initial attachment and spreading of osteoblasts preferentially over fibroblasts and that manipulation of the biopolymer could alter the level of attachment and spreading of cells.

Chitosan and osteoblasts

Chitosan is osteoconductive promoting new bone formation. Muzzarelli and coworkers (Muzzarelli et al., 1988b) applied chitosan in human periodontal defects and found that chitosan decreased fibroplasia and enhanced cell proliferation and tissue organization of periodontal soft tissues. Chitosan powders accelerated healing of periodontal pockets and large intrabony defects of extracted socket (Muzzarelli et al., 1993a). Methylpyrrolidinone chitosan promoted mineralization of bone osteoid formation in bone defect of rabbits (Muzzarelli et al., 1993b). Porous chondroitin-4-sulfate (CS)–chitosan sponges incorporated with platelet-derived growth factor-BB (PDGF-BB) induced the increasing of fetal ral calvarial osteoblasts migration and proliferation as compared with chitosan sponge alone and could gradually release of PDGF-BB from CS–chitosan sponge resulting in significant enhancement of osteoblast migration and proliferation (Park et al., 2000). Composite porous matrices of chitosan-PLLA with primary cultures of rat calvarial osteoblasts demonstrated the increased osteoblast attachment and improved bone forming capacity by increasing mechanical stability and biocompatibility (Lee et al., 2002). The other study detected the expression of osteoblastic genes of ostoeblsts *in vitro* using a low concentration of water soluble

chitosan oligomer and found that chiosan oligomer modulated the activity of osteoblastic cells in mRNA levels and chitosan controlled the genes concerning cell proliferation and differentiation (Ohara et al., 2004). ROS osteoblast-like cells were cultured on chitosan scaffolds for biocompatibility test. They found that the scaffolds supported attachment, spreading of cytoplasmic process demonstrating the potential applicability of the scaffolds in tissue engineering (Ho et al., 2004). Xu and Simon (In press) examined the biocompatibility of the porous calcium phosphate cememnt (CPC)–chitosan composite with an MC3T3-E1 osteoblast cell line. They revealed that this scaffold with pore size ranging from 165–271 μm facilitated osteoblasts cell adhesion and proliferation *in vitro*.

Chitosan in combination with other materials

Chitosan has been used in combination with other materials including, inorganic ceramics and bioactive polymers such as chitosan ascorbate gel (Muzzarelli et al., 1988b), tropocollagen, sodium alginate (Lai et al., 2003), *N*-carboxybutyl (Biagini et al., 1991) and methylpyrrolidinone (Muzzarelli et al., 1993b;Muzzarelli et al., 1993a). A combination of chitosan with other biomaterials was expected to enhance cell adhesion, differentiation, and tissue compatibility while maintaining their original physical properties and enhancing bone growth and tissue regenerative efficacy.

Chitosan ascorbate gel enhanced reconstruction of periodontal tissue in humans. It enhanced the normal cell proliferation and organization of reconstructed tissue. Chitosan was progressively reabsorbed while tooth mobility and pocket depths were significantly reduced (Muzzarelli et al., 1988b). Ito (1991) developed a self hardening mixture of chitosan and hydroxyapatite and proposed its use as a bone filling paste for the treatment of periodontal defects or augmentation of edentulous ridges. Kawakami and coworkers (Kawakami et al., 1992) reported that the chitosan-bonded hydroxyapatite paste enhanced osteoconduction when applied to the tibia of adult Japanese white rabbits as compared to controls. Lee and coworkers (Lee et al., 2000) developed chitosan/tricalcium phosphate (TCP) sponges for tissue engineering of fetal rat calvarial MC3T3-E1 osteoblasts. The author found that chitosan/TCP sponges supported proliferation and differentiation of osteoblasts on the sponges, as it was indicated by high ALP activities of cells and deposition of mineralized matrices on the sponges. The results suggested that the chitosan/TCP sponge can be used as a biodegradable matrix for tissue engineering. Zhao and coworkers (Zhao et al., 2002) studied biomimetic 3-D hydroxyapatite/chitosan-gelatin network composite scaffolds with

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similar composition to normal human bone. They examined the proliferation and function of neonatal rat calvaria osteoblasts on the scaffolds and found that osteoblasts attached and proliferated on the scaffolds. The osteoblasts synthesized ECM including type I collagen and proteoglycan-like substrate forming osteoid and bone-like tissue formation.

In study of chitosan and combination of chitosan and sodium alginate sponges, the chitosan alone showed the slowest dissolution profile, while the mixed systems showed a relatively rapid dissolution profile. Therefore, the use of combination of chitosan and alginate allowed for the manipulation of both mechanical and drug release properties of the sponges (Lai et al., 2003). Polyglycolide (PGA)-chitosan matrix was tested for biocompatibility and physical properties using fibroblast cells culture model. It was clearly demonstrated that this scaffold was degradable and biocompatible with high strength and porosity. Fibroblast cells attached and proliferated into the scaffolds (Wang et al., 2003b).

Collagen

Physical properties

Collagen is the major structural protein found in all multicellular organisms. It is the most abundant protein in mammals and the major fibrous element of tendon, cartilage, bone and skin in an animal body and also main component of natural ECM (Lee et al., 2001). It is a natural polymer that contains both acid and basic amino acid residues and may bear either a positive or negative charge depending on the environment (Nezu and Winnik, 2000). The chemical and physical structural characteristics of collagen, in forms like fibrils, native or denatured, crosslinked or not, has a marked influence on the morphology and physiology of cells (Yoshizato et al., 1984;Yoshizato et al., 1988;Nishikawa et al., 1987).

The collagen molecule is composed of three polypeptide chains, two alpha and one alpha$_2$ chains, which wrap in a triple helix with three left handed helical alpha chains twisted into a right handed super helix structure (Figure 2). The alpha chains are similar in structure and contain approximately 1000 amino acid residues where commonly every third molecule is glycine (Piez, 1984). The other amino acids are either leucine and proline, or hydroxyleucine and hydroxyproline (Miyata et al., 1992;Rao, 1995). To maintain this structure, hydrogen bonds and disulfide bonds must

remain intact or it results in a non-functional protein (Harkness, 1966). This triple helix arranged collagen is known as fibrillar collagen. The chemical and physical structural characteristics of collagen have a marked influence on the morphology and physiology of cells (Lee et al., 2001). The major characteristics of collagen, which are suitable for medical applications are easily absorbable in the body and has very low antigenicity (Fujioka et al., 1998;Rao and Alamelu, 1992).

Figure 2 Structure of collagen (Piez, 1984)

Collagen has high tensile strength and high affinity with water. It is non-toxic, biocompatible, and biodegradable (Friess, 1998;Maeda et al., 1999). Collagen can be prepared in a number of different forms including strips, sheets, sponges and beads. Collagen can be solubilized into an aqueous solution, particularly in acidic aqueous media, and can be engineered to exhibit tailor-made properties. Collagen is relatively stable due to its function as the primary structural protein in the body, but it is still liable to collagenolytic degradation by enzymes, such as collagenase and telopeptide-cleaving enzymes. Collagenase binds tightly to triple helices, and degrades collagen starting from the surface. The melting profile of the pepsin-solubilized collagen showed a biphasic transition, indicating that age-related decrease in thermal stability has implications for the mechanical strength and turnover of the bone collagen (Danielsen, 1990).

Collagen in biomedical application

Regarding its biocompatibility and safety due to biological characteristics, made collagen the primary resource in medical applications (Berthod et al., 1993). The main applications of collagen as drug delivery systems are collagen shields in ophthalmology, sponges for burns/wounds, mini-pellets and tablets for protein delivery, gel formulation in combination with liposomes for sustained drug delivery, as controlling material for transdermal delivery, and nanoparticles for gene delivery and basic matrices for cell

culture systems (Lee et al., 2001). Collagen scaffolds have been observed to promote cell and tissue attachment and growth. It was also used for tissue engineering including skin replacement, bone substitutes, and artificial blood vessels and valves (Chvapil, 1977;Huynh et al., 1999).

Collagen in drug delivery

Collagen sponges were found suitable for short-term antibiotic delivery, such as gentamicin (Wachol-Drewek et al., 1996) and it was proved that collagen sponges containing gentamicin reduced local infection without side effect when it was placed on abdominal wound (Vaneerdeweg et al., 1998;Vaneerdeweg et al., 2000) and was reabsorbed after a few days (Stemberger et al., 1997).

Collagen in wound dressing

Collagen sponges have the ability to easily absorb large quantities of tissue exudate, smooth adherence to the wet wound bed with preservation of low moist climate as well as its shielding against mechanical harm and secondary bacterial infection (Armstrong et al., 1986). Experiments using sponge implantation demonstrated a rapid recovery of skin from burn wounds by an intense infiltration of neutrophils into the sponge (Boyce et al., 1988). Coating of a collagen sponge with growth factor further facilitated dermal and epidermal wound healing. Furthermore, collagen is a highly thrombogenic material, as is demonstrated by its use as a hemostatic powder or sponge. Collagen induced blood coagulation, platelet adhesion and aggregation as well as activation of intrinsic blood coagulation (Miyata et al., 1992).

Collagen in tissue engineering

Recent developments have renewed interest in naturally occurring 3-D structures such as collagen-based biomaterials. Collagen-based biomaterials were initially developed and used in a wide range of applications, such as hemostatic agents (Miyata et al., 1992), cardiovascular implants (Auger et al., 1998;Chevallay and Herbage, 2000;Kuzuya and Kinsella, 1994), dressings for burns and wounds (Boyce, 1998), and materials for correcting soft-tissue defects (Miyata et al., 1992).

Non-crosslinked collagen has been reported as a good matrix for endothelial cell seeding *in vitro* (Schor et al., 1979). Berthod and coworkers (Berthod et al., 1993) showed that collagen and protein production inside collagen sponges by human fibroblasts were significantly increased compared with monolayer culture and collagen

gel. Sabbagh and coworkers (Sabbagh et al., 1998) demonstrated that collagen sponges support growth and differentiation of urothelial cells and suggested that the collagen sponges were suitable substrate for tissue engineering. In the cultured skin substitutes, the contracted collagen lattice was used as a support for epithelial growth and differentiation to replace pathological skin (Yannas et al., 1989).

Yamada and coworkers (Yamada et al., 1999) developed an allogenic cultured dermal substitute (CDS) prepared by plating fibroblasts onto a collagen sponge and subsequently freeze dried. The authors demonstrated that epithelialisation on the area of the donor site that was covered with allogeneic CDS was more rapid than that covered with a commercially-available freeze-dried porcine dermis. They concluded that collagen sponges fabricated from a 1% aqueous solution of atelocollagen provided a favorable environment for epithelialization. Kemp (2000) developed type I collagen lattice to organize the cells into a 3-D structure *in vitro*. The lattice supported growth and differentiation of epithelial cells and was a potential skin replacement material.

The major organic component of bone ECM is type I collagen. Type I collagen regulates and promotes expression of osteoblastic phenotypes (Lynch et al., 1995;Shi et al., 1996;Masi et al., 2000;Ignatius et al., 2005) and mineralization of ECM (Owen et al., 1990). Collagen-based scaffolds are frequently used as scaffold in bone tissue engineering (Stock and Vacanti, 2001).

Casser-Bette and coworkers (Casser-Bette et al., 1990) seeded the clonal osteogenic cells line MC3T3-E1 onto the 3-D collagen type I matrix. It was found that in 56-cultured days, the MC3T3-E1 cells grew and differentiated on 3-D structure of the scaffolds and mineralized ECM. Expressions of ALP and osteocalcin were found and a calcification of a newly synthesized collagen type I was resemble to osteoid surrounded osteocyte-like cells. The authors postulated that osteogenic differentiation and mineralization of ECM were promoted by type I collagen matrice resulting in bone like tissue formation. Lynch and coworkers (Lynch et al., 1995) reported that the temporal expression of genes characterizing stages of growth and differentiation of osteoblasts are altered when osteoblasts are grown on film of type I collagen. The 3-D cell culture of osteoblasts on type I collagen gel promoted expression of osteoblastic phenotypes in levels much greater than the levels of expressions in monolayer cell culture (Masi et al., 1992).

Pore size of scaffolds influence adhesion of cells on collagen-glycosaminoglycan (CG) scaffolds. Attachment of MC3T3-E1 cells on CG scaffold was decreased when pore size in the specific surface area of the scaffold was increased. It was indicated that growth and differentiation of cells were optimal at pore size ranging from 95.5–150.5 μm (O'Brien et al., 2005).

Collagen as carriers of osteogenic proteins

Collagen is used as bone substitutes due to its osteoconductive activity. Collagen sponges were used as carriers of osteoinductive protein such as recombinant human bone morphogenetic protein-2 (rhBMP-2) *in vivo* (Reddi, 2000;Geiger et al., 2003). A composite of rhBMP-2 and collagen was implanted beneath the cranial periosteum of 10-month-old rats. Active bone formation and a direct connection between augmented bone and host bone were found. The ossification process in the rhBMP-2/collagen was occured directly through intramembranous bone formation. The collagen fibers of carriers were found in the woven bone and were completely absorbed at 8 weeks. These results indicated that rhBMP-2/collagen is an effective material as a biological onlay implant, and the implant showed osteoinductive properties and was completely replaced by new bone. It was postulated that collagen carrier did not only play a role as a carrier of rhBMP-2, but also being anchorage for cell attachment and differentiation (Murata et al., 1999).

An absorbable collagen sponge (ACS) containing rhBMP-2 was tested in the rat model for the evaluation of the efficacy of rhBMP-2 as osteoconductive substance (Kimura et al., 2000). ACS impregnated with rhBMP-2 were embedded in extraction sockets and augmentation sites with endosseous implant insertion. The 3-year results demonstrated that endosseous implants placed in these areas were all stable with no radiographic or clinical complications. The results from this study suggested that rhBMP-2/ACS could be safely used in tooth extraction sites and in local ridge augmentation procedures and the regenerate bone could be functionally restored with endosseous implants (Cochran et al., 2000).

Crosslinking and physical properties of collagen scaffolds

The fast biodegrading rate and poor mechanical strength of the collagen scaffold are crucial disadvantages that limit the further use of this material (Lee et al., 2001). Cross-linking of the collagen-based scaffolds is an effective method to modify the biodegrading rate and optimize the mechanical property of the scaffolds. For this

reason, the cross-linking treatment to collagen has become one of the most important issues for the collagen-based scaffolds.

Currently, there are two different kinds of cross-linking methods employed in improving the properties of the collagen-based scaffolds, chemical and physical methods. The latter includes the use of photooxidation, dehydrothermal treatments and ultraviolet irradiation, which can avoid introducing potential cytotoxic chemical residuals of glutaraldehyde (GA) and sustain the excellent biocompatibility of the collagen materials. However, most of the physical treatments can not yield high enough cross-linking degree to satisfy the demand of tissue engineering. Therefore, the treatments by chemical methods are still necessary in almost all cases. The reagents used in the cross-linking treatment recently involve traditional GA, 1-ethyl-3-(3-dimethylaminopropyl) -carbodiimide (EDAC), polyglycidyl ether and polyepoxidic resins (Khor, 1997;Osborne et al., 1999;Courtman et al., 2001;Ma et al., 2004). GA is a kind of bifunctional cross-linking reagents that can bridge amino groups between two adjacent polypeptide chains and has become the predominant choice in tissue engineering because of its water solubility, high cross-linking efficiency and low cost (Jorge-Herrero et al., 1999).

Chitosan-collagen scaffolds for tissue engineering

Collagen and chitosan are amongst the most abundant polymers in nature. They both have intrinsic properties that provide a strong but manipulable scaffolding structure for many multi-cellular organisms. Collagen and chitosan do not exist together as blended polymer in nature, but the specific properties of each may be used to achieve unique structural and mechanical properties. Matrices compose of collagen and chitosan may create an appropriate environment for the regeneration of osteoblastic cells. The mechanical properties and biodegradation rates of the two natural materials especially in aqueous media are not particularly good. A common approach for changing the properties of these materials is the crosslinking them by various processes (Weadock et al., 1983;Draget et al., 1992).

Chitosan can function as a bridge to increase the cross-linking efficiency of glutaraldehyde in the collagen-based scaffolds owing to the large number of amino groups in its molecular chain. Hence, one can expect that less GA could be used in the presence of chitosan and the potential cytotoxicity of GA might be decreased (Ma et al., 2003). Collagen and chitosan should be crosslinked to make them applicable as biodegradable materials. Types I and III bovine collagen fibres and GAGs were linked

by ionic bonds to chitosan in which free amino groups of this polysaccharide interacted with carboxyl groups of collagen and carboxyl and sulphate groups of GAGs. After lyophilization, the sponge presented an alveolar structure with pores ranging between 50 and 150 μm in diameter (Berthod et al., 1994). It was clearly demonstrated that the incorporation of chitosan into a collagen scaffold increased the mechanical strength of the scaffold and reduced the biodegradation rate against collagenase (Taravel and Domard, 1996;Lee et al., 2004).

Chitosan and collagen composite scaffolds have gained interest in tissue engineering. Collagen-chitosan scaffolds in the ratio 6:4 crosslinked by GA in the form of interpenetrating polymeric network could be utilized as substrate to culture human epidermoid carcinoma cells (HEp-2) and could also be used as an *in vitro* model to test anticancerous drugs (Shanmugasundaram et al., 2001).

Collagen-chitosan scaffolds in the ratio of 9:1 crosslinking by GA was applied for skin tissue engineering. A presence of chitosan obviously improved the biostability of the collagen-chitosan scaffold under the GA treatment, where chitosan might function as a crosslinking bridge. A steady increase of the biostability of the collagen-chitosan scaffold was achieved when GA concentration is lower than 0.1%. Culture of human dermal fibroblasts demonstrated that the GA-treated scaffold retains the original good cytocompatibility of collagen to effectively accelerate cell infiltration and proliferation. A test in animal model revealed further that the scaffold could sufficiently support and accelerate the infiltration of fibroblasts from the surrounding tissue. All these results suggested that collagen-chitosan scaffold crosslinked by GA with enhanced biostability and good biocompatibility was suitable for dermal equivalent (Ma et al., 2003).

The other study used collagen-chitosan matrix ratio 1:1 crosslinked by EDAC in *N*-hydroxysuccinimide (NHS) and a 2-morpholinoethane sulfonic acid .(MES) buffer system for the regeneration of livers. The platelet deposition and hepatocyte culture experiments showed that collagen-chitosan matrix had excellent blood and cell compatibility. The results suggested that the collagen-chitosan matrix was a promising candidate matrix for implantable bioartificial livers (Wang et al., 2003a).

Aims of the study

Aims of this study were to investigate growth and differentiation of mouse osteoblasts on porous structure of chitosan-collagen sponges in a long term 3-D cell culture. Effects of amount of collagen in chitosan-collagen scaffolds to porous structure of the scaffolds were studied.

Hypothesis

It is hypothesized that

1. Chitosan-collagen composite sponges would support growth and differentiation of osteoblasts and
2. Collagen in chitosan-collagen sponges would improve osteoconductive property and porous structure of chitosan sponge.

Objectives of the study

The objectives of this study are to

1. Observe microstructure of chitosan-collagen sponges before cell seeding and monitor morphology and growth of cells on the sponges using scanning electron microscope (SEM).
2. Determine growth of MC3T3-E1 osteoblast cell line on the chitosan-collagen sponges using MTT assay.
3. Determine differentiation of MC3T3-E1 osteoblast cell line on the chitosan-collagen sponges by determining levels of ALP activity and mineralization of ECM measuring calcium content.