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In vitro evaluation of Malaysian Natural coral Porites bone graft substitutes (CORAGRAF) for bone tissue engineering: a preliminary study

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Abstract

Aim: An approach for three-dimensional (3D) bone tissue generation from bone marrow mesenchymal adult stem cells (BMSC-AS) was investigated. **Methods:** The BMSC-AS cells were induced to differentiate into osteogenic precursors, capable of proliferating, and subsequently differentiating into bone-forming cells. The differentiated cells were seeded on the surface of coral discs with a mean diameter 10 (±2) mm and a mean thickness 1 (±0.5) mm. The seeded scaffolds were characterized using von Kossa and Alizarin Red staining, electron and confocal microscopy and RT-PCR analysis. **Results:** The results demonstrated that BMSC-AS derived bone-forming cells attached to and colonized into coral scaffolds. Furthermore, these cells produced bone nodules when grown for 3-4 weeks in mineralization medium containing ascorbic acid and beta-glycerophosphate both in tissue culture plates and in scaffolds. The differentiated cells also expressed osteospecific markers when grown both in the culture plates and in 3D scaffolds. Osteogenic cells expressed alkaline phosphatase, osteocalcin, and osteopontin, but not a BMSC-AS cell-specific marker, *oct*-4. **Conclusion:** These findings suggest that Malaysian Natural coral Porites bone graft substitutes (CORAGRAF) with BMSC-AS cells can be used for in vitro tissue engineering to cultivation of graftable skeletal structures.

Keywords: *In vitro*, coral reef, CORAGRAF, 3 D scaffolds, bone tissue engineering

Introduction

Since Malaysia has a vast resource of corals along its coast, which are essentially composed of a calcium carbonate skeleton, systematic development of value added products for biomaterial applications using these corals has been undertaken. In 2002, the Malaysian National Tissue Bank (Universiti Sains Malaysia, Health Campus, Kubang Kerian, Malaysia) started processing and developing sea coral obtained from the Porites species in the east coast in peninsula Malaysia as a bone graft substitute named CORAGRAF as a consequence of the demand for cheaper implant materials. The production of this substitute has been complemented with proper evaluation and testing including toxicology, biocompatibility, mechanical properties, physicochemical characterization, and in vivo testing[1-4].

Natural coral graft substitutes are derived from the exoskeleton of marine madreporic corals. Researchers first started evaluating coral as potential bone graft substitutes in the early 1970s in animals and in 1979 in humans. The structure of the commonly used coral, Porites, is similar to that of cancellous bone and its initial mechanical properties resemble those of bone. The exoskeleton of these high content calcium carbonate scaffolds has since been shown to be biocompatible, osteoconductive, and biodegradable at variable rates depending on the exoskeleton porosity, the implantation site and the species. Although not osteoinductive or

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osteogenic, coral grafts act as an adequate carrier for growth factors and allow cell attachment, growth, spreading and differentiation. When applied appropriately and when selected to match the resorption rate with the bone formation rate of the implantation site, natural coral exoskeletons have been found to be impressive bone graft substitutes[5].

Different types of commercially available Coralline hydroxyapatite (CHA) are available as granules or blocks with 200 μm or 500 μm pore size. The biological evaluation and clinical indications of the use of commercially available CHA Interpore-200® and -500® have been reported to be osteoconductive rather than osteoinductive, as new bone formation is found in the pore surfaces which are in direct contact with bone but there is no incorporation of bone into the pores where the implant is not in contact with viable bone. Interpore- 500® is reported to be the resorbable version and is available as granules of 0.4-1.0 mm for oral surgical applications and 0.4-6.0 mm for periodontal applications.

It has been evaluated for use in atrophic mandibular ridge augmentation and also in bone reconstruction in periodontal sites where rapid vascularization of the implant material occurred and the newly formed fibrovascular tissue transformed into bone[6-8]. Interpore-500® has shown advantages over powdered HA as it demonstrated bioresorbability and the potential to stimulate the development of osseous tissue within the pores of the material demonstrating increased bone bonding and graft fixation[9]. Interpore- 200® is also available as blocks and granules. Histological data from studies in animals and from human trials in a variety of surgical procedures have revealed satisfactory results.

These implants were well-tolerated with significant amounts of bone infiltration showing no resorption or osteolysis beneath the implant and the material was strong enough to resist denture forces when used in tooth sockets[6,10-11]. Pro-Osteon 500® is considered as a viable option for the management of bone defects as it showed good osteogenesis in a one year follow up study in distal femur implantation in rabbits[11-12].

The outcome of a clinical study involving the follow up of 71 patients for 2.4 postoperative years after use of Pro-Osteon 500® to surgically replace bone tumors was very promising[13]. Biocoral®, a coralline calcium carbonate, showed good results as a bone substitute in clinical studies[14-15].

The material surface can influence cell reaction through changes in the cytoskeleton, a network of protein filaments extending through the cell cytoplasm within eukaryotic cells. The actin microfilament cytoskeleton is involved in the formation of cell processes, cell shape, and cell attachment. Microspikes or filopodia are thin projections of the plasma membrane supported by actin bundles. As the cell adheres to a substrate material filopodia are formed, and moved into place by actin acting upon the plasma membrane. The actin is observed in the filopodia as tight parallel bundles. Contractile stress fibers are seen once the filopodia are attached[16].

In our approach, the CORAGRAF developed from Malaysian sea coral with pore diameters very similar to a commercially available bone substitute, CHA. Apart from chemical and physical characterization of the materials, cytocompatibility is an equally important factor to consider for potential biomedical application of the grafting substrates used. To the best of our knowledge this is the first in vitro study to document the growth, differentiation, morphology and matrix mineralization of BMSC-AS (rat bone marrow mesenchymal cells) on the three-dimensional 3D bone tissue graft substitute named CORAGRAF.

Materials and methods

CORAGRAF Preparation

Natural Malaysian coral of Porites species with a pore size of 66.5 μm to 186.2 μm and porosity of about 42% based on a prior study[3] was used in this study. Briefly, dead Sea coral of Porites species had been harvested from Malaysian biodiversity, processed it using innovative techniques. Coral skeleton material were cleaned from any debris and washed with distilled water. Coral was cut and processed into granules and blocks with different dimension according to the requirement. For the purpose of this study coral was cut into discs with a mean diameter 10 (±2) mm and a mean thickness 1 (±0.5) mm.

Adult Stem Cell Culture

BMSC-AS [Rat bone marrow mesenchymal cells, (BMSC-AS)] were isolated and cultured using the method described by Maniatopoulos et al.[17]. Femora of male Spruge-Dawely rats were washed in culture medium á-Minimal Essential Medium (MEM; Gibco BRL) with 0.5 mg/ml gentamycin and 3 mg/mL fungizone (Sigma-Aldrich Corp., St. Louis, MO, USA). Epiphyses were cut off and diaphyses flushed out with 15 mL non-osteogenic culture medium. When colonies of ASs increased in size, but prior to the time they became multilayered and the colonies came in contact with one another (usually on day 12 of primary culture), the cells were subcultured by treatment with 0.25% trypsin in 1 mM EDTA for 5 min at 37°C. Trypsinization was arrested with the addition of medium, and the resulting cell suspension was centrifuged at 500 g for 5 min, resuspended and counted with a hemacytometer. To distinguish the ASs from the osteogenic cells, the ASs cells were transferred to tissue culture plates in ASs medium supplemented with ascorbic acid phosphate (50 μg/mL) and β-glycerol phosphate (10 mM) (mineralization medium). The resulting cells were incubated under the same condition for three weeks with a medium change every 2 days.

Seeding of Scaffold

The BMSC-AS were subjected to selective differentiation of osteogenic cells in the mineralization medium as described above and shown to produce bone nodules (see Results). The differentiated cells were treated with trypsin and the scaffolds were seeded as follows. The scaffold discs were placed in six-well tissue culture plates the discs were pre-soaked in 1 ml medium for 2 h and seeded with the osteogenic cells derived from BMSC-AS (a concentration of 200μL of 10^6 cells per mL). The cells were incubated for 0, 2, 4, and 8 h at 37æ%C in 5% CO_2 incubator before supplementing with 2.5 mL of the culture medium, and then incubated for 4 weeks with a medium change every 2-3 days. Seeded and unseeded controls included scaffolds seeded with and without BMSC-ASs cells, respectively, and were incubated in the same way in the mineralization medium.

The scaffolds were periodically checked under a phase contrast light microscope (Ziess) and one set for each of the scaffolds including controls (unseeded scaffolds) was sacriffced at intervals of 0, 1, 2, 3, and 4 weeks, and analyzed by confocal scanning electron microscopy for monitoring cell growth and production of bone nodules as well as expression of speciffc genetic markers.

Simultaneously, six well culture plates with 100μL of 10^6 cells per mL were incubated as above. One set of the wells was sacriffced at intervals of 0, 1, 2, 3, and 4 weeks and analyzed as above. All experiments were run in triplicates.

Electron microscopic studies

For transmission electron microscopy (TEM), the cells were fixed in 3%glutaraldehyde in PBS for 3 h at room temperature. They were then washed with PBS for 30 min, post-fixed with 1% osmium oxide in PBS for 45 min and washed overnight with PBS. The samples were dehydrated in ascending ethanol series (50%, 60%, 70%, 80%, 90%, 100%), treated with Epon 812 at ratios of 1:3, 1:1, and 3:1 for 4 h each and 100% overnight. The samples were analyzed using TEM (Zeiss, Germany).

A scanning electron microscope (Leica Cambridge S360 at 10 KV) was used to examine the specimens and the cells on the specimens. Cells cultured on COROGRAF discs specimens were rinsed with saline, fixed with 2.5% glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.3) at 4°C overnight. The specimens were then dehydrated through a graded series of alcohols (50%, 70%, 90%, and two changes of 100%), immersed in hexamethyldisilazane for 10 min 3 times and air-dried at room temperature. Samples were mounted on stainless stubs with double sticky tabs then sputter coated with gold and examined with scanning electron microscopy (Leica Cambridge S360 at 10 K).

Cytoskeletal Organization

The cytoskeletal organization of individual cells was examined on the GORAGRAF discs. The distribution of actin, a key protein in cellular structure, was observed over different incubation periods. Cell-GORAGRAF discs were washed in PBS, fixed and stained with a TRITC-Phalloidin (Molecular Probes) according to manufacture procedure. The samples were kept in dark place for 45-60 min. Then samples were washed five times with PBS, and finally, the samples were put on glass slides and mounted with cover slides with mounting solution (90% glycerol, 0.1× PBS, 92.5 μM p-phenylenediamine (Sigma-Aldrich Corp) using finger nail polish and clay to seal the slide and store at -20°C until use. The samples were visualized by confocal laser microscopy (Leica TCS SP II (Germany) examined with an argon (514/488 nm) and HeNe (543 nm) laser. By xyz mode scanning of sample at various focal planes along the Z-axis a three- dimensional data is set acquired for samples the location and assembly of actin were examined and correlations between the cytoskeletal organization and morphology of the cell were evaluated.

Extraction of RNA and RT-PCR

Cells were detached from the cell culture plates and collected by centrifugation. The RNA from the cells was extracted using Rneasy Kit (Qiagen Inc, Valencia, CA, USA) following the manufacturer's instructions. Analysis of the RNA samples was performed using the one-step RT-PCR kit (Qiagen GmbH). PCR conditions used were as follows: reverse transcription, 50æ %C, 30 min; Taq polymerase activation, 95æ %C, 1 5 min; then thermal cycling, 94æ %C, 30 s, 55æ %C, 30 s, 72æ %C, 30 s, for 35 cycles; followed by a single elongation step at 72æ %C, 10 min. The primer sequences and expected product sizes are listed in Table 1. RT-PCR products were analyzed by 1.5% agarose gel electrophoresis.

Results

Differentiation of AS cells

The BMSC-AS cells were plated in the mineralization medium containing beta-glycerophosphate and ascorbic acid. The BMSC-AS cells were differentiated into progenitor osteogenic cells. The isolated cells displayed morphological features similar to osteogenic cells (i.e., osteoblasts and osteocytes) as shown in Figure 1 Shows the characteristic cytoplasmic extensions contacting the adjacent osteocytes when grown for 4 weeks in the mineralization medium. Electron micrographs of the BMSC-AS cells differentiated into osteogenic cells are shown in Figure 2. The differentiated cells exhibit characteristics of differentiated cells including cellular organelles (Golgi apparatus and rough endoplasmic reticulum.

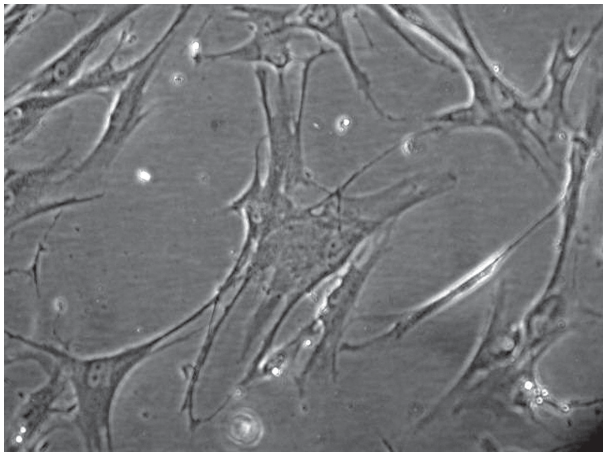

Fig. 1. Differentiation of BMSC-AS cells to osteogenic cells.

Production of Bone Nodules

Cells cultured in osteogenic media demonstrated a dramatic change in cell morphology from day 5 of induction, with the cells changing from a spindle-shaped morphology to a polygonal, spiculated morphology. The cells grown in osteogenic media demonstrated intense staining of calcium nodules with Alizarin red (Figure 3) .The osteogenic

Table 1. RT-PCR primers

oct-4	Sense GCAACTCAGAGGGAACCTCCT	62 bp
	Antisense TCTCCAACTTCACGGCATTG	
Alkaline phosphate	Sense AGGCAGGATTGACCACGG	138 bp
	Antisense TGTAGTTCTGCTCATGGA	
Osteocalcin	Sense CTTGGGTTCTGACTGGGTGT	212 bp
	Antisense GCCCTCTGCAGGTCATAGAG	
Osteopontin	Sense TCACCATTCGGATGAGTCTG	436 bp
	Antisense ACTTGTGGCTCTGATGTTCC	

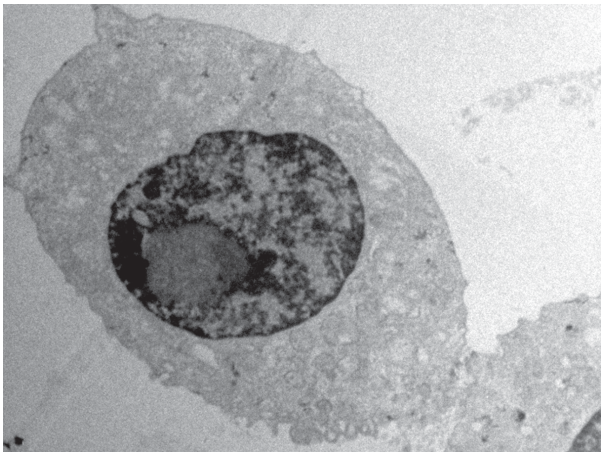

Fig. 2. Electron micrographs of BMSC-AS cells differentiated into osteogenic cells

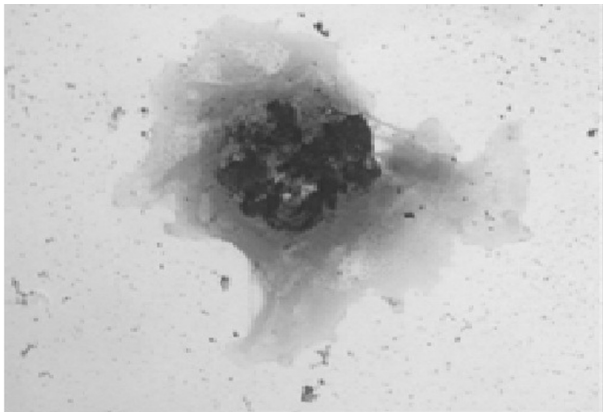

Fig. 3. The Alizarin Red stain.

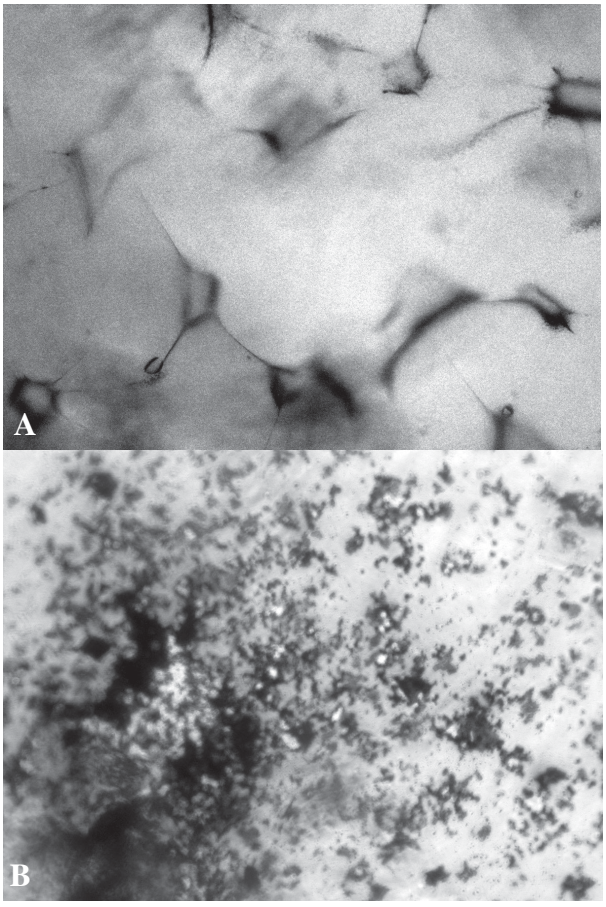

Fig. 4 A, B. Von Kossa staining showed spots of dark brown coloration (Production of bone nodules by the osteogenic cells seeded onto A)scaffolds and B)cultured on the plate)

cells grown for 3 to 4 weeks and subjected to von Kossa staining showed spots of dark brown coloration Figure 4 A, B. BMSC-AS cell, differentiated into cells other than osteogenic cells did not stain under the same conditions.

Growth of Osteogenic Cells in Scaffolds

Osteogenic cell growth, attachment, and invasion of scaffold were investigated by the scanning electron microscope. The results in Figure 5 A,B,C,D show an extensive growth of cellular biomass completely covering the scaffold as compared to the control-unseeded scaffold. Growth and invasion of the scaffolds by the osteogenic cells could be seen after 1 week of incubation in mineralization medium. No nodules were detected during the first 2 and 3 weeks in cell plates and scaffolds, respectively. After 4 weeks of incubation of seeded scaffolds, small nodular patches of excreted extracellular matrix (bone nodule structures) could be seen Figure 4 A, B. Production of bone nodules by the osteogenic cells seeded onto scaffolds and cultured on the plate was judged by the von Kossa staining, as well as the expression of osteospecific surface markers as determined by the RT-PCR. No nodular structures were found in control scaffolds.

Cytoskeletal Organization

To determine cytoskeletal network, immunofluorescence was performed on the substrate Cell-GORAGRAF discs over the 1st 2nd, 3rd and 4th week. Confocal Laser scanning microscopy evaluation of the specimens revealed that cell spreading at early culture times usually was associated with the formation of short cellular extension. After longer culture periods, many cells elongated and formed long processes extending away from the cell body. Different morphology cells were also found with variability in cell size and shape. In addition, spreading was associated with the formation of stress fibers. These stress fibers were only seen in the part of cell population Figure 6 A, B, C.

Molecular Characterization of the Osteoprogenitors

The differentiated BMSC-AS cell exhibit expression of several cell surface specific antigens molecular markers, such as, alkaline phosphatase (ALP), OC, and osteopontin (OP).This study investigated the expression of selected markers in differentiated cells producing bone nodules. The differentiated BMSC-AS cell not only colonized the scaffolds but also produced bone nodules as judged by the scanning electron micrography and von Kossa staining. The RT-PCR analysis of the transcripts of the cells colonizing the scaffold showed expression of osteospecific markers. The mRNA from these cells yielded RT-PCR amplified products of ALP, OC, and OP.

Discussion

Like any implanted biomaterials, the ideal scaffold should exhibit biocompatibility without causing an inflammatory response or foreign body/toxic reaction. Strong bonding with the host bone, active bone ingrowth into the graft, and bioabsorbability are equally desirable. Although scaffolds can be constructed from numerous materials, the

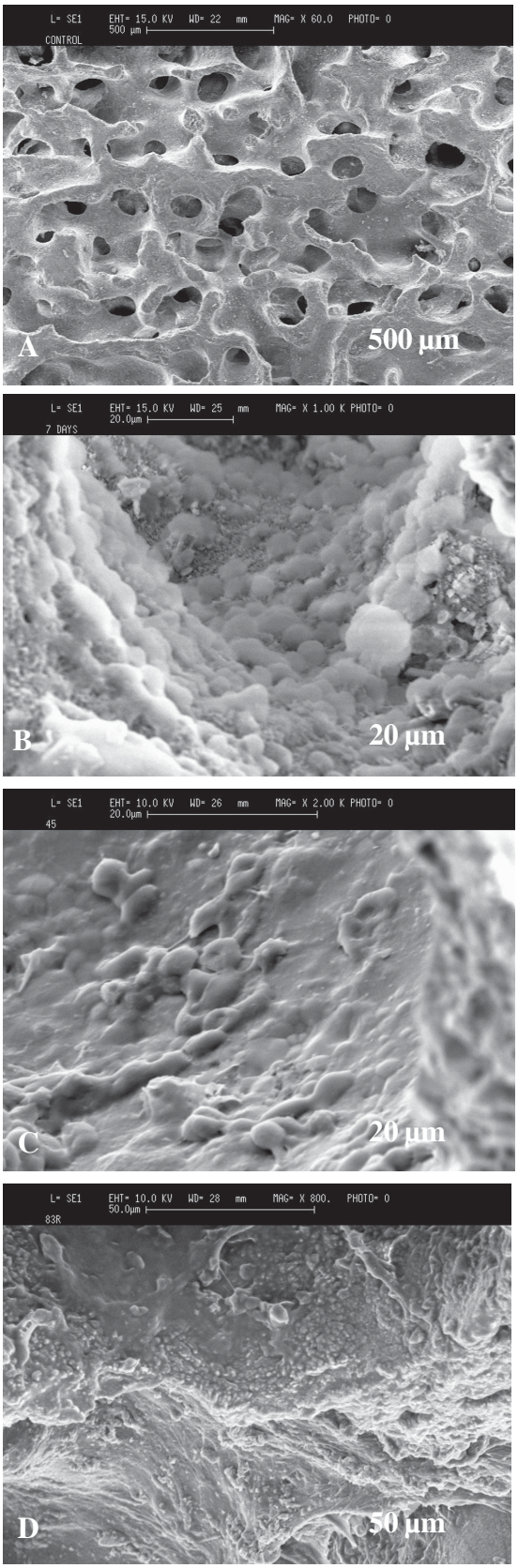

Fig 5. Scanning electron micrographs of scaffold seeded and unseeded with osteogenic cells and incubated in mineralization medium. (a)Control unseeded coral scaffold. (b) Seeded scaffold after 1 week of incubation. (C, D Seeded scaffold after 4 weeks of incubation.

primary materials studied mostly revolve around polymers such as polylactic acid, polyglycolic acid,polyurethane, and a number of copolymers[18].

Researchers have also focused on the use of natural coral graft to synthesize bone-like scaffolds. Rationales for the use of natural coral graft from the fact that natural coral are composed primarily of calcium carbonate (99%) in the form of aragonite and the remaining 1% is composed of simple amino acids in addition, the structure of the commonly used coral, Porites, is similar to that of cancellous bone and its initial mechanical properties resemble those of bone. The exoskeleton of these high content calcium carbonate scaffolds has since been shown to be biocompatible, osteoconductive, and biodegradable at variable rates, depending on, the exoskeleton porosity, the implantation site and the species. Coral grafts act as an adequate carrier for growth factors and allow cell attachment, growth, spreading and differentiation. When applied appropriately and when selected to match the resorption rate with the bone formation rate of the implantation site, natural coral exoskeletons have been found to be impressive bone graft substitute. Coral has been shown to possess all the principal of an adequate bone graft substitute, with the exception of its lack of osteoinductivity and osteogenesis, which can be provided by adding growth factors such as bone morphogenetic proteins and bone marrow cells. The addition of growth factors or bone marrow cells to coral grafts were found in general to improve bone formation when compared to implantation of coral alone. Coral scaffolds thus act as good carriers of growth factors and good supports for cell transplantation into a bony site[5]. Adult stem cells can differentiate to cells of the osteogenic, adipogenic, chondrogenic and myogenic lineages under appropriate conditions and in addition, Adult stem cells can migrate to sites of injury, inflammation, and tumors. These properties of Adult stem cells make them attractive candidates for use in regenerative medicine as well as for delivery vehicles for site-specific therapy[19-21]. In tissue engineering, the microenvironment provided by the scaffold must support cell attachment; proliferation and differentiation; neo tissue generation and correct 3D organization[22].

The ultimate goal of this study is to evaluate the GORAGRAF bone graft substitutes as scaffold to be used in bone tissue engineering. The results of this preliminary experiment, showed that BMSC-AS cells were differentiated into osteoblasts. The alizarin red and von Kossa staining documented that mineralization occurred in the cell layers during osteoblastic differentiation of the BMSC-AS cells in the mineralization cultures. The osteoblastic differentiation of BMSC-AS cells was confirmed also by investigation of gene expression for alkaline phosphate, osteocalcin and osteopontin which are markers for osteogenic differentiation. The progenitors of marrow stroma are suggested as a source for cell-based therapies and tissue engineering.

In this way, it is of great importance that uniform results in osteogenic capacity are obtained after each cell selection. From previous studies, it is known that a great biological variation exists between the different heterogeneous primary cell populations[7,18]. This variation in bone forming capacity of bone marrow cells has to be solved before a reliable bone construct can be made. Therefore, we choose for selecting osteoprogenitor cells from the marrow stromal cell population by using specific cell surface receptors[23-24].

BMSC-AS cells derived osteoprogenitor cells seeded on coral scaffold attached to and colonized the coral scaffolds. These results

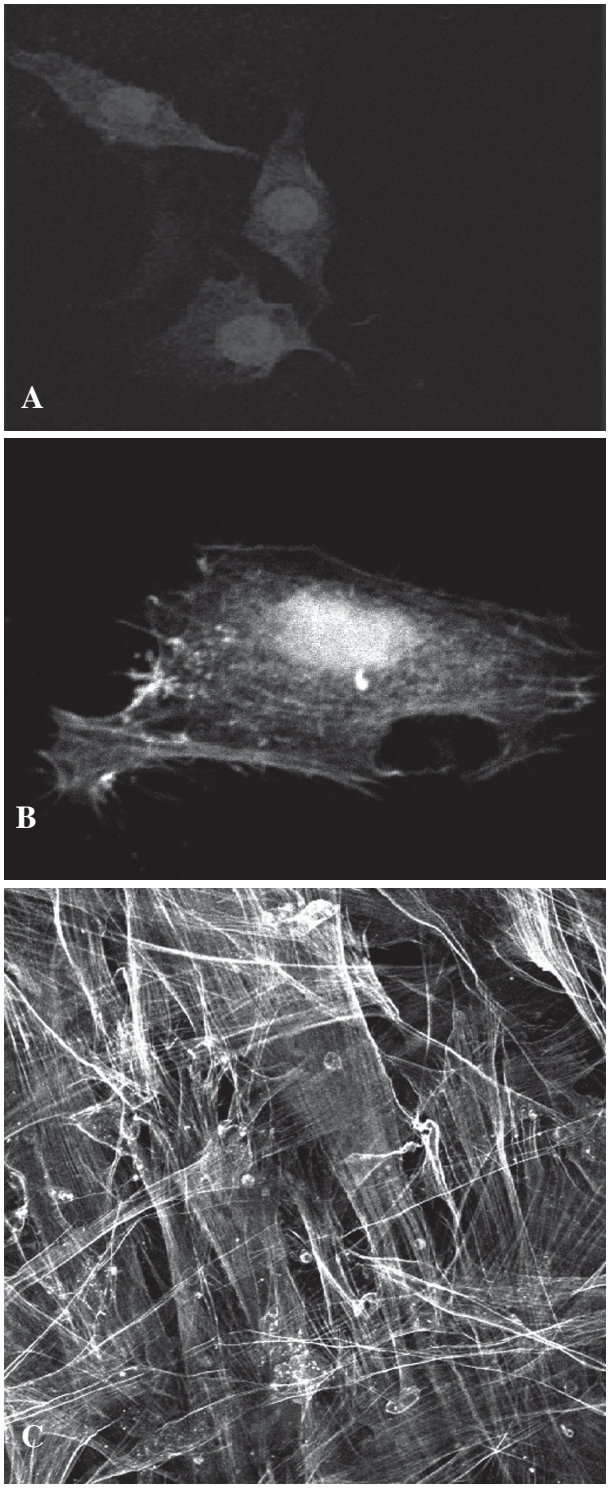

Fig 6. Confocal Laser Scanning Microscopy micrographs of scaffold seeded with BMSC-AS cells differentiated into osteogenic cells A) cells in the 1st week showed short cellular extension B) stress fibers extending from the cells . C) many cells elongated and formed long processes extending away from the cell body(X 20).

show that BMSC-AS cells can be used for tissue engineering of bone structures/grafts. In vitro grown bone grafts could facilitate skeletal reconstructions in cases involving defects created by tumor resection, injury. In the current study, BMSC-AS cells have been induced to differentiate into osteoprogenitor cells in vitro. These cells were utilized to evaluate GOROCRAF scaffold. Initial cellular attachment on GOROCRAF scaffold was random with cells distributed within the pores with cellular morphology varying from a spherical to slightly flattened appearance, with roughened 'blebs' evident on the surface, and protrusions of processes initiating cellular anchorage on the surface. This is typical of initial cellular interaction with biocompatible materials[25-27]. Similar cellular morphology was observed during the initial 2 h of cellular anchorage in previous study[26,28]. Rajaraman *et al.*[28], postulated that the roughened surface morphology was caused by the increase in total surface area of harvested cells as they round up from the flattened state in response to the treatment with trypsin. The initial point of contact between the cell and the substratum has been shown to be a random process that is largely determined by the distribution of adhesion proteins adsorbed onto the surface of the biomaterials. The results of scanning electron microscopy support the arrangement and attachment of AS cell on the surface, indicated that coral scaffold was able to support normal osteoblast cell growth, with cell rapidly spreading on the coral surface as well as in side the pores. The micrographs demonstrate the bioactive properties of coral with the preferential anchorage of AS cells to exposed coral discs. CLSM showed an increase in focal adhesion with coral. Integrin proteins are located within focal contacts, and are part of a signal transduction pathway from the extracellular matrix absorbed onto a material surface to the cell nucleus. The integrins form part of the many inter-and intra-cellular signal pathways affecting cell embryology (proliferation and differentiation). They act as organization centers for actin cytoskeleton and the extracellular matrix. This signaling pathway can exert effects globally as well as to a single cell as the cytoskeleton can exert forces onto the substratum, thus orientating the matrix. In the current study, the cytoskeletal filaments were visible in cells at different period of incubation. Previous studies have demonstrated that osteoblast-like cell behaviour such as cell attachment efficiency, spreading and actin stress fibre formation as well as cell migration is dependent on the material's surface chemistry[29-30]. Perinpanayagam *et al.*[30] investigated the extent of cytoskeletal organization in AS osteoprogentor cells. Cellular proliferation and subsequent colonization of AS osteoprogentor cells was evident after one week of culture, with the uppermost strut surfaces of coral scaffold, partially covered with cells. Initial lateral attachment of cells, spanning the inner wall of the pores, is clearly visible at this stage. This is consistent with previous observations of cellular migration across macroporous structures on the surface of hydroxyapatite[31-32]. By the 4th week, the surface of the pores were covered with a canopy of multilayered cells moving inwardly in concentric circles, which is highlighted due to shrinkage of the cells, an artifact caused by dehydration of the samples during SEM processing. Spherical morphology was shown by some cells on the surface of the cellular canopy on of hydroxyapatite.

Ideal scaffold materials for bone tissue engineering should mimic the extracellular matrix of bone tissue. Therefore, CORAGRAF scaffolds have been developed and have enabled osteoblast growth on the external surface and internal porous spaces *in vitro*. In the present study, it was possible to achieve *in vitro* remodeling of the scaffolds through the activities of osteoblasts-like cells.

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