Biocompatibility and *in vitro* tests

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The Bone tissue

Complex mineralized living tissue, having the property of marked rigidity and strength while still maintaining some degree of elasticity.

**Trabecular bone**
15 – 25% volume calcified.
Filled with bone marrow.
**Metabolic function**

Bone is continuously in a **remodeling process**: (resorption of old bone and formation of new bone)
Maintenance of bone tissue integrity and mineral homeostasis

**Cortical bone**
80 – 90% volume calcified.
**Mechanical and protective functions**

Bone is **highly vascularized**
Supriment of oxygen, nutrients, progenitor cells, growth factors
Removal of metabolism products
The bone microenvironment: The bone cells

Calcified matrix
Collagen type I (~ 90%)
Glycoproteins, Proteoglycans,
Growth factors, cytokines, …
… Hydroxyapatite
The Bone tissue: Bone regeneration (time line)

Inflammation

- Hematoma/inflammatory response/Angiogenesis
- Migration of bone precursor cells
- **Repair:** formation of an immature bone (primary stabilization)
- **Remodeling:** replacement of the immature bone by the mature bone (Occurs slowly over months to years and is strongly influenced by local mechanical stress placed on bone)

Bone formation

- Bone formation is always preceded by vascular invasion
- Osteogenesis occurs in the vicinity of newly formed blood vessels

Remodeling

(coordinated activity of OC and OB)

Regeneration of the form, structure and function
Incorporation / replacement of a bone graft

Similar to the regeneration process that occurs with a bone fracture

Following implantation
Inflammatory response/Angiogenesis
Protein adhesion to the material surface
Adhesion of osteoprogenitor cells to the material surface (via protein layer)
Formation of an immature bone
Remodeling phase: OC/OB activity

Bone formation
intimate interaction
angiogenesis/osteogenesis

Remodeling

Material
New bone
Blood vessel
Osteoblasts

Osteoclasts
Osteoblasts
Cell culture models to address bone/biomaterial interaction

Relevant cell types and cell types interactions

Scaffold

Angiogenesis/ osteogenesis

Remodeling

Endothelial cells

Osteoblasts

Osteoclasts

Osteoblasts
Cell culture models of the bone/biomaterial interface

**Osteoblast (OB) cell cultures**
- Bone formation
- Mesenchymal stem cells
- Pre-Osteoblasts
- Osteoblasts
- Mineralized matrix

**Osteoclast (OC) cell cultures**
- Bone resorption
- Hematopoietic stem cells
- Pre-Osteoclasts
- Osteoclasts
- Mineralized matrix

**Endothelial cell (EC) cultures**
- Angiogenesis

**EC/OB Co-cultures**
- Angiogenesis/
  osteogenesis

**OC/OB Co-cultures**
- Resorption/
  formation
**Cell cultures: equipment**

Safety Cabinet

**Incubator**
37 °C; humidified atmosphere
5% CO₂ / ar

Culture flasks and plates

Appropriate culture medium
Osteoblastic cell cultures
Osteoblastic cell cultures

Representative model of the osteoblastic differentiation

**Osteoblastic differentiation**

- Mesenchymal stem cell
- Osteoprogenitor
- Preosteoblast
- Osteoblast

**Decreasing proliferation**

**Increasing differentiation**
Osteoblastic cell cultures

- Culture of a bone marrow suspension
- Outgrowth from bone explants
- Comercial MSC

37 °C; 5% CO₂/air
1 – 2 weeks
(70 – 80% confluency)

Subculture
(to expand the cells)

Standard culture conditions:
Culture medium: alfa-MEM; DMEM
10% fetal bovine serum
Penicillin / Streptomycin;
Anphotericin B

50 mg/ml ascorbic acid
Dexamethasone (10 nM)
b-glycerophosphate (10 mM)

Osteoblastic differentiation

Characterization of the cell behaviour:
- Cell adhesion to the material substrate
- Cell viability/Proliferation (MTT, ADN, Protein)
- Apoptosis
- Cell cycle
- Morphology/F-actin cytoskeleton
- Focal adhesion points
- Expression of osteoblastic genes
  (Runx-2; Col-1; ALP; OC; RUNKL; OPG; …)
- Functional activity
  Alkaline phosphatase activity
  Formation of a mineralized matrix
- Intracellular signalling pathways

Biochemical, histochemical, immunohistochemical and molecular methodologies; SEM, CMSM
Osteoblastic cell cultures: Proliferation / differentiation pathway

Human bone marrow-derived osteoblastic cell cultures

Characterization of the cell behaviour

Cell proliferation (SEM images)

Adhesion and spreading SEM images

Gene expression profile

Runx-2; Col-1; ALP; OC; OPG; RANKL; ...

Alkaline phosphatase staining

Matrix mineralization (SEM)
Osteoblastic cell cultures: Proliferation / differentiation pathway

Human bone marrow-derived osteoblastic cell cultures

Inverse relationship between proliferation and differentiation
Osteoblastic cell cultures: cells / biomaterials interactions

Human bone marrow-derived osteoblastic cell cultures

Cell adhesion: standard tissue culture plates

Cell adhesion to HA substrates with different topographies; 30 min

Cell morphology

Culture plates
Degradable ceramic

CLSM of cells stained for F-actin cytoskeleton (green) and nucleus (red)

Gene expression profile

Runx-2; Col-1; ALP; OC; OPG; RANKL; .....
Osteoblastic cell cultures

Human bone marrow-derived osteoblastic cell cultures

Formation of a cellular mediated mineralized matrix in several biomaterials
Osteoblastic cell cultures

Illustrative studies

Ceramic composites

- Bonelike®
- Glass/Si3N4 composites

Biomaterials, 26: 485-493 (2005)

Macroporous ceramics


Collagen substrates

Connective Tissue Research, 50: 336-346; 2009

Chitosan hybrid membranes

Osteoblastic cell cultures

Illustrative study

Micro and Nanostructured macroporous ceramic scaffolds

Osteoblastic cell cultures

Illustrative study

Collagen-nanohydroxyapatite biocomposite scaffolds
Osteoblastic cell cultures

Illustrative study

Bioceramics/carbon nanotubes (CNT) composites (conductive substrates) Electrical stimulation

HA/Glass  HA/Glass/CNT

Microimaging

HA/Glass  HA/Glass/CNT

Nanoscale; 04 (2015)


J Biomedical Nanotechnology; 10:725-743 (2014)

Nanotechnology 25; 145602 (2014)
Osteoblastic cell cultures

Illustrative study

Guided proliferation of osteoblastic cells on patterned surfaces


*Dental Materials, 28:1250-1260 (2012)*


*Microsc Microanal 16:670-67 (2010)*
Osteoclastic cell cultures
Osteoclastic cell cultures

Representative model of the osteoclastic differentiation

Osteoclastic differentiation

Hematopoetic mononuclear precursor cells (PBMC) (peripheral blood)

OC precursor TRAP(-) → PreOC TRAP(+) → OC TRAP(+) → Maturation → Resorption

Active OC
TRAP(+)
Ruffled membrane
Vitronectin receptors
Calcitonin receptors
CATK
Actin ring
Bone resorption

Increasing differentiation
**Osteoclastic cell cultures**

**Peripheral blood**

- Buffy coat
- Plasma
- Erythrocytes

**Buffy coat**

**Magnetic separation of CD14+ cells**

**Characterization of the cell behaviour:**

- Cell adhesion to the material substrate
- Total protein content
- Apoptosis
- Morphology
- Formation of actin rings
- Immunostaining of Calcitonin and Vitronectin receptors
- Expression of osteoclastic genes (c-myc; c-src; TRAP; CATK, CA; …)
- Functional activity
  - TRAP activity
    - Formation TRAP+ multinucleated cells
  - Resorption activity
- Intracellular signaling pathways

**Obtention:**

- Isolation of the peripheral mononuclear cells from a buffy coat (Buffy coat + PBS) + Histopaque: mixt of monocytes, platelets and lymphocytes
- Magnetic separation of CD14+ cells
- Cell culture (2 x 10^6 cell/ml); 21 days
  - a-MEM; 10% human AB serum;
  - 1% glutamine; 30 mg/ml ascorbic acid
  - 25 ng/ml MCSF; 30 ng/ml RANKL
Osteoclastic cell cultures

Formation of multinucleated cells (TRAP staining)

Characterization of the cell behaviour

Expression of osteoclastogenic genes
Osteoclastic cell cultures

Illustrative studies

TRAP staining in different biomaterials

Hydroxyapatite seeded with OC cells

Multinucleated cells

Actin rings

Vitronectin receptor
Modulation of bone cell behaviour by surface topography in Hydroxyapatite substrates

Illustrative study

Acta Biomaterialia 8:1137-45 (2012)
Endothelial cell cultures
Endothelial cell cultures

Representative model of angiogenesis

1. Degradation of the matrix
2. Migration
3. Proliferation
4. Lumen formation
5. Increase in the permeability
Endothelial cell cultures

Human umbilical vein endothelial cells (HUVECs)

Obtention:
Endothelial cells isolated from umbilical vein
Culture in 1% gelatine pre-coated substrates
Medium M199; 20% fetal bovine serum
1% glutamine; penicillin/streptomycin
Trypsin / EDTA solution (70 – 80% confluency)
• First subculture (2x 10^4 cell/ml)
  1% heparin; 1 mg/ml EGFS; 7 days

Commercial endothelial cells of different origins
Microvascular endothelial cells
Appropriate culture medium for angiogenic differentiation

Characterization of the cell behaviour:
• Cell adhesion to the material substrate
• Cell viability/proliferation. Pattern of cell growth
• Apoptosis
• Cell cycle
• Morphology / F-actin cytoskeleton
• Immunostaining of PECAM-1, VE-caderin, vWB
• Expression of endothelial genes (PECAM-1, VE-caderin, factor vWB)
• Functional activity
  Production of NO
  Formation of tubular-like structures
Endothelial cell cultures

Characterization of cell behaviour

Circular pattern of cell proliferation

Capillary-like tube formation

PECAM-1

factor vWB

VE-cadherin

Capillary-like tube formation
Endothelial cell cultures

Formation of tube-like structures after the addition of an extracellular matrix (Matrigel)
Co-cultures of endothelial and osteoblastic cells
Co-cultures of endothelial and osteoblastic cells

**Angiogenesis/Osteogenesis**

- **OB/EC interaction**

Separation of the two cell populations (flow cytometry)

**Characterization of each population for typical phenotype features**
Co-cultures of endothelial and osteoblastic cells

Illustrative study

Immunostaining of osteoblast cells, endothelial cells and co-cultures

Formation of tubular-like structures

Cell proliferation; 45:320-334 (2012)
Co-cultures of endothelial and osteoblastic cells

Illustrative studies

Macroporous granules of nanostructured-hydroxyapatite agglomerates

Co-cultures of osteoblastic and endothelial cells
Co-cultures of osteoblastic and osteoclastic cells
Co-cultures of osteoblastic and osteoclastic cells

Hematopoetic mononuclear precursor cell (PBMC)

OB/OC interaction

Separation of the two cell populations (flow cytometry)
Characterization of each population for typical phenotype features
Co-cultures of osteoblastic and osteoclastic cells

Illustrative study

Vitronectin and Calcitonin receptors

Gene expression

VNR

CTR

SEM
Representative Cell culture models:

To address the cytocompatibility of biomaterials regarding cells involved in the bone regeneration events

To perceive key Biomaterial/Cell interactions, to understand cellular recognition of material surfaces, and specific cellular events leading to efficient new bone growth

….. To exploit/optimise relevant cellular/material interactions to improve bone regeneration events
**In vitro models**

**Advantages**
- Information on the molecular and cellular behaviour in controlled experimental conditions
- To address specific aspects of the cellular behaviour in the absence of the in vivo complexity

**Limitations**
- Alteration of the cell phenotype with the culture time
- Absence of the integrated molecular, cellular and tissue in vivo complexity

*In vitro* observations can not be extrapolated to *in vivo*

First stage of biological response to biomaterials
• Laranjeira MS, Fernandes MH, Monteiro FJ. Reciprocal induction of human dermal microvascular endothelial cells and human mesenchymal stem cells: time-dependent profile in a co-culture system. Cell Proliferation 2012;