THE ROLE OF NITRIC OXIDE ON THE PROLIFERATION OF HUMAN OSTEOBLASTS (HOS CELLS) STIMULATED WITH HYDROXYAPATITE

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by

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ABSTRACT

Hydroxyapatite (HA) as a ceramic material is widely used for orthopaedic and dental implants, since this biomaterial has ability to stimulate osteoblast functions *in vitro* and *in vivo*. However, the exact mechanism by which HA accelerates osteoblast function, thereby stimulating rapid bone formation, remains far from clear. It is well known that bone formation is tightly regulated by nitric oxide (NO) via its effects on osteoblast and osteoclast function. Therefore, the overall aim of the present studies was to elucidate the possible regulation of NO in HA-stimulated HOS cell proliferation. The present study used a human osteoblast cell line (HOS cells), since this cell line is known to mimic the functions of normal human osteoblasts, thereby representing the biological response of normal osteoblasts in humans.

The first experiments were carried out to delineate whether HA-stimulated HOS cell proliferation is regulated by endogenous NO. The results showed that following contact with HA, elevated NO production and HOS cell proliferation were observed. Exogenous L-arginine further promoted HA-stimulated osteoblast NO production and proliferation. Furthermore, cell proliferation and NO production by HA-stimulated HOS cells in the presence of anti-human integrin αV antibody were reduced. Similarly reduction of cell proliferation and NO production by HA-stimulated HOS cells was observed when the cells were incubated with eNOS, but not nNOS and iNOS, inhibitor. Deletion of NO production by a NO scavenger resulted in suppressed HA-stimulated osteoblast proliferation.

The effect of exogenous NO on HA-stimulated HOS cell proliferation was then determined. The results showed that exogenous NO up to 20 μM enhances HA-

cell proliferation was abolished by the presence of NO scavenger but only partially reduced by eNOS inhibitor. The effect of exogenous NO on HA-stimulated HOS cells proliferation was also reduced when the cells were pre-treated with anti-human integrin αV antibody.

The next experiments were to determine whether the regulation of NO on HA-stimulated HOS cell proliferation was via its ability to enhance prostaglandin E_2 (PGE_2) production. The results showed that the production of PGE_2 by HA-stimulated HOS cells was augmented by exogenous NO. The presence of NO scavenger suppressed the production of PGE_2 by HA-stimulated HOS cells with or without the presence of exogenous NO. The presence of eNOS inhibitor only partially suppressed the production of PGE_2 by HA-stimulated HOS cells with or without the presence of exogenous NO. Again, the production of this prostaglandin by HA-stimulated HOS cells with or without the presence of NO was reduced when the HOS cells were pre-treated with anti-human integrin αV antibody. The production of this prostaglandin and cell proliferation by HA-stimulated HOS cells with or without the presence of NO was reduced when the HOS cells were pre-treated with indomethacin (a COX-1 and COX-2 inhibitor) or nimesulide (a COX-2 inhibitor) but not aspirin (a COX-1 inhibitor), suggesting COX2-mediated PGE_2 synthesis. HA-stimulated cell proliferation with or without the presence of NO was also decreased, when HOS cells were pre-coated with anti-human EP4 receptor antibody. .

The next experiments were to assess whether the regulatory role of NO on HA-stimulated HOS cell proliferation involves the cyclic nucleotide [cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP)] activity. The results of the present study showed that HA-stimulated HOS cells with or without the

cAMP but not cGMP analog augmented HA-stimulated HOS cell proliferation with or without the presence of NO. An adenylyl cyclase but not a guanylyl cyclase inhibitor suppressed HA-stimulated HOS cell proliferation with or without the presence of NO. In the presence of carboxy PTIO or L-NIO, the levels of cAMP in HA-stimulated HOS cells with or without the presence of NO were reduced. However, in the presence of carboxy PTIO or L-NIO, both endogenous and exogenous NO in HA-stimulated HOS cells were still able to induce the production of cAMP. The presence of anti-integrin αV antibody in HA-stimulated HOS cells also reduced the production of cAMP. However, exogenous of NO was still able to slightly increase the levels of cAMP in the cultures of anti-integrin αV-coated cells incubated on the HA surfaces. HA-stimulated HOS proliferation with or without the presence of exogenous NO was enhanced by forskolin, an adenylyl cyclase activator, and IBMX, a phosphodiesterase (PDE) inhibitor. The proliferation of HA-stimulated HOS cell proliferation with or without the presence of exogenous NO was significantly reduced when the cells were pre-treated with KT5720, a protein kinase A (PKA) inhibitor.

In conclusion, the present study showed that upon stimulation with HA, HOS cells proliferated and produced NO in an integrin αV molecule and eNOS dependent mechanism and that HA-stimulated HOS cell proliferation was regulated by endogenous NO in an autocrine fashion. Exogenous NO augmented HA-stimulated HOS cell proliferation independent on endogenously synthesized NO but partially dependent on the interaction of HOS cell surface integrin αV molecule and HA. The binding between HOS cell surface integrin αV and HA surface increased synthesis of PGE_2 via COX-2 activities. The released PGE_2 would then bind on EP4 receptor in an autocrine mechanism. In this respect, NO up-regulated the COX-2-mediated PGE_2 synthesis.

pathway which was controlled by PDE activity. Nitric oxide did amplify cAMP activity, thereby enhancing the HA-stimulated HOS proliferation. Therefore, the results of the present study demonstrate, for the first time, that the role of NO on HA-stimulated HOS cell proliferation may be via its ability to up-regulate the production of COX2-mediated PGE_2 synthesis and the cAMP-PKA pathway.

ABSTRAK

Hidroksiapatit (HA), yang merupakan bahan seramik, digunakan dengan meluas didalam bidang ortopedik dan implantologi pergigian kerana keupayaannya untuk meyebabkan stimulasi fungsi 'in vitro' dan 'in vivo' sel osteoblas. Walaubagaimanapun, mekanisma dimana HA menggalakkan fungsi sel osteoblas, iaitu pembentukkan tulang masih tidak jelas. Nitrik oksida (NO) meregulasi pembentukkan tulang melalui kesannya terhadap fungsi sel osteoblas dan osteoklas. Maka, tujuan keseluruhan kajian terkini adalah untuk mengetahui kemungkinan regulasi NO terhadap proliferasi sel HOS yang telah distimulasi oleh HA. Kajian ini telah meggunakan sel induk osteoblas manusia (sel HOS) kerana sel-sel ini dapat mewakili respons biologikal sel osteoblas normal manusia.

Eksperimen yang pertama bertujuan untuk mengetahui jika NO endogenus memainkan peranan dalam regulasi proliferasi sel HOS yang telah distimulasi oleh HA. Keputusan yang diperolehi menunjukkan bahawa dengan pendedahan terhadap HA, peningkatan dalam produksi NO dan prolifearsi sel HOS diperhatikan. L-arginin eksogenus pula dapat mempromosi produksi NO dan proliferasi sel osteoblas yang telah distimulasi oleh HA. Proliferasi sel dan produksi NO oleh sel HOS yang telah distimulasi oleh HA turut berkurangan dengan kehadiran antibodi anti-human integrin αV. Kesan pengurangan yang sama turut diperhatikan apabila sel-sel itu diinkubasi dengan perencat eNOS dan bukan perencat nNOS atau iNOS. Penghapusan produksi NO oleh perencat NO telah menyebabkan perencatan proliferasi sel osteoblas yang telah distimulasi oleh HA.

Pengaruh NO eksogenus terhadap proliferasi sel HOS yang telah distimulasi oleh HA dikenalpasti. Keputusan yang diperolehi menunjukkan bahawa NO eksogenus, sehingga 20μM dapat meggalakkan proliferasi sel HOS yang telah distimulasi oleh HA. Pengaruh ini terus terhapus sepenuhnya dengan kehadiran perencat NO tetapi mengalami separa pengurangan dengan kehadiran perencat eNOS. Pengaruh NO eksogenus ini juga turut berkurangan apabila sel-sel itu telah terlebih dahulu didedahkan kepada antibodi anti-human integrin αV.

Eksperimen seterusnya adalah untuk mengenalpasti regulasi NO terhadap proliferasi sel HOS yang telah distimulasi oleh HA melalui keupayaannya untuk menggalakkan penghasilan prostaglandin E2 (PGE_2). Keputusan yang diperolehi menunjukkan, produksi PGE_2 oleh sel HOS yang telah distimulasi oleh HA adalah dipengaruhi oleh NO eksogenus. Kehadiran perencat NO menyebabkan suppresi penghasilan PGE_2 oleh sel HOS yang telah distimulasi oleh HA, tanpa dipengaruhi oleh kehadiran atau ketidakhadiran NO eksogenus. Kehadiran perencat eNOS pula hanya menyebabkan suppresi separa penghasilan PGE_2. Selain itu, penghasilan prostaglandin oleh sel HOS yang telah distimulasi oleh HA dengan atau tanpa kehadiran NO juga didapati berkurangan apabila sel HOS dirawat terlebih dahulu dengan antibodi anti-human integrin αV. Penghasilan prostaglandin dan proliferasi sel HOS yang telah distimulasi oleh HA dengan atau tanpa kehadiran NO didapati berkurangan apabila sel-sel HOS dirawat terlebih dahulu dengan indomethacin (perencat COX-1 & COX-2) ataupun nimesulide (perencat COX-2) tetapi bukan dengan aspirin. Keputusan ini mencadangkan sintesis PGE_2 dimediasikan oleh COX-2. disamping itu, proliferasi sel yang telah distimulasi oleh HA menjadi kurang dengan atau tanpa kehadiran NO apabila sel-sel HOS menjalani rawatan terlebih dahulu dengan antibodi anti-human reseptor EP4.

Eksperimen eksperimen seterusnya adalah untuk menentukan samada regulasi NO keatas proliferasi sel HOS yang telah distimulasi oleh HA melibatkan aktiviti neukleotida siklik [cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP)]. Keputusan daripada ujikaji ini telah membuktikan bahawa sel-sel HOS yang telah distimulasi oleh HA dengan atau tanpa kehadiran NO meregulasikan had cAMP tetapi bukan cGMP. Penambahan analog eksogenus cAMP telah meningkatkan lagi proliferasi sel-sel HOS yang telah distimulasi oleh HA dengan atau tanpa kehadiran NO. Keadaan ini tidak dapat diperhatikan apabila analog eksogenus cAMP ditambahkan. Perencat 'adenyl cyclase' tetapi bukan perencat 'guanyl cyclase' didapati telah mengurangkan proliferasi sel HOS yang telah distimulasi oleh HA dengan atau tanpa kehadiran NO. dalam kehadiran karboksi PTIO atau L-NIO, kuantiti cAMP di dalam sel-sel HOS yang telah distimulasi oleh HA dengan atau tanpa kehadiran NO juga didapati berkurangan. Walaubagaimanapun, kehadiran karboksi PTIO atau L-NIO, kedua-dua endogenus dan eksogenus NO didalam sel-sel HOS yang telah distimulasi oleh HA masih berupaya untuk memulakan penghasilan cAMP. Penghasilan cAMP juga kelihatan berkurangan dengan kehadiran antibodi anti-human integrin αV didalam sel-sel HOS yang telah distimulasi oleh HA. Sementara itu, kuantiti cAMP didalam kultur sel-sel yang diselaputi oleh anti-integrin αV dan diinkubasi pada permukaan HA, menunjukkan peningkatan kecil dengan kehadiran NO eksogenus. Forskolin, sejenis activator siklase adenil dan IBMX, sejenis perencat fosfodiesterase meningkatkan proliferasi sel-sel HOS yang telah distimulasi oleh HA dengan atau tanpa kehadiran NO eksogenus. Walaubagaimanapun, proliferasi sel-sel tersebut berkurangan apabila dirawat dengan KT5720, sejenis perencat protein kinase, sebelum ujikaji.

Kesimpulannya, kajian ini menunjukkan bahawa sel-sel HOS apabila distimulasi oleh HA mengalami proliferasi dan menghasilkan NO didalam molekul integrin αV

melalui mekanisma cNOS. Disamping itu, proliferasi sel-sel ini juga dikawal oleh NO endogenus dalam bentuk autokrin. NO eksogenus telah menambahkan proliferasi sel-sel HOS yang telah distimulasi oleh HA tanpa bergantung sepenuhnya kepada NO endogenus yang disintesiskan. Walaubagaimanapun, proliferasi sel-sel tersebut sedikit sebanyak kepada interaksi diantara molekul integrin αV pada permukaan sel HOS dan HA. Perikatan di antara molekul integrin αV yang terdapat pada permukaan sel-sel HOS dengan permukaan HA telah meningkatkan sintesis prostaglandin melalui aktiviti COX-2. Prostaglandin yang dihasilkan kemudiannya akan terikat kepada reseptor EP4 melalui mekanisma autokrin. Dalam konteks ini NO meregulasi sintesis prostaglandin yang diperantarai oleh COX-2. selain itu proliferasi sel-sel HOS yang distimulsai oleh HA bergantung kepada laluan cAMP-PKA yang sebenarnya dikawal oleh aktiviti PDE. Nitrik oksida juga membantu aktivit cAMP dengan menambahkan proliferasi HOS yang distimulasi oleh HA. Oleh yang demikian, kajian ini menunjukan untuk yang pertama sekali bahawa pengaruh NO keatas proliferasi sel-sel HOS yang distimulasi oleh HA mungkin adalah melalui regulasi sintesis prostaglandin yang diperantarai oleh COX-2 dan juga laluan cAMP-PKA.

CHAPTER 1

INTRODUCTION AND LITERATURE SURVEY

1.1 HYDROXYAPATITE

Ceramics used for the repair and reconstruction of diseased or damaged parts of human body are termed as bioceramics. In the past two decades, the utilization of calcium hydroxyapatite and tricalcium phosphate as fillers, spacers, and bone graft substitutes for orthopaedic as well as maxillofacial surgery has received great attention, primarily because of their biocompatibility, bioactivity, and osteoconduction characteristics (Damien and Parsons, 1990). Attentions have been particularly placed on the fabrication of bioceramics with "porous" configuration allowing the tissue to infiltrate, which further enhances the implant-tissue attachment (Ohgushi, *et al.*,1992; Klein, *et al.*, 1994; Fabbri, *et al.*, 1994; Fabbri, *et al.*, 1995; Le Huec, *et al.*, 1995; Liu, 1996) (see Fig. 1.1)

Hydroxyapatite (HA) is one of the ceramic materials commercially used for orthopaedic and dental implants, since it enhances osteoblast proliferation and differentiation, thereby inducing bone formation. However, high degree of brittle and low strength seems to be a major drawback of this material. Hydroxyapatite is the major component, and an essential ingredient, of normal bone and teeth. It makes up bone mineral and the matrix of teeth and gives rigidity of bones and teeth. It is chemically similar to the mineral component of bones and hard tissues in mammals and, hence, is one of the frequently used bioceramics for bone and dental tissues reconstitution. It has excellent biocompatibility with hard tissues (Wozney and Rosen, 1998; Suchanek and Yoshimura, 1998), and high osteoconductivity and bioactivity despite its low degradation rate (LeGeros, *et al.*,. 1995., Asashina, *et al.*, 1997; Suchanek and Yoshimura, 1998; Ducheyne and Qiu, 1999; Burg, *et al.*, 2000; Green, *et al.*, 2002), mechanical strength and osteoinductive potential (Asashina, *et al.*, 1997, Burg, *et al.*, 2000; Reddi, 2000). It has neither antigenicity nor cytotoxicity (Burg, *et al.*, 2000). It is

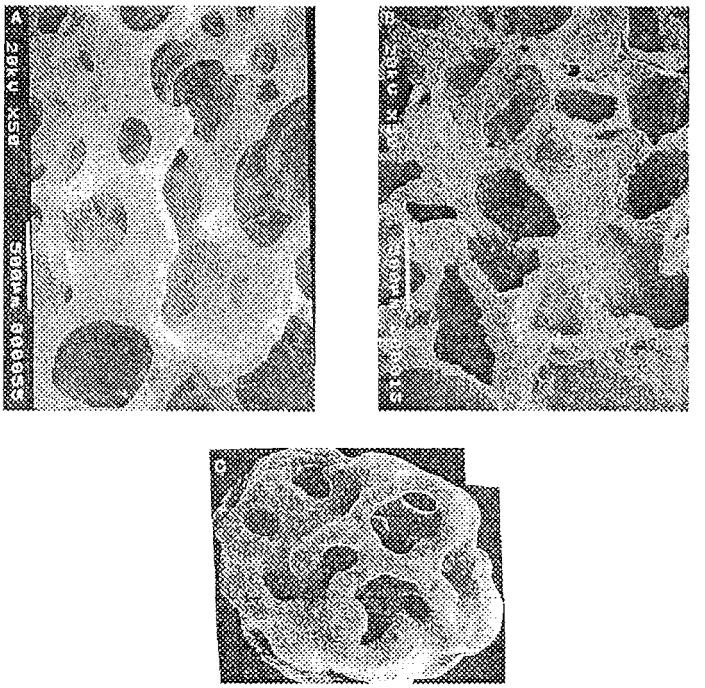

Fig.1.1 Scanning electron micrograph of bovine bone-derived hydroxyapatite (A), biphasic calcium phosphate (B) and coralline hydroxyapatite (C). (Figure taken from LeGeros Z (2002). *Clin Orthop Related Res* 395:91-98)

one of few materials that are classed as bioactive, since it supports bone in growth and osseointegration when used in orthopaedic, dental and maxillofacial applications. The chemical nature of hydroxyapatite lends itself to substitution, since it is not uncommon for non-stoichiometric hydroxyapatites to exist. The most common substitutions involve carbonate, fluoride and chloride substitutions for hydroxyl groups, while defects can also exist resulting in deficient hydroxyapatites.

Hydroxyapatite consists of Ca^{2+} ions surrounded by both PO_4^{2-} and OH^- ions, and has the chemical formula $Ca_5(OH)(PO_4)_3$. It is also known as tricalcium phosphate and calcium hydroxyapatite (Sivakumar,1998) and used for implant fixation in hard tissue prosthetics. A thin layer of HA is applied to the surface of a metal substrate, which provides the bioactivity of composite. Previous studies on HA-coated implants showed good bone fixation due to its good osteogenic response. (Koeneman, *et al.*, 1990). However, particulate debris at the bone prosthesis interface has been found to

cause a foreign response that is destructive to the surrounding tissues. The poor bonding strength of HA to the substrate results in loss of fixation of the prosthesis at the metal–HA interface. In the development of bone substitutes, a high porosity level is required for the following considerations.

1. Porous materials have a large surface area to volume ratio, resulting in significant bioresorption, which induces high bioactivity.
2. Interconnected pores permit tissue ingrowth and thus anchor the prosthesis to the surrounding bone, preventing loosening of implants.
3. Interconnected porosity acts like an organization of vascular canals that can ensure the blood and nutrition supply for the bone.

1.1.1 Chemically Derived Synthetic Ceramic

Hydroxyapatite is $Ca_{10}(PO_4)_6(OH)_2$ (Burwell, 1994; Sivakumar, 1998) and a stoichiometric compound with a Ca/P mole ratio of 1.(Schimandle, *et al.*, 1995; Sivakumar, 1998). It is formed by precipitation of Calcium nitrate and ammonium dihydrogen phosphate. (Sivakumar, 1998). Hydroxyapatite alone has been found to be insufficient for formation of bone in numerous studies. (Chappard, *et al.*, 1993; Burwell, 1994; Begley, *et al.*, 1995; Emery, *et al.*, 1996; Johnson, *et al.*, 1996; Rueger, *et al.*, 1998; Sandhu and Boden, 1998).

Rahimi, *et al* (1997) have used coral based hydroxyapatite as a bone graft substitute in foot and ankle surgery with excellent results in an 8-year study. Shahgaldi (1998) used coral based hydroxyapatite for restoration of osteochondral defects. Boden (1999) found that hydroxyapatite (coral based) mixed with autograft as graft extender but nor bone marrow aspirate resulted in postero-lateral fusion of the spine. Passuti, *et al* (1989) used macroporous, synthetic, hydroxyapatite for adolescent scoliosis for 12 patients with a 24-month follow-up. This study found certain levels of ceramic

resorbtion with new bone formation and fusion in all cases. Ransford, *et al* (1998) in a randomized, prospective trial, at centers in UK and France, found no difference between autografts and synthetic hydroxyapatite (Triosite) in 341 patients of scoliosis. Gouin (1995) followed up 19 of 21 patients of bone defects following tumors, non-unions and osteotomies, treated with synthetic hydroxyapatite. The ceramic was used alone in 6 cases, with bone marrow in 12 cases and with autograft in five. All defects healed and 17 of them had a union time of three months. Toth (1995) found that the porous calcium phosphate ceramics mixed with autograft in the ratio of 70 (ceramic): 30 (autograft) were effective for anterior cervical interbody fusion in goat. Itokazu (1996) reported that tibial plateau fractures receiving interporous hydroxyapatite as a bone graft substitute united with full function. Bucholz, *et al* (1989) reported that graft with interporous hydroxyapatite as bone graft substitute results in similar to that with autografts.

In an experimental study comparing the various bone graft substitutes, Johnson *et al* (1996) found that hydroxyapatite alone gave poor results. Only one out of nine defects united. When mixed with bone marrow aspirate, it gave results similar to autografts, but not as good as autografts. They also noted that presence of hydroxyapatite, which is not resorbed, made radiological assessment of union almost impossible

1.1.2 Properties

Hydroxyapatite is a thermally unstable compound, decomposing at temperature from about 800-1200°C depending on its stoichiometry. Generally, dense hydroxyapatite does not have the mechanical strength to enable it to succeed in long term load bearing applications. Hydroxyapatite coatings are often applied to metallic implants (most commonly titanium/titanium alloys and stainless steels) to alter the surface properties and the recipients well accept hydroxyapatite-type materials. On the

other hand, HA- uncoated materials would act as a foreign body which, in turn, would be isolated from the surrounding tissues. To date, the only commercially accepted method of applying hydroxyapatite coatings to metallic implants is plasma spraying. Hydroxyapatite may be applied in forms of powders, porous blocks or beads to fill bone defects or voids. The bone filler provides a scaffold and induces the rapid filling of the void by naturally forming bone. It also becomes part of the bone structure and reduces healing times compared to the situation.

1.2 HYDROXYAPATITE AND OSTEOBLAST

The surface of implant materials presented to cells can be considered as a foreign chemical species with reactive sites. The end groups of polymer chains may also interact with reactive groups such as protein or carbohydrate molecules in serum. When a material is implanted in vivo, it is immediately covered with a thin layer of extracellular fluid, and it is through this layer that the cells interact with the implant material (Kasemo and Lausmaa, 1994). The bonding established varies depending on the forces, with van der Waals attractions through to covalent bonding (Kasemo and Lausmaa, 1994; Takahiro, *et al.*, 1997). Cells placed in contact with a biomaterial surface may show a range of responses from induction of an inflammatory response through to perception of the material as 'tissue-like' invoking no reaction. The nature of the cellular response determines whether the implant becomes encapsulated with fibrous tissue, or initiates bone growth, if indirect contact with bone (Kasemo and Lausmaa, 1994). There are four possible responses that may occur; tissue death (toxic materials), fibrous tissue formation (inert materials), interfacial bond formation (bioactive materials), or replacement by surrounding tissue (biodegradable materials) (Hench, 1988).

1.2.1 Attachment And Spreading

Interaction of cells and HA requires specific interaction of adhesion molecules and extracellular matrix. Immediately after implantation, implant surfaces are markedly changed by the adsorption of proteins and other components from exposure to blood at the implant site. Comparing with other implant materials such as titanium, HA has a strong adsorptive property for protein due to the ionic strength and crystallinities of this material (Kandori, *et al.*, 2005; Yang, *et al.*, 2005). Indeed, HA adsorbed serum-derived fibronectin, vitronectin and integrin molecules much stronger than titanium (Matsuura, *et al.*, 2000; Kilpadi, *et al.*, 2001), suggesting that HA may have higher affinity than other implant materials for extracellular matrix (ECM) adsorption. Osteoblasts cultured in serum-free medium failed to attach in HA surfaces (Okamoto, *et al.*, 1998) suggesting that serum ECM adsorbed onto HA surface is pre-requisite for osteoblast attachment on HA surface. Further studies revealed that RGD peptides are the specific ECM site where osteoblasts are attached (Okamoto, *et al.*, 1998; Matsuura, *et al.*, 2000; Itoh, *et al.*, 2002).

Since osteoblasts attach on ECM adsorbed on HA surface, the cells require specific protein receptors which bind with ECM. Following osteoblast-HA interaction, the expression of osteoblast surface integrin αV and βV subunits was increased (de Ruijter, *et al.*, 2001). Matsuura and colleagues demonstrated that when osteoblasts were pre-coated with anti-integrin αV antibody, these cells failed to attach and spread on HA surfaces in serum-containing culture medium, suggesting that osteoblast surface integrin αV molecules may bind on serum-coated HA surface (Matsuura, *et al.*, 2000). Thus, it seems to suggest that interaction of osteoblast integrin αV and RGD peptide of ECM may result in the induction of signal transduction which lead to cell attachment and spreading on the surface of HA.

The surface of HA is highly reactive and can bind strongly with bone (Gruen, 1979; Posner and Betts,1975). Cells react to HA presented within the material surface topography. Topography can be subdivided into macro and micro-topography; with macro-topography dealing with the physical configuration of the implant (e.g. screw threads), and micro-topography resulting from surface roughness or texture (Mohan, *et al.*, 1999; Curtis and Riehle, 1999). 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 (Alberts, *et al.*, 1989; Amos and Amos, 1991).

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 (Alberts, *et al.*, 1989). 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 fibres are seen once the filopodia are attached (Cooper, 1997). Cells use topography for orientation, alignment, and migration by recognising surface features and reacting to them, possibly by reshaping their actin filaments in filopodia, resulting in contact guidance (see Fig. 1.2). When the microspikes find a suitable site, focal adhesions and mature actin fibres are formed. The probing of the microspikes can be influenced by surface discontinuities (Clark, *et al.*, 1990; Walboomers, *et al.*, 1999).

Various biomaterials have been studied as tissue replacements for bony skeletal defects originated from trauma, tumor resection and skeletal abnormalities. The commonly used bone grafting materials included autografts, allografts, xenografts and synthetic composites (Costantino and Friedman, 1994). Although autografts are most

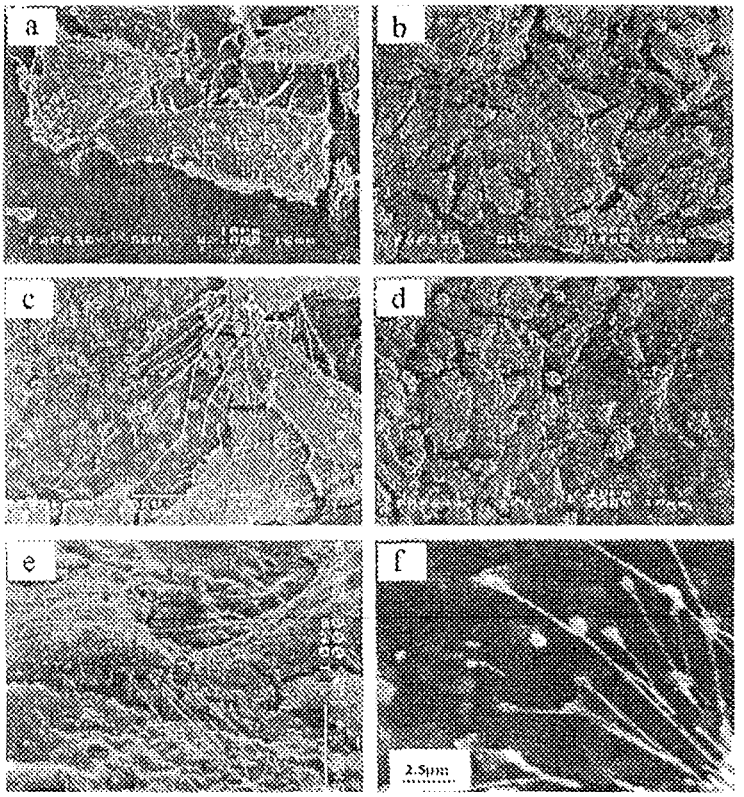

Fig. 1.2 Scanning electron micrograph of human osteoblasts on HA-coated poly cement. Cells attached on poly cement showed a flattened shape (a and b). Cells attached on HA-poly cement showed extension of osteoblast filaments which adhered on exposed HA surface (c-f). Extended anchorage cells was formed by the cells to adhere on HA surface (f). Figure is taken from Dalby MJ, *et al* (2001). *Biomaterials* 22:1739-1747.

suitable for bone regeneration, they have to be dissected from the other bone tissues of the host. It causes donor site morbidity and is usually difficult to form the desired shape. On the other hand, allografting and xenografting have the disadvantages of causing immune reaction and transferring pathogens. Collagen and hydroxyapatite have been used as the bone-filling materials in orthopedic surgery (Bell and Beirne, 1988; Hahn, *et al.*, 1988; Marouf, *et al.*, 1990).

The collagen fibers are known to serve as the scaffold for tissue repair (Pachence, 1996), whereas hydroxyapatite is well compatible and osteoconductive for bone regeneration. While the dense hydroxyapatite disc is not biodegradable, particulate hydroxyapatite can be removed and remodeled in the host (Pohunkova and Adam,

1995). Unfortunately, direct implantation of hydroxyapatite particles resulted in the dislocation of material within the tissue. To eliminate the undesired mobility, hydroxyapatite powders are often mixed with collagen (Sugaya, *et al.*, 1989), gelatin (Nagase, *et al.*, 1989) or fibrin glue (Wittkampf, 1988), when used as bone grafts. Although most of the collagen matrices are prepared in a slab form, spherical composites of collagen and hydroxyapatite are more versatile in biomedical applications. Other than being used for cell culturing, spherical gel beads have greater flexibility in filling different geometric cavity with closer packing than gels with nonspherical shapes. Microspheres composed of collagen and hydroxyapatite are also injectable for repairing tissue defect. Collagen gel beads have been prepared by discharging a suspension of crude collagen fibers into liquid nitrogen (Dean, *et al.*, 1989). The collagen used in this process contains the amino-terminal telopeptides which could induce immune response of the host.

The body produces biological HA for use as a structural component in bone. In cortical (compact) bone, HA crystals are found within collagen as needles oriented in the direction of the.bers (Kaplan, *et al.*,1994, Narasaraju and Phebe, 1996; Shackelford, 1999). The needles are nanodimensional, generally10–60nm long and 2–6nm wide (Narasaraju and Phebe, 1996), and are bound to collagen fibers (Guyton, 1991). Synthetic HA has the same chemical composition as biological HA and thus mimics many properties of natural bone (Jarcho, 1992). When used to coat an orthopedic or dental implant, synthetic HA provides a surface for the anchorage-dependent osteoblasts to deposit calcium-containing mineral. This promotes osseointegration of an implant, stabilizing it, and preventing motion-induced damage (Jarcho, 1992; Willmann, 1993; Cabanela, 1999; Soballe, 1999). HA has been labeled ''osteoconductive'' due to its ability to bind strongly with natural bone tissue (Dalby, 2001). Despite these optimal properties, synthetic HA is limited in use due to high in vivo solubility and poor

mechanical properties, such as low impact resistance (Jarcho, 1992; Willmann, 1993; Soballe. 1999). Several researchers have attempted to remedy these problems by doping synthetic HA with small amounts of impurities. Biologic al HA crystals *in vivo* also contain many impurities, e.g., substitutions of K+, Mg2+, Na+, CO_3^{2-}, and F^-, which usually reflect dietary history, but can also indicate exposure to environmental hazards, e.g. Pb2+ and Sr2+ (Guyton, 1991; Kaplan, et.al.,1994; Narasaraju and Phebe, 1996). These ionic substitutions can alter properties of HA (such as solubility and mechanical) either causing or inhibiting normal function, e.g., excessive fluoride can weaken bone (Kaplan, *et al.*, 1994; Martin, 1999).

Webster, *et al.*, (2002) reported that doping HA with 2mol% of Zn2+ significantly increased osteoblast adhesion as compared to undoped HA. Furthermore, Ito, *et al.*, (2000), determined that doping with Zn2+ in amounts between 0.6 and 1.2wt% enhanced the proliferation of mouse osteoblast-like cells in a tricalcium phosphate/HA composite ceramic. A similar study also found Zn2+ to be an *in vitro* inhibitor of osteoclastic bone resorption (Ito, *et al.*, 2001). Moreover, doping with 2 mol% of Mg2+ significantly enhanced osteoblast adhesion as compared to undoped HA.

However, Serre *et al.*, (1998), discovered that the addition of 20 wt% Mg2+ reduced the osteoconductivity of an apatite sample with osteoblast-like cells, indicating that too much substitution exceeded the potential benefits of doping. Webster, *et al.*, (2002), also reported that osteoblast adhesion on HA doped with 2 mol% yttrium (Y3+) was 28% greater than on undoped HA. This response was greater than osteoblast adhesion to other HA formulations in that study, including Zn2+ and Mg2+ as well as Cd-doped HA formulations.Greater amounts of added Y3+ achieved greater adhesion results, but the effect seemed to level off in the range of 5–7 mol% (Webster, *et al.*, 2002).

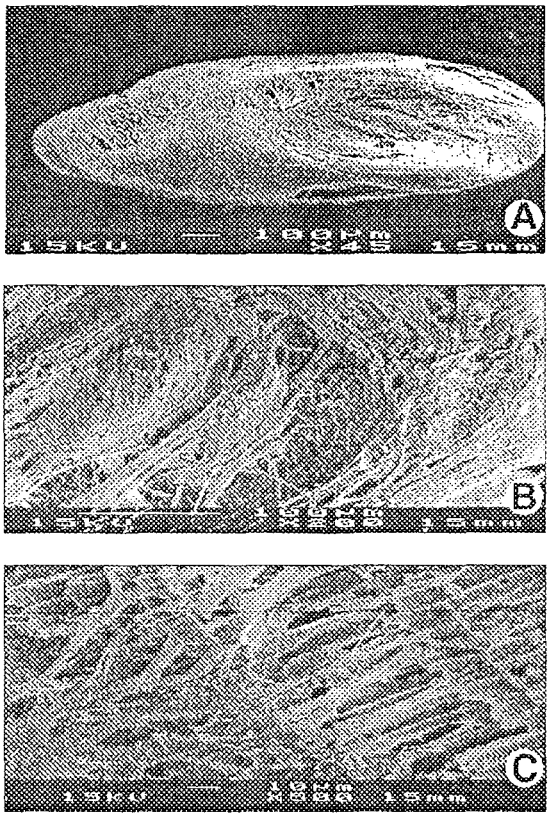

Fig. 1.3 Scanning electron micrograph of osteoblasts on HA granule. (A) HA surface covered by cells; (B) Cells on the surface of HA; (C) Cells in the pores of particle. Figure is taken from Turhani D, *et al.* (2005). *J Oral Maxillofac Surg* 63:793-799.

1.2.2 Proliferation And Differentiation

In general, following osteoblast attachment on the HA surface, osteointegration is inititated by the formation of non-collagen proteins such as osteopontin and bone sialoprotein at the surface of material.. Calcium phosphate is then attached to one or both proteins and crystallization by inducing collagen production is then occurred. This process is subsequently followed by mineralization collagen matrix (Kubler, *et al.*, 2004), suggesting that osteoblast proliferation and differentiation is highly up-regulated by HA (Fig. 1.3). Increased osteoblast differentiation induced by HA has, indeed, been well documented. For example, Hott *et al* (1997) found that collagen synthesis and calcium uptake by human osteoblasts were increased on HA surface. Increased cell

proliferation and the production of type I collagen, osteocalcin, osteonectin, osteopontin, alkaline phosphate and bone sialoprotein by human osteoblasts on the surface of HA, suggesting that human osteoblast proliferation and differentiation to form calcified tissue are enhanced by HA (Phan, *et al.*, 2003; Knabe, *et al.*, 2004; Ogata, *et al.*, 2005;Turhani, *et al.*, 2005). HA ceramics also induced mesenchymal cell differentiaton to osteoblasts (Norman, 1994; Okumura, *et al.*, 1997; Cong, *et al.*, 2001).

It appears to suggest that the ability of HA to induce osteoblast differentiation may be via the production of growth factors such as transforming growth factor-β (TGF-β). Boyan *et al.*, (2003) demonstrated that increased tendency of human osteoblast differentiation on HA rough surface is highly associated with increased production of this cytokine. Bigi and colleagues also found that the peak production of this cytokine HA-stimulated human osteoblast was paralleled with that of type 1 collagen by these cells (Bigi, *et al.*, 2005). These previous results have also been confirmed by Clarke *et al.*, (2004) who showed that when HA was incorporated with recombinant TGF-β and then implanted in the proximal tibia of rats, rapid bone formation occurred at the implanted site, suggesting that this cytokine may accelerate osteoblast proliferation and differentiation, and hence, bone formation at the HA implanted site.

1.2.3 Role of Bone-related proteins

When osteocytes contact with HA surfaces, cell behavior could be altered by the surface topography, chemistry and energy (Zreiqat, 1996; Zreiqat, *et al.*, 1999; Zreiqat, *et al.*, 1999; Zreiqat and Howlett, 1999; Zreiqat, *et al.*, 2003; Knabe, *et al.*, 2003). By bonding to specific receptors in osteoblast membrane, these extracellular factors start to chain reaction inside cells, which eventually result in the alteration in bone-related gene and protein expression and the consequence is the changes in osteoblast proliferation

and differentiation. Sometimes, these changes in gene expression are favorable for osteoblast function (Zreiqat, *et al.*, 2002). However, these changes may be detrimental to osteoblast differentiation and function, which may result in the failure of implant. Alkaline phosphatase, osteocalcin, osteonectin, bone sialoprotein and type I collagen are some of the most important genes expressed by osteoblasts which can collectively contribute to the formation of the osseous matrix and controlled calcification (Mundlos, 1997).

Different bone matrix proteins have different biological functions in bone formation. Type I collagen is the major bone matrix protein, which binds to hydroxyapatite crystal to provide bone with enough biomechanical strength. Alkaline phosphatase, a membrane-bound protein, is the marker of early osteogenesis and it functions to provide free calcium and phosphate for hydroxyapatite crystal growth and calcium balance in the body (Owen, *et al.*, 1990; Strauss, *et al.*, 1990; Gerstenfeld , *et al.*, 1990; Sodek, *et al.*, 1991; Pockwinse, *et al.*, 1992). Osteocalcin is responsible for calcium ion binding and is believed to be the marker of late stage of bone formation. Experiment results also showed that osteocalcin could down-regulate bone mineralization by inhibiting the formation of hydroxyapatite crystal in bone (Romberg, *et al.*, 1986). Osteonectin, a multifunctional protein widely expressed in many tissues, is a collagen-binding protein and functions as a regulator to the osteoblast proliferation (Termine, *et al.*, 1981). Bone sialoprotein is responsible for the nucleation of HA crystal and therefore, it is very important to bone mineralization (Oldberg, *et al.*, 1988). All these bone related genes are highly expressed in the process of osteogenesis in a stepwise fashion and they play a crucial role in osteogenesis.

1.2.4 Factors Associated With HA-Osteoblast Interaction

Critical to the long-term success of orthopaedic and dental implants is the development of a stable direct connection between bone and surface implant, which must be structural and functional (osteointegration). The establishment and maintenance of osteointegration depend on wound healing tissues, repair and remodelling. The tissue response to an implant involves physical factors, depending on the implant design, surface topography, and chemical factors associated with the composition and structure of the material. To improve a direct implant fixation to bone, several strategies have been developed focusing the attention on the surface of materials. Chemical modifications have been realised by covalent attachment of an organic monolayer anchored by a siloxane network (Sukenik, *et al.*, 1990) and immobilising specific adhesive peptides, like arginine, glycine, aspartic acid, serine (RGDS) (Dee, *et al.*, 1996). Surface roughness has been modified by different techniques, because it has been demonstrated that the osteoblastic cells tend to attach more rapidly to surfaces with a rougher microtopography (Lincks, *et al.*, 1998; Martin, *et al.*, 1995) increasing the bone apposition (Jinno, *et al.*, 1998; Vercaigne, *et al.*, 1998).

Moreover, the implants have been coated with different materials like calcium phosphate ceramics, bioactive glass (Matsuda and Davies, 1987; Hayashi, *et al.*, 1989; Barth, *et al.*, 1990; Vrouwenvelder, *et al.*, 1993; Oliva, *et al.*, 1998; Wheeler, *et al.*, 1998), diamond-like carbon and amorphous C-N film (Du, *et al.*, 1998). Many studies have been carried out to develop stable hydroxyapatite coatings for their capacity to establish a bond with tissues in vivo, and to promote rapid attachment and cell growth in vitro (De Lange, *et al.*, 1990; Bagambisa, *et al.*, 1990; Faucheux, *et al.*, 1994). Various techniques, such as plasma spray (De Groot, *et al.*, 1987). Ion beam assisted deposition (Cui, *et al.*, 1997), radiofrequency magnetron sputtering (Hulshoff, *et al.*, 1995), have been used to produce coatings on implants. Some drawbacks have been noticed

regarding the long-term performance of the obtained coatings: coating resorption, poor mechanical properties, high thickness, non-homogeneity, lack of adherence (Kangasniemi, *et al.*, 1994).

Substantial amount of work has been done to explore how sintering temperature may influence HA ceramic properties and its bioresponses after implantation. Sintering temperature can change the crystallinity of HA ceramics and its bioactivity, and thus, when sintered at different temperature, they possess different biomechanical strength and other physical properties (Ruys, *et al.*, 1995), osteogenicity (Wang , 1990) and initial bonding behavior with bone (Kitsugi, *et al.*, 1987; Kitsugi, *et al.*, 1988). Sintering temperature of HA ceramics can also affect physiological changes in cells, as demonstrated by the ionic concentration changes in monocytes in vitro (Laquerriere, *et al.*, 2001). When the biological behavior of sintered and unsintered HA were compared, totally different properties in protein absorption has been reported. At the molecular biology level, different alkaline phosphatase and osteocalcin protein expression were reported with different sintering temperatures (Villareal, *et al.*, 1988). When they were implanted in vivo, different biological responses were also observed (Wang, 1990). Although many studies have explored how sintering temperature may influence the osteoblast behavior, little is known about the molecular levels that determines the interaction between the different HA ceramics and their interaction with bone forming cells.

1.3 NITRIC OXIDE

Nitric oxide (NO) is a gaseous molecule generated from L-arginine under catalization of nitric oxide synthase (NOS) and produced by cells such as macrophages, endothelial cells, and hepatocytes. This free radical gas plays a crucial role on the nervous, vascular and immune system (Mayer and Hemmens, 1997).

In 1978, it was found that nitric oxide (NO) is produced from L-arginine amino acid by nitric oxide synthase (NOS), which is present in endothelial cells and other tissues (Marletta, 1993). Studies revealed the role of NO in relaxation of smooth muscles of arteriole walls, inhibition of platelet aggregation, and interneuronal transaction (Marletta, 1993; Moncada, *et al.*, 1991). Nitric oxide is released from macrophages and osteoclasts during cell to cell interactions and acts as a cytotoxic molecule to kill intracellular microorganisms and tumor cells. Nitric oxide also plays a role in bone resorption (Henry, *et al.*, 1991).

NO is synthesized in mammalian cells by a family of three NO synthases (NOS). It is not known whether additional mammalian NOS isoforms exist, but the failure of homology based molecular cloning approaches to identify novel NOS cDNAs makes it unlikely that newly discovered members of the mammalian NOS gene family will bear significant structural similarity to the current trio of isoforms (Michel, *et al.*, 1995). As for any newly described gene family, an accepted nomenclature of the NOS isoforms has evolved only as novel information becomes more generally established. The initial NOS nomenclature reflected the early observations that NO synthesis was not characteristic of unactivated inflammatory cells, but could become induced upon immunoactivation, hence the term iNOS. This prototypic "inducible" iNOS was contrasted to a "cNOS" activity that was constitutively expressed in certain characteristic cell types (neuronal, endothelial). However, it is now known that the levels of gene expression of both eNOS and nNOS may also be induced under different physiological conditions (e.g., hemodynamic shear stress or nerve injury), and conversely, that iNOS may function as a "constitutive" enzyme under physiological conditions in some cells (Guo, *et al.*, 1995).

NO is a free radical gas which was discovered to be a physiological mediator in 1987 after its identification as the moiety responsible for Endothelium-derived

relaxation activity (Palmer, *et al.*, 1987). Since then, NO has emerged as a pleiotropic mediator in several tissues and organ systems (Moncada and Higgs, 1993). Whilst initial work focused on the effects of NO on the vascular and nervous systems, an accumulating body of evidence has now emerged to suggest that NO plays an important role as a paracrine and autocrine mediator of bone cell activity in response to diverse stimuli, such as cytokine activation, sex hormone deficiency and mechanical strain.

1.3.1 Synthesis And Metabolism Of NO

Nitric oxide is generated by the nitric oxide synthase (NOS) group of enzymes which combine molecular oxygen with the terminal guanidino nitrogen of the amino acid L-arginine to yield NO and L-citrulline as a co-product (Palmer, 1993) (see Fig.1.4) . Under aerobic conditions, NO rapidly reacts with oxygen to yield the stable metabolites nitrate (NO_3) and nitrite (NO_2). Since the half-life of NO is in the order of seconds, direct measurement is difficult, but can be achieved by chemiluminescent techniques (Kikuchi, *et al.*, 1993). More commonly, however, NO production is assessed indirectly, by measuring the conversion of radio-labelled L-arginine to L-citrulline (Bredt and Snyder, 1990), or by measuring accumulation of NO_3 and NO_2 in biological fluids such as plasma-urine and synovial fluid (Green, *et al.*, 1982).

Three isoforms of NOS have been identified that share some homology at the nucleotide and amino acid level: a neuronal form (nNOS or NOS I) (Bredt, *et al.*, 1991), an endothelial form (ecNOS or NOS III) (Robinson, *et al.*, 1994) and an inducible form (iNOS or NOS II) (Lowenstein, *et al.*, 1992) (see Fig. 1.5). The NOS enzymes, while similar in function, differ substantially in terms of regulation\ tissue distribution and catalytic activity. Both ecNOS and nNOS are constitutively expressed at low levels in their tissues of origin, and for this reason are sometimes collectively referred to as 'constitutive' NOS (cNOS) enzymes (Marletta, 1993). Since their original discovery, it

has become clear that expression of both ecNOS and nNOS is not restricted to vasculature and neural tissues; both have been detected in tumour cell lines (Jenkins, *et al.*, 1994), in gut, muscle, spleen, thymus (Salter, *et al.*, 1991) and in bone-derived cells such as osteoblasts and osteoclasts (Brandi, *et al.*, 1995; Evans, *et al.*, 1997) This raises the possibility that low levels of NO production derived from the cNOS enzymes may play a more widespread regulatory role than has generally been appreciated.

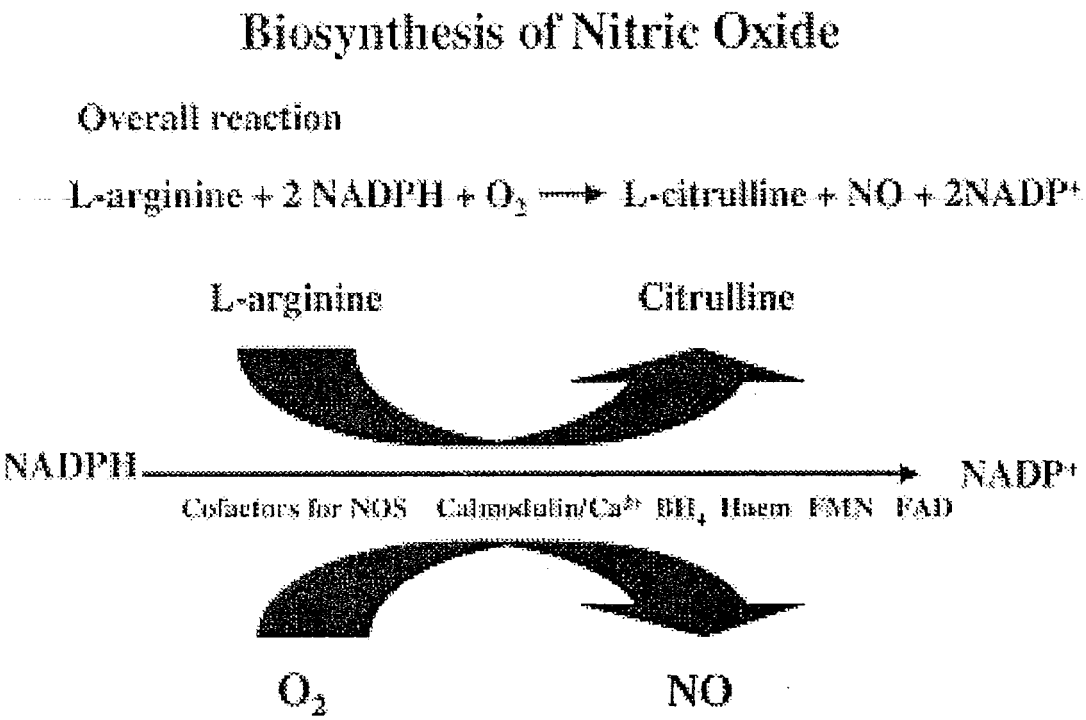

Fig. 1.4 Biosynthesis of nitric oxide from L-arginine. Nitric oxide synthases catalyzed this reaction. [Figure taken from Bruckdorfer R (2005). *Mol Aspect Med* 26:3-31].

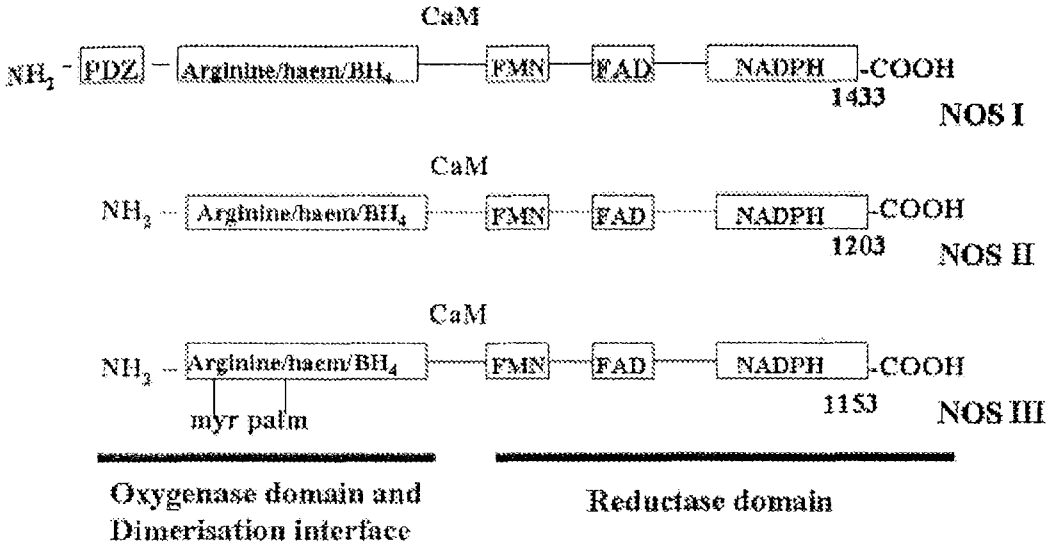

Fig. 1.5 Structure of nitric oxide synthase (NOS) isoforms. NOS I = nNOS; NOS II = iNOS; NOS III = eNOS. BH_4 = tetrahydrobiopterin; CaM = calmodulin; FAD = flavin adenine dinucleotide; FMN = flavin mononucleotide; NADPH = nicotine adenine dinucleotide phosphate. [Figure taken from Bruckdorfer R (2005). *Mol Aspect Med* 26:3-31].

The activity of the cNOS enzymes is primarily regulated at a post-translational level by changes in free intracellular Ca^{2+} concentration (iCa^{2+}), which binds to calmodulin associated with the enzyme to induce a conformational change that increases catalytic activity (Marletta, 1993). This pathway of NO synthesis is characterized by low-output NO production (picomolar concentrations) that can be rapidly modulated by external stimuli which alter iCa^{2+}. Relatively little is known of the factors which regulate ecNOS and nNOS expression at a transcriptional level. The bovine ecNOS gene promoter contains both oestrogen-response elements and shear stress-responsive elements (Venema, et.al., 1993) suggesting that these stimuli may play a role in regulating ecNOS transcription. In keeping with this\ increases in ecNOS mRNA have been found in endothelial cells after exposure to physiological concentrations of oestrogen (Weiner, *et al.*, 1994; Hayashi, *et al.*, 1995), and in response to shear stress (Uematsu, *et al.*, 1995).

While iNOS also contains a binding site for calmodulin, changes in iCa^{2+} do not substantially alter catalytic activity because the calcium-calmodulin complex is tightly bound to the enzyme under normal physiological conditions. Rather, regulation of iNOS principally takes place at the level of gene transcription. The iNOS synthetic pathway is activated by pro- inflammatory cytokines such as interleukin-1 (IL-1), tumour necrosis factor (TNF), interferon (IFN) gamma and endotoxin in a variety of cell types, whereas glucocorticoids and the anti-inflammatory cytokines IL-4, IL-10 and transforming growth factor (TGF)-β are inhibitory (Nathan, 1992; Moncada and Higgs, 1993). Sequence analysis of the iNOS promoter in rodents and humans has shown the presence of response elements (RE) which are activated by several cytokines, including TNF-RE, IFN-RE, NF*k*B, AP-1, GAS and NF-IL-6 (Nunokawa, et.al., 1994; Xie, *et al.*, 1994), and molecular studies have shown that transcription driven by the iNOS promoter is markedly upregulated by cytokines and endotoxin in minigene constructs linked to reporter assays (Xie, *et al.*,1993; Lowenstein, *et al.*, 1993). Interestingly, studies of the human iNOS promoter have shown substantial differences from the murine promoter in terms of its inducibility by cytokines. Although the proximal region of the human promoter is similar to that of the murine one, the human gene requires much more 5' sequence (3,8-16 kb) for cytokine inducibility *in vitro* (de Vera, *et al.*, 1996), suggesting that upstream sequences may be crucial for effective induction by cytokines in man. Such differences in genomic organization may partly be responsible for differences in the ease with which cytokines can induce NO production in different species. For example, the combination of endotoxin and multiple cytokines does not effciently activate the iNOS pathway in human macrophages *in vitro* (Albina, 1995), whereas rodent macrophages can be readily induced to produce large amounts of NO upon exposure to only one of these stimuli. Different cell types also differ in their ability to produce NO after cytokine stimulation; human primary osteoblast cultures

require combinations of two or three cytokines for significant induction of NO (Ralston, *et al.*, 1994), whereas human chondrocytes can be induced by single cytokines such as IL-1 and TNF to produce NO (Hickery, *et al.*, 1994; Grabowski, *et al.*, 1995). In all cell types, however, combinations of cytokines are generally more potent inducers of NO than single cytokines (Nussler, *et al.*, 1992; Ralston, *et al.*, 1994; Grabowski, *et al.*, 1995; Ralston and Grabowski, 1996) and, irrespective of the primary stimulus, the iNOS pathway is thought to be capable of generating much larger quantities of NO (nanomolar range) over a more prolonged time frame than the cNOS enzymes (Moncada and Higgs A, 1993; Palmer, 1993)

1.3.2 Mechanisms Of NO Action

Nitric oxide is a reactive molecule and, as such, has many potential molecular targets. In vascular smooth muscle and platelets, NO is thought to act by binding to the haem moiety of soluble guanylyl cyclase (sGMP) (Ignarro, *et al.*, 1986; Ignarro, *et al.*, 1987). This induces a conformational change which increases enzyme activity, resulting in increased production of cyclic GMP. The elevation in cGMP levels causes activation of a cascade of cGMP- dependent protein kinases which mediate smooth muscle relaxation and inhibition of platelet adhesion. It is of interest that conformational changes induced by binding of NO to the haem moiety of the NOS enzymes inhibit enzyme activity\ thereby providing an autoregulatory loop by which increased NO levels act to limit NO production (Rogers and Ignarro, 1992).

Other biological effects of NO are mediated by its ability to react with sulphydryl residues and iron-sulphur centres in proteins. It is thought that the former interaction is responsible for the down- regulation of *N*-methy-D-aspartate (NMDA) receptor activity (Garthwaite, *et al.*, 1988), thus providing a neuroprotective effect, and the latter for the inhibitory effect of high NO concentrations on cell growth via the

interaction with mitochondrial aconitase, a critical enzyme in the Krebs cycle. Protein nitrosylation provides a further mechanism of NO action. Nitrotyrosine is readily detected in situations characterized by increased production of NO (e.g. in rheumatoid synovium) (Blake, *et al.*, 1996), and the inhibitory effects of NO on DNA synthesis and cell division are partly mediated by nitrosylation of a tyrosyl radical at the catalytic site of ribonucleotide reductase (Lepoivre, *et al.*, 1992). Since tyrosine is a crucial residue in several signalling molecules, this action of NO could have widespread effects on cell signaling. Finally, NO can react with oxygen-derived free radicals such as superoxide anions to form a cascade of highly reactive species, including the peroxynitrite anion and the hydroxyl radical (Stamler, *et al.*, 1992). Production of such toxic moieties may contribute to the tissue damage that is a characteristic feature of an inflammatory response by inducing lipid peroxidation and protein nitrosylation (Halliwell and Gutteridge, 1990; Lipton, *et al.*, 1993). The interactions of NO with free radicals may have consequences for the control of osteoclast function, since superoxide and hydrogen peroxide stimulate osteoclastic bone resorption (Garrett, *et al.*, 1990; Hall, *et al.*, 1995; Fraser, *et al.*, 1996), and osteoclasts generate superoxide during the process of bone resorption (Steinbeck, *et al.*, 1994).

1.4 NITRIC OXIDE AND OSTEOBLAST

1.4.1 Expression Of NOS Isoforms In Bone And Bone-Derived Cells

Immunohistochemical studies of whole bone and cultured bone-derived cells have shown evidence of widespread ecNOS expression in bone marrow stromal cells, osteoblasts, osteocytes and osteoclasts (Brandi, *et al.*, 1995; Evans, *et al.*, 1996; Chow, *et al.*, 1996), and ecNOS transcripts have been found in freshly isolated bone, osteoblasts and bone marrow cultures by reverse transcription-polymerase chain

reaction (RT/PCR) assays. While nNOS mRNA has also been detected in whole bone and bone marrow cultures by RT/PCR (Pitsillides, *et al.*, 1995; Evans, *et al.*, 1996), this isoform has proved more difficult to detect in bone and cultured bone-derived cells by immunohisto-chemical techniques. Schmidt, *et al.*, (1992) failed to detect evidence of nNOS in whole bone. More recent studies using different antibodies, however, have shown evidence of nNOS expression in bone lining cells and osteocytes (Chow, *et al.*, 1996). Although low levels of iNOS transcripts have been detected in whole bone and cultured osteoblasts by RT/PCR, iNOS protein has not been found by immunostaining in bone sections or in cultured osteoblasts under basal conditions. The inducible isoform has, however, been detected in growth plate chondrocytes *in vivo* and in bone marrow macrophages *in vitro*, even in the absence of cytokine stimulation (Evans, *et al.*, 1996). This may well explain the presence of iNOS transcripts in whole bone and the relatively high levels of NO production which have been found under basal.

Most bone cells can be induced to produce iNOS in response to stimulation with cytokines and/or endotoxin (Ralston, *et al.*, 1994; Lowik, *et al.*, 1994; Ralston, 1995; Ralston and Grabowski, 1996; Van't Hof and Ralston, 1997). As in other tissues, multiple cytokines have stimulators of NO production in bone cells than single been found to be more potent cytokines and rodent cells to be more responsive to cytokines than human cells (Damoulis and Hauschka, 1994; MacPherson, *et al.*,1995). In contrast, the calciotropic hormones parathyroid hormone (PTH) and $1,25(OH_2)D_3$ do not stimulate NO production significantly (Ralston, *et al.*, 1994; Ralston, *et al.*, 1995) and, in one study, $1,25(OH_2)D_3$ was found to inhibit cytokine-induced NO production slightly (Lowik, *et al.*, 1994). It is currently unclear whether osteoclasts are capable of producing iNOS. Studies in bone marrow co-cultures show that the majority of cytokine-induced NO derives from osteoblasts (Van't Hof, 1996), failed to detect iNOS protein or mRNA in cultured osteoclasts (Evans, *et al.*, 1996). Nonetheless, iNOS