BIOCOMPATIBILITY STUDIES OF HUMAN FETAL OSTEOBLAST CELLS

CULTURED ON GAMMA TITANIUM ALUMINIDE

By

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ABSTRACT

There is a continuous search of new implant materials with better biocompatibility and physiological properties than the current commercially pure Ti and Ti-6Al-4V alloys. Gamma titanium aluminide (γTiAl) appears to have excellent potential for bone repair and replacement. The response to γTiAl implant is expected to be similar or better than those of Ti-6Al-4V. Human fetal osteoblast cells were cultured on the surface of γTiAl and Ti-6Al-4V discs with variable surface roughness for both SEM and immunofluorescent analysis to detect the presence of collagen type I and osteonectin, proteins of the bone extracellular matrix. Results show that cell growth and attachment on γTiAl was normal compared to that of Ti-6Al-4V, suggesting that γTiAl is not toxic to osteoblasts. Normal growth and the presence of collagen type I and osteonectin was observed on γTiAl and Ti-6Al-4V. The results obtained suggest γTiAl is biocompatible with the osteoblasts.

RESUMEN

Existe una búsqueda continua de materiales nuevos para ser usados en implantes con mejores propiedades de biocompatibilidad y fisiológicas que los actuales Ti comercialmente puro y aleaciones Ti-6Al-4V. Gamma titanium aluminide (γTiAl), aparenta tener excelente potencial para reparar y reemplazar huesos. Se espera que la respuesta a implantes de γTiAl sea similar o mejor que la de Ti-6Al-4V. Células de osteoblastos fetales humanas fueron cultivadas en la superficie de discos de γTiAl y Ti-6Al-4V con rugosidades variables para realizarles estudios de MER y análisis de inmunoflorescencia para detectar la presencia de colágeno tipo I y osteonectina, proteínas de la matriz extracelular del hueso. Los resultados muestran que el crecimiento celular y la adhesión en γTiAl eran normales al compararlos con células creciendo en Ti-6Al-4V, sugiriendo que γTiAl no es tóxico para los osteoblastos. El crecimiento normal y la presencia de colágeno tipo I y osteonectina fue observado en células cultivadas en γTiAl y Ti-6Al-4V. Los resultados obtenidos sugieren que γTiAl es biocompatible con los osteoblastos.

To my loving husband and wonderful family…

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CHAPTER I

INTRODUCTION

In biomaterials science, biocompatibility refers to the different effects of material on the surrounding tissue, determining the material's ability to be used as implants in the human body. These materials have various applications and can be used as dental and cardiovascular implants like catherers, heart valves and stents (help circulation by maintaining arteries and veins open), and in bone repair and replacement. Metals currently used in medical applications include: stainless steel, Cr-Co alloys, commercially pure (cp) Ti and its alloys.

Titanium and its alloys are very popular biomaterials due to their high strength, low weight and excellent corrosion resistance, but they suffer from low fracture toughness and poor wear properties. Ti-6Al-4V is the most common titanium alloy used in bone repair and replacement. Studies have shown that although Ti-6Al-4V has high strength, low weight and good corrosion resistance it suffers from poor shear strength and can cause seizing because of a high coefficient of friction in both bone-metal and metal-metal interfaces.

There is a continuous search for new materials for use in implants with better biocompatibility properties than the current cp Ti and Ti-6Al-4V alloys. A new Ti alloy, gamma titanium aluminide (γTiAl) originally designed for aerospace applications, has excellent properties compared to Ti-6Al-4V, with possible biological implant applications. This titanium alloy has superior corrosion resistance, high specific strength and rigidity, and lower density than currently used Ti alloys. It has been frequently

reported that titanium oxide debris is formed in Ti implants causing an immunological reaction resulting in loosening of the implants at the metal-bone interface. γTiAl implant may preferentially form aluminum oxide instead of titanium oxide, which will resist wear and loss of particles avoiding the response of immune system. Preliminary studies were performed using γTiAl in *in vivo* models on rats showing cell attachment and bone tissue formation.

The main objective of this research was to study *in vitro* cell attachment and bone tissue formation of human fetal osteoblast cells hFOB 1.19 cultured in the presence of γTiAl with different surface roughness in order to determine its biocompatibility. This was established by detecting the presence of collagen type I and osteonectin, both proteins of the bone extracellular matrix, using Scanning Laser Confocal Microscopy. Cell attachment per se was qualitatively checked using Scanning Electron Microscopy. Ti-6Al–4V was the material selected to compare the results obtained with γTiAl.

CHAPTER II

LITERATURE REVIEW

Bone Composition

The skeletal system is composed of specialized forms of connective tissue, such as cartilage and bone. Cartilage is a strong nonvascular structure that forms the framework that supports certain organs, the articulating surfaces of bones and the greater part of fetal skeleton (1). Cartilage can be found as three types: hyaline, elastic and fibrocartilage. The bone is a hard vascular connective tissue that consists of cells and an extracellular matrix. It has a dense rigid outer shell of compact bone, the cortex, and a central medullary or cancellous zone of thin interconnecting narrow bone trabeculae (2). The extracellular matrix has a wide variety of functions. These functions include support, protection, calcium reservoir and homeostasis.

The cellular component of the bone consists of three kinds of cells: osteoclasts, osteoblasts and osteocytes (3). Osteoclasts are large multinuclear cells derived from monocytes important in bone remodeling and can be found on the surface of the bone. Osteoblasts are cuboidal cells found on the surface of the new bone where they synthesize the bone matrix. As osteoblasts make the bone matrix, they become trapped as the matrix calcifies; these trapped osteoblasts are known as osteocytes (1). Osteocytes are flatenned, discoid cells found inside of the bone and are responsible for bone maintenance. They are interconnected by long branches and can sense pressure or cracks in the bone, directing osteoclasts to dissolve the bone. The cooperation that exists between osteoblasts and osteoclasts is responsible for the formation, remodeling and

repair of a bone, as well as for the long-term maintenance of calcium and phosphate homeostasis of the body (1).

Bone differs from other connective tissues by the mineralization of its extracellular matrix, which is 50% organic and 50% inorganic matter. The organic component of the bone matrix is 90% collagen (type I and to a lesser extent collagen type V). The other 10% of the matrix contains ground substance in the form of glucosaminoglycans (hyaluronic acid, chondroitin sulfate and keratin sulfate), small glycoproteins such as osteocalcin, osteonectin and osteopontin, and several sialoproteins (3). The inorganic component of the bone is mainly formed by calcium hydroxyapatite crystals.

The cells of all the skeletal tissues have close structural and functional relationships and a common origin from primitive mesenchymal cells (2). Mesenchymal connective tissue has vascularized areas (centers of ossification) where the cells differentiate into osteoprogenitor cells, which differentiate into osteoblasts. Osteoblasts are large cells with abundant basophilic cytoplasm, a large Golgi apparatus and a pale stained nucleus with prominent nucleolus. The osteoblasts secrete the collagen and the ground substance that forms the initial unmineralized bone or osteoid. The calcification process is initiated when the osteoblasts secrete the matrix vesicles, which are rich in alkaline phosphatase (3). The newly formed bone is called primary or woven bone, the arrangement of collagen fibers lack the precise orientation present in older bone.

Bone Repair

When a fracture occurs, dense connective tissue and newly formed cartilage cover the fracture site to produce a fibrocartilaginous callus to stabilize and bind the fractured bone. Meanwhile, osteoprogenitor cells differentiate into osteoblast that deposit new bone adjacent to both ends of the fracture site, invading the callus and replacing it with a bony callus. While compact bone is being formed, the bony callus is removed by the action of osteoclasts, and the gradual remodeling restores the bone to its original shape (3). Bone repair can last from 6 to 12 weeks, but the healing process can be accelerated by the use of external materials such as plates and screws.

History of Biomaterial Implants

Dental Implant Studies

Ancient Egyptian and South American civilizations experimented with re-implanting lost teeth with hand-shaped ivory or wood substitutes. In the 18th century lost teeth were sometimes replaced with extracted teeth from other human donors, but the success rate was low due to the strong immune reaction of the receiving individual. In 1809, Maggiolo fabricated a gold implant, and placed it into fresh extraction sockets to which he attached a tooth after a healing period. A physician named Harris attempted the same procedure in 1887 using a platinum post, instead of a gold post. Edmunds was the first in the US to implant a platinum disc into a jawbone, to which a porcelain crown was fixed; this was presented at the First District Dental Society of New York in 1886. Further implantation attempts were made experimenting with different metal alloys and porcelain formulations; however the long-term success rates were very poor.

In early 1900’s a vanadium steel was the first metal alloy developed specifically for human use (4). As soon as the first bone plate implants were introduced, surgeons identified problems with the material and design that resulted in premature loss of implant function, such as mechanical failure, corrosion, and poor biocompatibility. These difficulties still remain the three critical issues in today's biomedical implants and devices. By 1924 a study by Zierald showed that implant materials caused a series of reactions in the tissues surrounding the implant. Iron and steel dissolved rapidly and provoked erosion of the adjacent bone. Copper and nickel embedded in the bones caused substantial discoloration of tissues around the implants. The metals, which did not produce discoloration, like gold, silver, or pure aluminum, were all too soft or weak for most applications.

In 1926 18% chromium, 8% nickel stainless steel was used in surgical applications. This material was more resistant to corrosion in body fluids than the vanadium steel. Later in 1926, 18-8SMo stainless steel, also known as 316 stainless steel, was introduced; it contained a small percentage of molybdenum, improving the corrosion resistance in salt water (4).

In 1937 Strock placed the first successful oral implants at Harvard University and published a paper on the physiological effects of a cobalt-chromium-molybdenum alloy (vitallium) in bone, placing a series of implants into test animals and humans. Samples were immediately implanted after an extraction of a tooth and no post-operative complications or reactions were recorded, showing a remarkable tissue tolerance to the vitallium implants.

In 1947 possible applications for titanium surgical implants were considered, since the pure metal had excellent inertness in the seawater environment, so resistance to corrosion seemed likely in the human environment (4). A few surgical implants were made into human subjects and when removed the excellent corrosion resistance was confirmed. In the 1950's the carbon content of 316 stainless steel was reduced from 0.08% to 0.03% resulting in better corrosion resistance; this metal became known as 316L stainless steel.

In 1952 Per Ingvar Branemark, an orthopedic surgeon heading a Swedish research team in the Laboratory of Vital Microscopy at the University of Lund, Sweden, studied the microscopic healing events in bone of rabbits. Dr. Branemark coined the term osseointegration when he discovered that the titanium metal cylinders that were screwed into the rabbit's thighbone had fused to the bone, thus showing the ability of living tissue to integrate with titanium. Osseointegration has been described as a direct structural and functional connection between living bone and the surface of a load carrying implant.

In 1982, the Toronto Conference on Osseointegration in Clinical Dentistry laid down the first parameters on what is to be considered the successful implant treatment within the stringent confines of the scientific community. This conference also catalyzed the acceptance and use of dental implants in North America.

Hip Implant Studies

In 1890, in Germany, Gluck is said to have performed the very first total hip replacement. His experimental prosthesis consisted of a carved ivory ball and socket, fixed with a glue composed of colophony or rosin, pumice powder, and plaster of Paris.

Due to problems with infections he cautioned others against this type of surgery. For decades surgeons and scientists were unable to find a material biocompatible with the body and that was strong enough to withstand the tremendous forces that could be used to treat arthritis. In 1925, M.N. Smith-Petersen, M.D., a surgeon in Boston, molded a piece of glass into the shape of a hollow hemisphere that could fit over the ball of the hip joint and provide a new smooth surface for movement (5). The glass was biocompatible, but could not withstand the stress of walking. In 1936 scientists manufactured a cobalt-chromium alloy, strong and resistant to corrosion, which was almost immediately applied to orthopedics. In 1938 Wiles (6), in London, first introduced the idea of a total hip prosthesis of stainless steel consisting of a femoral component (secured to the neck of the femur by a bolt) and the acetabular component (anchored to a buttress plate by screws). He inserted the device in six patients with Still's disease. The results of this study were reported in 1950; loosening and breakage of both the bolt and the screws that held the components in place had been observed. Frederick R. Thompson of New York, and Austin T. Moore of South Carolina, separately developed replacements for the entire ball of the hip. These implants only addressed the problem of the arthritic femoral head (the ball), while the diseased acetabulum (hip socket) was not replaced (5). While very popular in the 1950's, results remained unpredictable and arthritic destruction of the socket persisted, patients developed pain due to the loosening of the implant. By 1938, Dr. Jean Judet and his brother, Dr. Robert Judet, of Paris, attempted to use an acrylic material to replace arthritic hip surfaces, but in the end it came loose. Their idea served as the basis for Dr. Edwarc J. Haboush from the Hospital for Joint Diseases in New York City, where he utilized a "fast setting dental acrylic" to actually glue the prothesis to the

bone.

In England John Charnley pursued effective methods of replacing both the femoral head and acetabulum of the hip. In 1958, he replaced the eroded arthritic socket with a Teflon implant which did not work; he then tried polyethylene and obtained great results. To fix the polyethylene socket and the femoral implant to the bone, he used polymehtylmethacrylate, also known as bone cement, to firmly secure the artificial joint to the bone. By 1961, Charnley was performing the surgery regularly with good results.

Today over 100,000 hip replacements are performed annually in the United States using the principles of a low friction arthroplasty (surgical replacement of a joint) with a polyethylene socket and metal femoral prosthesis. Since the cement fixation breaks down over time, implants with textured surfaces have been developed to allow bone to grow into them (5). These have been used experimentally in animals and are now being used in humans.

Knee Implant Studies

The first attempt at total knee arthroplasty was a prosthesis with a hinge fixed to the bones with stems into the medullary canals (the hollow marrow cavity). The hinges provided good short-term pain relief but limited motion, and after a few years the prosthesis showed severe problems with loosening and infection. During this time, surgeons were trying to treat arthritis by placing a metal spacer between the bones of the knee to eliminate the rubbing of irregular surfaces on each other. McKeever (1957) and MacIntosh (1958, 1964), achieved some success but these were not predictable, and many patients continued with significant symptoms. Surgeons at Massachusetts General

Hospital made a prosthesis in the shape of the femoral half of the knee joint, which helped relieve symptoms but the results were also not predictable nor always lasting.

In 1968, a Canadian orthopedist, Frank Gunston, developed a metal on a plastic knee replacement secured to the bone with cement, which was the first metal and plastic knee and the first implant with cement fixation (5). In 1972, an Englishman, John Insall, M.D., designed a prosthesis made of three components which would resurface all three surfaces of the knee: the femur, the tibia and the patella (kneecap). This became the prototype for current total knee replacements. Each surface was fixed with bone cement and the results were outstanding.

Approximately 150,000 knee replacements are performed annually in the United States. The goal is to achieve greater knee motion and strength while improving patient function. Cementless fixation using a prosthesis with a textured, porous surface into which bone can grow is currently being used in patients with promising results (5).

Implant Materials

Biomaterials are used to repair, assist or replace living tissue or organs that are functioning below an acceptable level. Biomedical implant reliability depends on the corrosion, wear and fatigue resistance of the materials used to make the implant. The most important characteristics of implant metals are biocompatibility, strength (yield, tensile and fatigue), and corrosion resistance. The material should not be toxic, cause an allergic response when placed *in vivo*, cause changes in plasma proteins or enzymes, cause an immunological reaction nor have carcinogenic, teratogenic or mutagenic effects. Since the early 1900s, metal alloys have been developed for these applications providing

improved physical and chemical properties, such as strength, durability and corrosion resistance. Biomaterials include a wide variety of materials, such as ceramics, polymers, and metals (7).

Ceramics

Ceramics are inorganic, nonmetallic compounds with great strength and stiffness, resistance to corrosion and wear, and low density which makes them candidates for a wide range of biomedical applications. Ceramics can be used as implants in dentistry such as crowns and dentures. The orthopedic field utilizes ceramics for joint and bone segment replacement and temporary bone repair devices. Ceramics are also used as coatings for implants made of other materials to provide a biocompatible interface between the tissue and the implant. An important use for ceramics has been as pressure sensors in ultrasounds and MRIs (8).

Polymers

Polymers have been used in the augmentation and repair of the human body with great success (8). There is a wide range and sheer number of polymers used as biomaterials. The appropriate polymer should be chosen based on the body and tissue reactions, the mechanical and thermal properties and its synthesis. Typical polymers and biomedical applications include:

* Polymethylmethacrylate (PMMA): bone cement, contact lenses
* Polytetrafluoroethylene (PTFE): artificial vasculature
* Polyurethane: facial protheses, blood/device interfaces

* Polyvinylchloride (PVC): blood vessels, gastrointestinal grafts, heart components
* Polydimethylsiloxane (PDMS): ear/ear parts, heart components, bones and joints.
* Polyesters: lungs, kidneys, livers, blood vessels.
* Nylons: joints, blood vessels, kidney dialysis.

Metals

There are three major classes of metals used in orthopedics today: stainless steel, cobalt-chromium alloys and titanium (as alloys and commercially pure). In addition, dental casting alloys are based on precious metals (gold, platinum, palladium or silver), nickel and copper and may contain smaller amounts of many other elements, added to improve the alloys' properties (Table 1). Orthopedic applications of metal alloys include arthroplasty, osteosynthesis and in spinal and maxillofacial devices. Metallic alloys are also used for components of prosthetic heart valve replacements, pacemaker casings and leads. Small metallic parts may be used in a wide range of other implants, including skin and wound staples, vascular endoprostheses, filters and occluders. Dental applications of metals and alloys include fillings, prosthetic devices (crowns, bridges, and removable prostheses), dental implants and orthodontic appliances.

Element	Composition (% Weight)					
	Stainless Steel F55 or F56 Wrought	**Stainless Steel A296** Cast	**Co-Cr F75** Cast	**Co-Cr F90** (Vitallium) Wrought	**Titanium pure F67** Cast/ Wrought	**Ti-6Al-4V F136** Cast/ Wrought
W				14 - 16		
Co			57.4 - 65	46 - 53		
Cr	17 - 20	16 – 18	27 - 30	19 - 21		
Ni	10 – 14	10 – 14	2.5 max	9 - 11		
Mo	2 – 4	2 – 3	5 – 7			
Fe	59 – 70	62 – 72	0.75 max	3.0 max	0.5 max	0.25 max
C	0.03 max	0.06 max	0.35 max	0.05 – 0.15	0.10 max	0.08 max
Al						5.5 – 6.5
V						3.5 – 4.5
Ti					99+	88.5 - 92
Mn	2.0 max	2.0 max	1.0 max	2.0 max		
P	0.03 max	0.045 max				
S	0.03 max	0.03 max				
Si	0.75 max	1.0 max	1.0 max	1.0 max		
O					0.45 max	0.13 max
N					0.07 max	0.05 max
H					0.015 max	0.015 max

Table 1. Composition of different metals used as surgical implant materials. [Adapted from Black, J. (9)]

In order for these materials to perform successfully, they must have physical properties that allow the material to perform the function for which it was implanted, and the material must be biocompatible or unable to affect adversely the physiological environment. The biomaterial must be accepted by the organism without causing negative response, must not be toxic or carcinogenic, must be chemically stable, and resistant to corrosion and fatigue. The corrosion resistance of metals and alloys is a basic property related to the easiness with which these materials react with a given environment.

Of the three families of metal alloys used today, stainless steel alloys 316 and 316L are probably the oldest. Stainless steel is easily machined, has a low content of impurities, and a high strength and ductility. Because of the femoral component fractures with the early designs, stainless steel is no longer routinely used. From the standpoint of erosion, biocompatability, and fatigue life, stainless steel is inferior to other super alloys (4). Stainless steels may corrode inside the body under certain circumstances, such as a highly stressed and oxygen depleted region, making it suitable for use only in temporary implant devices, such as fracture plates, screws and hip nails (5).

There are two types of cobalt chromium alloys; CoCrMo or vitalium (used in dentistry and in making artificial joints) and CoNiCrMo (used for making the stems of prosthesis for heavily loaded joints such as the knee and hip). They have a higher corrosion resistance than the iron-based alloys, and are resistant to fatigue and to cracking caused by corrosion, yet have failed because of fatigue fracture. The abrasive wear properties of the CoNiCrMo alloy are similar to the CoCrMo alloy and both have excellent corrosion resistance (5). The superior fatigue and ultimate tensile strength of the

wrought CoNiCrMo alloy make it suitable for the applications that require long service without fracture or stress fatigue (4).

Titanium alloys are the newest family of orthopedic alloys. They are required to be comprised of at least the following elements, titanium, aluminum, and vanadium, for approval by the FDA. Not only are the titanium alloys the newest, but they are also rapidly becoming the most widely used alloys in implantable devices today. Titanium and its alloys are very popular biomaterials due to their high strength, low weight and excellent corrosion resistance, but they suffer from low fracture toughness and poor wear properties (10). Their excellent corrosion resistance is due to the formation of a solid oxide film on the surface that passivates the material (11). Titanium alloys have a strong affinity for oxygen promoting the formation of a stable and tightly adherent protective oxide layer on their surface. Since this oxide layer is in direct contact with biological tissue, the chemical composition and stability of this surface oxide layer is important and will affect implant success. Some disadvantages of titanium alloys are their low fracture toughness, poor wear properties and high coefficient of friction.

The most common titanium alloys used in bone repair and replacement are Ti-6Al-4V and commercially pure Titanium (cpTi). Studies have shown that although Ti-6Al-4V has high strength, low weight and good corrosion resistance, it suffers from poor shear strength and can cause seizing because of a high coefficient of friction in both bone-metal and metal-metal interfaces (12).

Despite the emergence of many new high-tech polymers used in implants, the orthopedic metal industry has continued to flourish with the creation of stronger and more durable alloys, which are less likely to be rejected by the body once implanted.

Adverse Effects of Metal Implants

A major concern with all joint replacements is the degradation of the metals used (13). In the biomedical field there is a concern about the possible propensity for cobalt-chromium to cause cancer. Cobalt-chromium consists of the elements cobalt, chromium, nickel and molybdenum. There is a concern that the corrosion of cobalt-chrome in the wet, salty surroundings of the human body may be sending toxins streaming into the body, possibly causing cancerous tumors (14). Even though only fifteen tumors have ever been reported at the site of an implant, many more could exist and go unreported (partially due to the age of most patients). Although these concerns have met some strong opposition in the industry, many companies are pushing towards safer materials. Such materials include titanium, inert fiber-reinforced composites, and ceramics. Studies involving titanium have illustrated that this material is generally well tolerated in the body.

Some other effects of corrosion exist in the implant materials. Skin conditions such as dermatitis have been reported from exposure to nickel. Cobalt shows signs of causing anemia by inhibiting iron from being absorbed into the blood stream. Ulcers and central nervous system disturbances have been detected as a result of chromium. Aluminum present in some implant materials may cause epileptic effects and Alzheimer's disease. Most of these side effects were reported from testing done outside the body in a site different from the implant (4), illustrating the possible hazards associated with the corrosion of implant materials inside the body.

These problems are well illustrated by the problems faced by the Bone Tumour service at the Royal National Hospital. As a result of the large amount of bone being

excised during tumour surgery, many, if not most patients, require massive prosthesis implants. Of particular concern with massive prosthesis is the wear and release of metallic ions from titanium alloy. The reason for this is that the shaft of the prosthesis and the intra medullary stem are made from titanium alloy, which can be worn either by rotation of the stem in the cement mantle or by soft tissue rubbing the part that replaces the bone (15). This leads to the generation of fine wear debris. This debris, which in part is derived from the titanium oxide layer, which develops on the alloy surface or is composed of small titanium alloy particles, discolors the tissues such that on revision of massive prosthesis the interface around the shaft is often discolored (16).

Titanium Implant Studies

Implant surface characteristics will determine the initial events on the bone–implant interface and its possibility for success. There are a great variety of parameters of toxicity, such as cell death, reduced cell adhesion, altered cell morphology, reduced cell proliferation and biosynthetic activity that give warnings regarding material toxicity (17). Bone remodeling and mineralization processes at the interface are fundamental elements of a good implant-bone fixation and therefore of the success of a prosthesis (18). In the case of unsuccessful implants nonmineralized connective tissue forms at the interface of the implant leading to loosening. In a successful implant, osseointegration occurs and the implant is directly connected to living bone.

Ti-6Al-4V and cpTi are among the most common used implant materials. Titanium is a bioinert and biocompatible metal, which induces bone formation on its surface after implantation. Their passive surfaces promote high stability and high *in vitro*

corrosion resistance. Poor wear resistance can induce a continuous release of metal debris or corrosion products that can accumulate or migrate. Dissolution of titanium into the human body can induce the release of osteolytic cytokines involved in implant loosening.

Interactions in the bone-implant interface are critical to the success or failure of an implant (19, 20). Mesenchymal cells have been shown to be capable of differentiating into fibrolast, chodroncytes or osteoblasts based upon the concentrations and presence of local factors (21, 22). In areas of high oxygen tension, osteogenesis is favored, whereas in areas of low oxygen tension, chondrogenesis is favored (23). Environmental factors at implant site will determine cell phenotypic expression, therefore influencing implant stability. *In vitro* and *in vivo* studies have confirmed the presence of an interfacial zone comprised of noncollagenous, proteoglycan containing, cement like material, between the metal substrate and the mineralized extracellular matrix produced by osteoblast-like cells in culture (24-26). The presence of mineralized bone deposited directly on the metal surface, and lined on the external side by a rim of osteoblast secreting bone matrix, suggest that early direct bone growth on the implant surface could improve the implant anchorage in bone (27). The quality and intensity of bone cell response to a Ti implant appears to depend on factors linked to the nature of the material and on the ability of bone cells to interact with the implant surface and microenvironment (7).

Bone cells are sensitive to material morphology, using this characteristic for orientation and migration, thus affecting the healing process and the success of the implant. Surface properties of the implant that influence the behavior of cells of mesenchymal origin such as osteoblasts, can be grouped into four interrelated categories: composition, surface energy, topography, and surface roughness (28). Topographic

features with dimensions around the cell size exhibit strong effects on cell guidance and shape regulation (29). Surface roughness affects chondrocytes and osteoblast proliferation, differentiation, and matrix synthesis *in vitro* (20). Bone-like tissue can be grown, in culture, directly on solid discs of commercially pure titanium, and the mineralized tissue formed in culture obviously follows the contours on the surface of the metal disc created by the 600-grit surfacing treatment (24). Also, to achieve firmer and earlier direct implant fixation to the bone, several methods of modifying the implant surface, such as porous coatings and calcium phosphate ceramic coating, have been tried (30). Although these coatings can present satisfactory clinical results, some problems have been recognized: decreased fatigue strength of porous- coated substrates; increased release of harmful metal ions from the large surface areas of porous coatings; separation of the coatings; dissolution and resorption of the coating; migration of wear and separated particles from coatings into the joint space, accelerating articular surface wear (30, 31). Other methods for creating a rough implant surface include polishing the surface using grit or diamond paste, and blasting it with a pressurized spray of particles, such as aluminum oxide (Al_2O_3). While coating methods are an addition technique, surface-blasting is a subtraction technique that can create a more uniform and controlled surface roughness (32), without the coating complications.

Several studies have been conducted using titanium implants of both cpTi and Ti alloys with different roughness in order to determine if surface properties affects expression of a differentiated cell phenotype and integration of implant in human body with variable results. Nevertheless, no consensus has been reached about the optimal surface roughness of bone implants.

In vitro experiments by Keller et al (33) using cp Ti and Ti-6Al-4V with grooved (600 grit paper), rough (sandblasted) or smooth (polished with 1μ diamond paste) surfaces showed no significant differences between both metal specimens, but the highest level of attachment was observed for the rough surface, in agreement with other investigators (34). Osteoblastic alkaline phosphatase activity is a recognized parameter of cellular differentiation on the implant surface (35), as well as extracellular matrix protein synthesis. Rough titanium surfaces increased alkaline phosphatase specific activity and calcification in cultures of embryonic chick osteoblasts (36). Human osteoblast-like MG-63 cells grown on Ti disks with different surface roughness exhibited differential responses with respect to cell morphology, proliferation, alkaline phosphatase activity, RNA synthesis, and protein and proteoglycan production (37). Cells cultured on rougher surfaces tend to exhibit attributes of more differentiated osteoblasts than did those cells cultured on smoother surfaces for comparable periods of time, including reduced cell numbers and increased alkaline phosphatase-specific activity (38). Also several investigators have concluded that a rough implant surface increases both apposition and interfacial strength *in vivo* (30, 31, 39-45), since the percentage of direct bone-implant contact is positively correlated with the increasing roughness of the implant surface (43).

Experiments using osteoblast-like cells cultured on titanium plates with three different surface roughness showed that roughness of titanium surfaces affects neither the proliferation of these cells nor the bone growth (46). This agrees with other investigators who have concluded that there is no marked difference in bone reaction to the different implant material and the roughness of the surface was found to have no effect on implant success (47, 48).

Studies using osteoblast-like cells, SaOS-2 have concluded that cell proliferation is higher on the smooth surfaces, while synthesis of extracellular matrix proteins was more abundant on rougher surfaces (49). While other studies have indicated no negative effects on the rate of bone growth using different surface roughness, they observed a different bone growth pattern and higher osteoconductivity as a result of the higher surface roughness (50). Cell morphology studies of human osteoblasts on stainless steel, cobalt chromium alloy, cpTi, Ti-6Al-4V, and Ti6Al7Nb showed that cells attached and grew on all test substrates in a time-dependent manner, without signs of disturbing influence from any of the materials (51). On the smooth surfaces the cells showed a flattened fibroblast-like morphology while on titanium alloys with a rough, sandblasted surface a three-dimensional growth was induced (51).

Other studies have found that a significant correlation is established between surface roughness and cell growth, the smoother the surface the better osteoblasts proliferation (52). Also investigators have stated that the lower the surface roughness the better the osteointegration (18). Anselme et al. (53, 54) observed lower proliferation and adhesion on rough surfaces compared to smooth ones with cells more spread on smooth surfaces than on rough ones, contrary to previous reports on *in vitro* tests (33, 34, 55-57).

Surface roughness has been found to induce the release of growth factors and cytokines in the adhering osteoblasts, active on cell growth and phenotype differentiation (7). Kieswetter et al (58) demonstrated that roughness modulates cytokine and growth factor production by MG63 osteoblast-like cells. Surface roughness in Ti has a profound effect on the profile of genes expressed by bone cells and suggest that improvements in the biological activity and possibly the clinical efficacy of these materials could be

achieved by selective regulation of gene expression mediated via modification of surface roughness (59). Study of gene expression of the transcription factor Cbfa1 and BSPII of osteoblast cells cultured on cpTi implant discs with grooved (treated with 600 grit) and roughened (sandblasted) surfaces suggested that osteoblast gene expression and mineralization were affected by roughened implant surface microtopographies during osseointegration of dental implants (60).

In addition to the effect of surface topography of the implant material, its surface chemistry plays a significant role in implant success. Implant surface can be treated using different components, such as hydroxyapatite that provides a coat on the surface allowing an increase in cell attachment. Studies of Ti-6Al-4V with a coat of fluorohydroxyapatite with two different roughness showed variable results: some showed excellent cell attachment and growth in the two test materials, irrespective of the type of surface roughness (61), while others observed inhibition in bone mineralization due to chemical changes of material during manufacturing process (18). Lavos-Valereto et al (62) performed *in vitro* and *in vivo* studies using coated and uncoated samples of Ti-6Al-7Nb, another Ti alloy, and found high osseointegration for both surfaces. The use of TiO_2 particles as blasting material will increase roughness without adding foreign elements to the surface. The use of other blasting material, such as $Al_2O_{3,}$ represents a potential risk of dissolution of aluminum ions into host tissue and inhibition of bone mineralization, while it may also change the materials biocompatibility. A study by Wennerberg (45) compares the effects of surface roughness and the biological outcome of both blasting materials *in vivo*. This study found better bone fixation for a rougher surface compared

to a smoother one, but no difference between TiO_2 blasted and Al_2O_3 blasted implants. These results sustained his previous findings (63).

Gamma Titanium Aluminide

There is a continuous search for new materials for use in implants with better biocompatibility and/ or physical properties than the current cp Ti and Ti-6Al-4V alloys. A new Ti alloy, gamma titanium aluminide (γTiAl) originally designed for aerospace and automotive applications (64), has excellent properties compared to Ti-6Al-4V, with possible biological implant applications. Common compositions of this alloy are Ti-48Al-2Cr-2Nb and Ti-46.5Al-4(Cr-Nb-Ta)-0.1B (64). Gamma titanium aluminide is available as sheets or rods under different commercial names. This titanium alloy has superior corrosion resistance, high specific strength and rigidity, and lower density (3.7 gm/cc) than currently used Ti alloys (4.2 gm/cc). It has been frequently reported that titanium oxide debris is formed in Ti implants causing an immunological reaction resulting in loosening of the implants at the metal-bone interface (65). γTiAl implant may preferentially form aluminum oxide, which will resist wear and loss of particles avoiding the response of immune system.

Titanium oxide debris is formed as a result in fretting at the femoral head/acetabulum interface in Ti alloy implants causing an immunological reaction resulting in loosening of the implants at the bone-metal interface. The formation of aluminium oxide in γTiAl (which is harder and more stable than titanium oxide) is likely to eliminate or reduce the immunological reaction, thus reducing the probabilities of implant loosening. Also, some studies have shown that Ti-6Al-4V implants could

possibly release of vanadium, considered as a toxic element (66), which may give rise to biocompatibility problems, altering the stability of this alloy and its viability as a biomaterial (13, 67). The use of γTiAl as a substitute for Ti-6Al-4V will eliminate the presence of vanadium, thus reducing its possible effects in the bone-implant interface. Preliminary studies were performed using γTiAl implants in *in vivo* models in rats with cell attachment and bone tissue formation, demonstrating a favorable tissue response and its potential to be used as implant material (68). A study evaluating the corrosion resistance of γTiAl in a body fluid environment which has been performed in order to verify its potential as a biomaterial shows that γTiAl exhibits a corrosion resistance similar toTi-6Al-4V (69).

CHAPTER III

MATERIALS AND METHODS

Cell line

Human osteoblast cell line hFOB 1.19 (CRL-11372) (ATCC, Manassas, Virginia) was cultured in 90% Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham (DMEM) (Sigma-Aldrich, St. Louis, Missouri) with 2.5 mM L-Glutamine and 15mM Hepes, without phenol red, suplemented with 0.3 mg/mL G418 (Calbiochem, San Diego, California) and 10% Fetal Bovine Serum (FBS) (Hyclone, Logan, Utah). Cells were grown in 25 cm^2 plastic culture flasks (Corning, Corning, New York) and incubated at 33 ºC until confluence. At confluence, cells were washed three times with phosphate buffered saline (PBS) (137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO4, 1.4mM KH_2HPO_4) and harvested using trypsin-EDTA (Gibco, Gaithersburg, Maryland) at 37 °C for 5 min. Cells were pelleted by low-speed centrifugation (3300 rpm) for 7 minutes, and subcultured in a 1:3 ratio. Cells were stored in liquid nitrogen in 72% culture medium, 20% FBS and 8% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, Missouri).

Titanium alloy disks

γTiAl and Ti-6Al-4V disks were prepared with three different surface roughness: polished with 600 grit papers (roughest surface), 3 micron (3μ) diamond paste and Mastermet® (Buehler, Lake Bluff, Illinois) (smoothest surfaces). Disks were 1 cm in diameter and 0.1 cm in height. Polishing was performed mechanically and manually to induce parallel orientation of residual grooves. Metal disks were cleaned using deonized

water, absolute alcohol and again deonized water. Titanium disks were sterilized by autoclave at 5 psi and 121 ºC and placed in 35 mm tissue culture plates (Corning, Corning, New York).

Adhesion tests

Cells were cultured on γTiAl and Ti-6Al-4V disks at a density of 1 x 10^4 to 4 x 10^5 cells per mL. Cell behavior and growth were studied for three different surface roughness of both γTiAl and Ti-6Al-4V; grit 600 polished, 3μ diamond paste and Mastermet®. Cell cultures were observed under light microscope in order to study cell growth and appearance. For the Scanning Electron Microscopy analysis (Table 2), samples were incubated for 21 days at 37 ºC using a cell growth control (cells growing on a glass coverslip), negative control group (metal disks incubated with culture media but without cells), a positive control group (cells cultured on Ti-6Al-4V), and the experimental group (cells cultured on γTiAl). For immunofluorescent labeling analysis samples were divided into four different groups (Table 3): Group I was incubated for 7 days at 37 ºC, Group II was incubated for 14 days at 37 ºC, Group III was incubated for 21 days at 37 ºC and Group IV was incubated for 28 days at 37 ºC. For each experimental group a cell growth control, a negative control and a positive control were used.

Incubation Period	Sample	Culture Surface
21 days at 37 ºC	Cell Growth Control	Glass Coverslip *
	γTiAl	600 grit
		3μ Diamond
		Mastermet
	Ti-6Al-4V	600 grit
		3μ Diamond
		Mastermet

* Cultured with cells only

Table 2. Sample preparation of control and, γTiAl and Ti-6Al-4V metal disks with different surface roughness for scanning electron microscopy analysis. Samples of each metal were cultured in the presence and absence of hFOB 1.19 osteoblast cells in order to study cell attachment and behavior.

Group	Sample	Culture Surface
I **7 days at 37 ºC**	Cell Growth Control	Glass Coverslip*
	γTiAl	600 grit
		3μ Diamond
		Mastermet
	Ti-6Al-4V	600 grit
		3μ Diamond
		Mastermet
II **14 Days at 37 ºC**	Cell Growth Control	Glass Coverslip *
	γTiAl	600 grit
		3μ Diamond
		Mastermet
	Ti-6Al-4V	600 grit
		3μ Diamond
		Mastermet
III **21 days at 37 ºC**	Cell Growth Control	Glass Coverslip *
	γTiAl	600 grit
		3μ Diamond
		Mastermet
	Ti-6Al-4V	600 grit
		3μ Diamond
		Mastermet
IV **28 days at 37 ºC**	Cell Growth Control	Glass Coverslip *
	γTiAl	600 grit
		3μ Diamond
		Mastermet
	Ti-6Al-4V	600 grit
		3μ Diamond
		Mastermet

* Cultured with cells only

Table 3. Sample preparation of control and, γTiAl and Ti-6Al-4V metal disks for immunofluorescent labeling. Samples of each metal were cultured in the presence and absence of hFOB 1.19 osteolast cells in order to study the expression of collagen type I and osteonectin, both proteins of the bone extracellular matrix.

Scanning Electron Microscopy

Samples were washed carefully with PBS and fixed overnight in 4 % glutaraldehyde buffered in PBS at 4 ºC. After washing three times with PBS the samples were dehydrated in graded alcohol ranging from 10% to 100% ethanol for 10 min each. Ethanol 100% solution was changed three times every 10 min. After critical point drying (EMS 850) (Electron Microscopic Science, Washington) samples were mounted on stubs and were sputtered coated with gold in EMS 550X (Electron Microscopic Science, Washington). Samples were then examined with a JEOL JSM-5410 LV SEM (JEOL, Japan) at 10 KV using variable magnification (75, 150, 500, 1500 and 5000X).

Immunofluorescent labeling

I. Fluorescein and Rhodamine labeling

Cells were fixed using 3.7% formaldehyde in PBS at room temperature for 10 min and washed twice with PBS. Cells were permeabilized and non-specific staining was blocked by incubation in blocking solution [0.1% BSA, 0.05% saponin, 5% normal goat serum (NGS) in PBS] for 30 min at room temperature. Blocking solution was removed and 40 μL per sample of primary antibody diluted in blocking solution was added. Primary antibodies used were monoclonal anti-human collagen type I (mouse) and polyclonal anti-human osteonectin (rabbit), both from Calbiochem (San Diego, California). After 1.5 to 2 hours incubation at room temperature, samples were washed three times with blocking solution for 15 min each and followed by the addition of 40 μL of secondary antibody diluted 1:100 in blocking solution. Secondary antibodies used were anti-mouse IgG (goat) with Rhodamine conjugate and anti-rabbit IgG (goat) with

Fluorescein conjugate (FITC), both from Calbiochem (San Diego, California). Samples were incubated in the dark for 1 hr at room temperature and washed three times with PBS in the dark for 15 min each (Figure 1). Samples were mounted in 20 x 40 mm coverslips with Fluorescent Mounting Media (Oncogene, San Diego, California) and stored at 4 ºC in the dark. Samples were observed using a Confocal Laser Scanning Microscope (Fluo View™ 300 Confocal Microscope) (Olympus, USA).

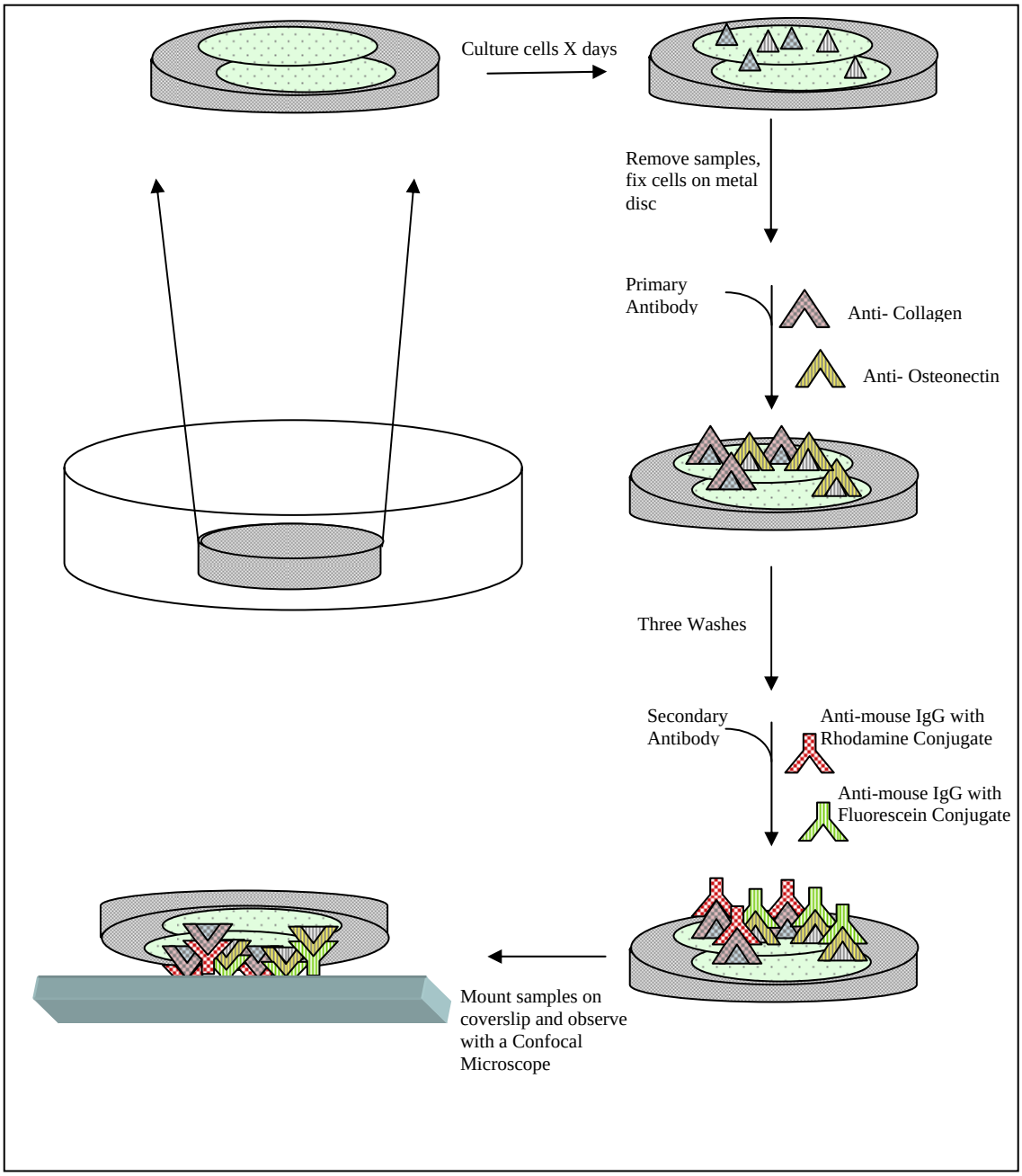


Figure 1. Immunofluorescent labeling assay for the detection of the proteins collagen type I and osteonectin from hFOB 1.19 osteoblast cells cultured on γTiAl and Ti-6Al-4V metal disks.

II. Cy5 and Rhodamine labeling

Cells were fixed using 3.7% formaldehyde in PBS at RT for 10 min and washed twice with PBS. Immunofluorescent labeling analysis was performed in order to detect the presence of collagen type I and osteonectin in samples cultured at 37 ºC for 7, 14, 21 and 28 days and observed with a Confocal Laser Scanning Microscope (Fluo View™ 300 Confocal Microscope) (Olympus, USA). Cells were permeabilized and non-specific staining was blocked by incubation in blocking solution for 30 min at room temperature. Blocking solution was removed and 40 μL per sample of primary antibody monoclonal anti-human collagen type I (mouse) (Calbiochem, San Diego, California) diluted in blocking solution was added. After 1.5 to 2 hours incubation at room temperature, samples were washed three times with blocking solution for 15 min each and followed by the addition of 40 μL of secondary antibody anti-mouse IgG (goat) with Rhodamine (Calbiochem, San Diego, California) diluted 1:100 in blocking solution. Samples were incubated in the dark for 1 hr at room temperature and washed three times with blocking solution in the dark for 15 min each. A second primary antibody, monoclonal anti-osteonectin (mouse) (Zymed Laboratories, San Francisco, California) was diluted and 40 μL of dilution was added to each sample. The samples were incubated 1.5 to 2 hours in the dark. The samples were washed three times with blocking solution for 15 min each in the dark and 40 μL of secondary antibody anti-mouse IgG with Cy5 conjugate (Zymed Laboratories, San Francisco, California) were added. After 1 hour incubation, the samples were washed three times with PBS, mounted in 20 x 40 mm coverslips with Fluorescent Mounting Media (Oncogene, San Diego, California) and stored at 4 ºC in dark. Samples were observed using the Confocal Laser Scanning Microscope.

CHAPTER IV

RESULTS AND DISCUSSION

Cell culture

The hFOB 1.19 cells were cultured until they reached confluency, and were subcultured and preserved for future applications. Osteoblast cells are one of the primary cells involved in osseointegration, having a direct impact in bone-implant interface. Cell attachment, proliferation and differentiation of bone cells in the presence of implant material can be achieved with various culture systems that include primary culture systems, nontransformed cloned cell lines, osteosarcoma cell lines and immortalized cell lines. The advantage of cell line systems over primary cell culture systems is their reproducibility; the disadvantage, however, is that it is not a natural biosystem (35). Human osteoblast cell line hFOB 1.19 (ATCC, Manassas, Virginia) express a temperature sensitive mutant of simian virus 40 (SV40) large T antigen (40). Activation of the large T antigen occurs at a temperature of 37 °C or less, preferably around 33 ºC to 36 °C, and results in the rapid cell division of human fetal osteoblastic cells. Increasing the temperature of incubation of the cell culture above 37 °C results in the inactivation of the large T antigen and little or no cell division. Differentiation occurs at elevated temperatures (Table 4). The use of an established cell line, such as like hFOB 1.19 is better than the use of a transformed osteosarcoma or cells obtained directly from tissue in that these cells have the ability to differentiate into mature osteoblasts expressing the normal phenotype (70) and can be subcultured for a long period of time since they are immortalized. hFOB 1.19 cells appear to be relatively undifferentiated cells programmed

to differentiate upon reaching confluence into cells that possess the full spectrum of osteoblast-associated features (40).

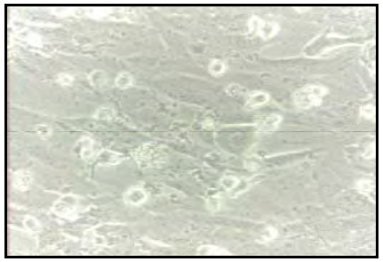
	Temperature	
	33 °C	**37 °C**
Proliferation	++	
Alkaline Phosphatase	+	++
	Cell Density	
	Subconfluent	**Confluent**
Proliferation	++	+-
Alkaline Phosphatase	+	++
Mineralization/ Nodule Formation		++
Matrix Production		++

Table 4. Effects of temperature and cell density on hFOB 1.19 cell functions. Adapted from Harris (71).

Adhesion tests

After the incubation periods described for each group of experimental samples, cells were observed under the light microscope. Since light is not transmitted through the metal disc, only the appearance of the cells surrounding the metals disks was observed. Cell growth in the presence of γTiAl appeared the same as the cells growing on the coverslips (control). Osteoblast cells grew evenly on the surface of the culture plates and touched the sides of the metal disks. Cells appeared to be attached directly to the metal, with cells using the sides of the metals disks as growing surfaces for both γTiAl and Ti-6Al-4V (Figure 2). The presence of an inhibition halo between the metal disks and the cells was not observed. An inhibition halo is characteristic of the presence of a toxic substance in the cell culture. These results lead us to speculate that γTiAl is not toxic and toxic particulate is not liberated from its surface when used for cell culture. Cells cultured in the presence of γTiAl had the same appearance as cells cultured in the presence of the currently used implant material Ti-6Al-4V. A better comparison of cell behavior and attachment in the presence of both metals can be further obtained after Scanning Electron Microscopy and Immunofluorescent labeling analysis.

Control- Cells on Coverslip



Surface Treatment

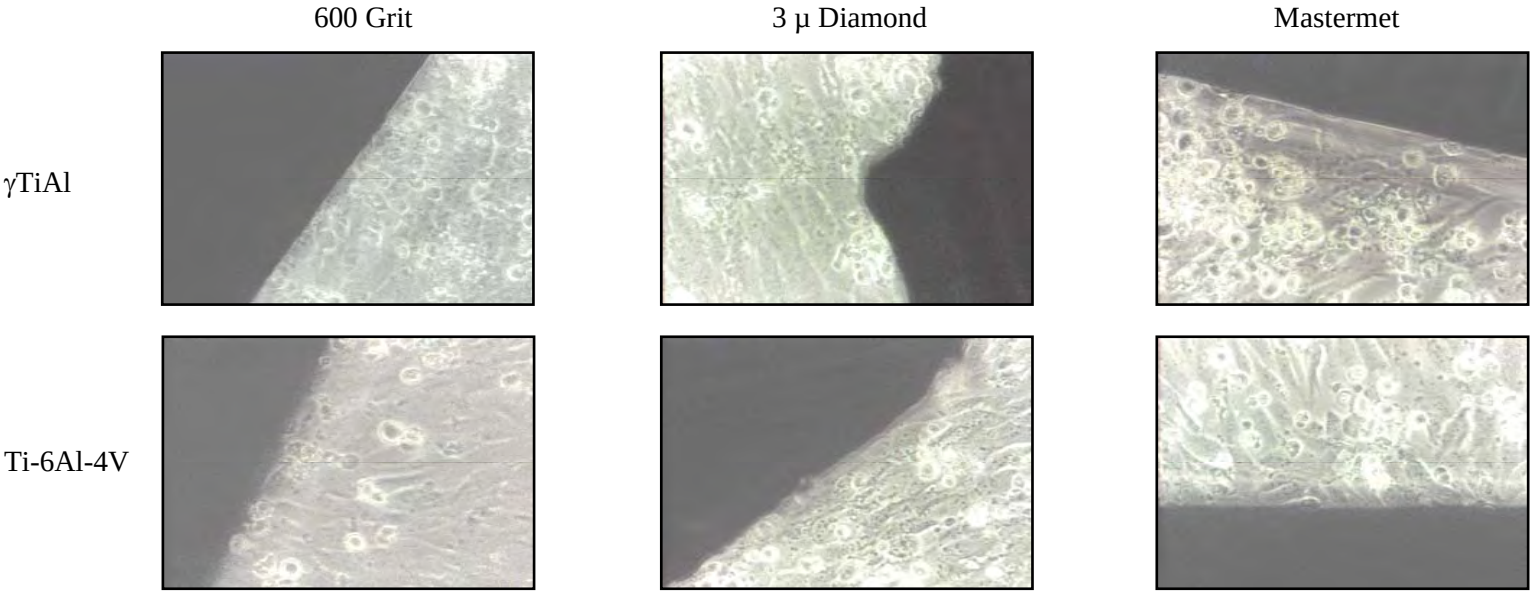


Figure 2. Light microscopy study of osteoblast cells hFOB 1.19 cultured at 37 °C for 21 days. In all metal surfaces the cells appeared to be growing evenly touching the sides of the metal disks (black region).

Scanning Electron Microscopy analysis

Differences in cell attachment and behavior were observed for hFOB 1.19 cells cultured on γTiAl and Ti-6Al-4V. Scanning electron microscopy analysis showed that hFOB 1.19 cells grew on the surface of both γTiAl and Ti-6Al-4V disks. The cell growth control sample allowed us to assay for cell confluence, normal growth, and attachment under *in vitro* conditions [Figure 3 (A and B)]. The polishing marks are evident on the surface of the negative control metal, which confirms the rough and smooth surfaces of the metals used. The presence of parallel grooves on the negative controls are more evident in samples polished with 600 grit of both γTiAl and Ti-6Al-4V [Figure 3 (C, D, E and F)] confirming the presence of a rough surface, while samples polished with 3μ diamond paste and Mastermet have a smoother appearance. Scanning electron microscopy of the negative control samples demonstrates that the presence of cell culture media and serum does not cause deposition of mineral or salts on the metal surfaces which could alter surface topography, therefore allowing us to make a comparison between the surface of each metal implant and the cell layer appearance.

There is an evident variation in osteoblasts cell appearance when cultured on the roughest to the smoothest surface for both metal surfaces. Cells cultured on the smoothest surfaces of both γTiAl and Ti-6Al-4V [Figure 3 (I, J, M, N, U, V, Y and Z)] have a similar, if not identical appearance to that of cells grown on coverslips (control) [Figure 3 (A and B)] forming a continuous and confluent cell layer. Thickness of the cell layer or the presence of a multilayer is expected, although it can not be observed, since cell growth is continuous and smooth. Cells cultured on the roughest surface also achieved confluency, but the appearance of the cell layer was different from that of the control.

Due to the topography of the metal surface, cells formed multiple growth layers. The presence of multiple cell layers is evident for hFOB cells cultured on samples polished with 600 grit for both γTiAl [Figure 3 (E and F)] and Ti-6Al-4V [Figure 3 (Q and R)]. In general, cell layer on the more polished surfaces had a smoother appearance, while cell layers grown on the roughest surfaces have a rough appearance.

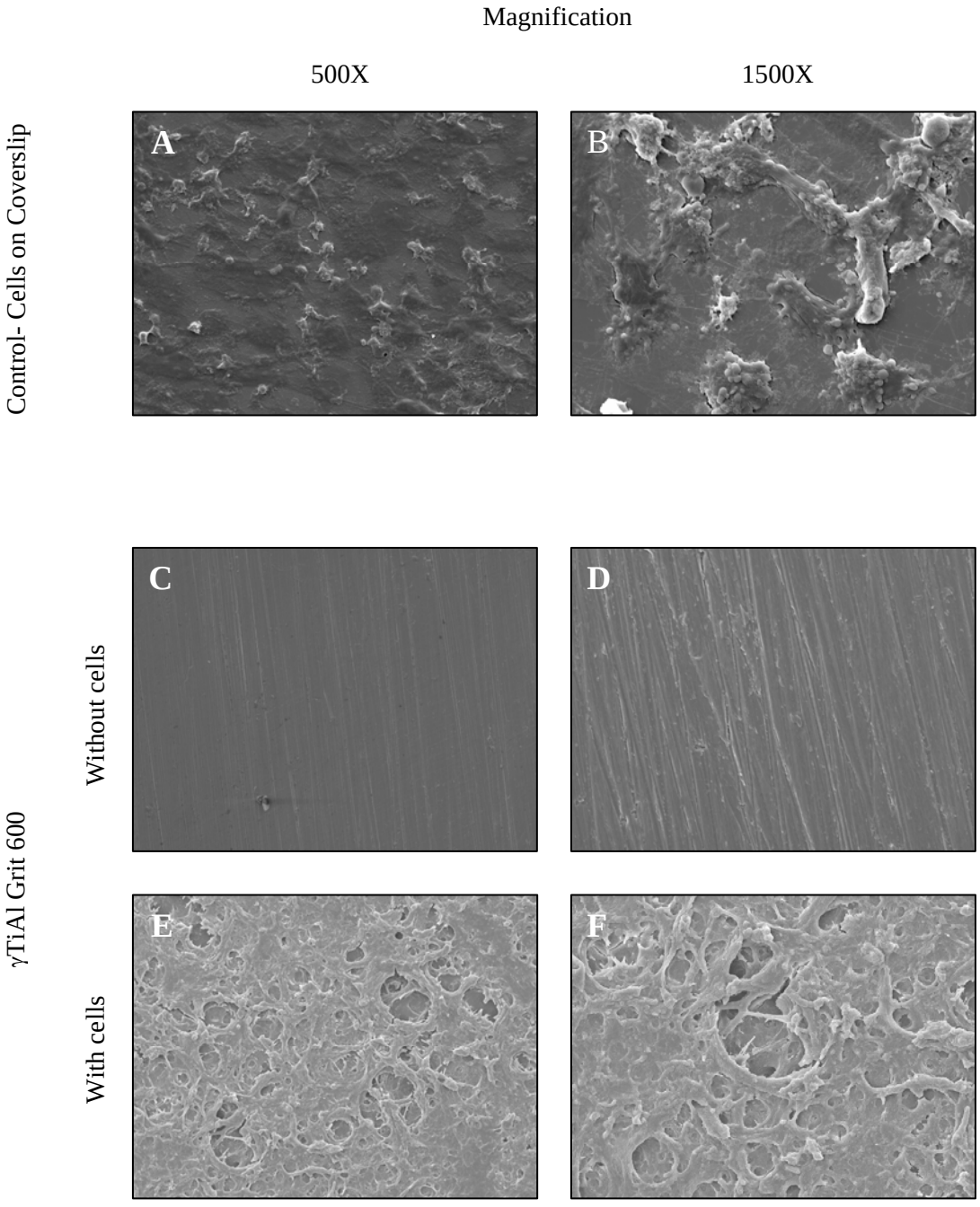


Figure 3. Scanning electron micrographs (A- Z) of control and, γTiAl and Ti-6Al-4V metal dicks with different surface roughness, cultured for 21 days at 37 ºC with and without hFOB 1.19 cells.

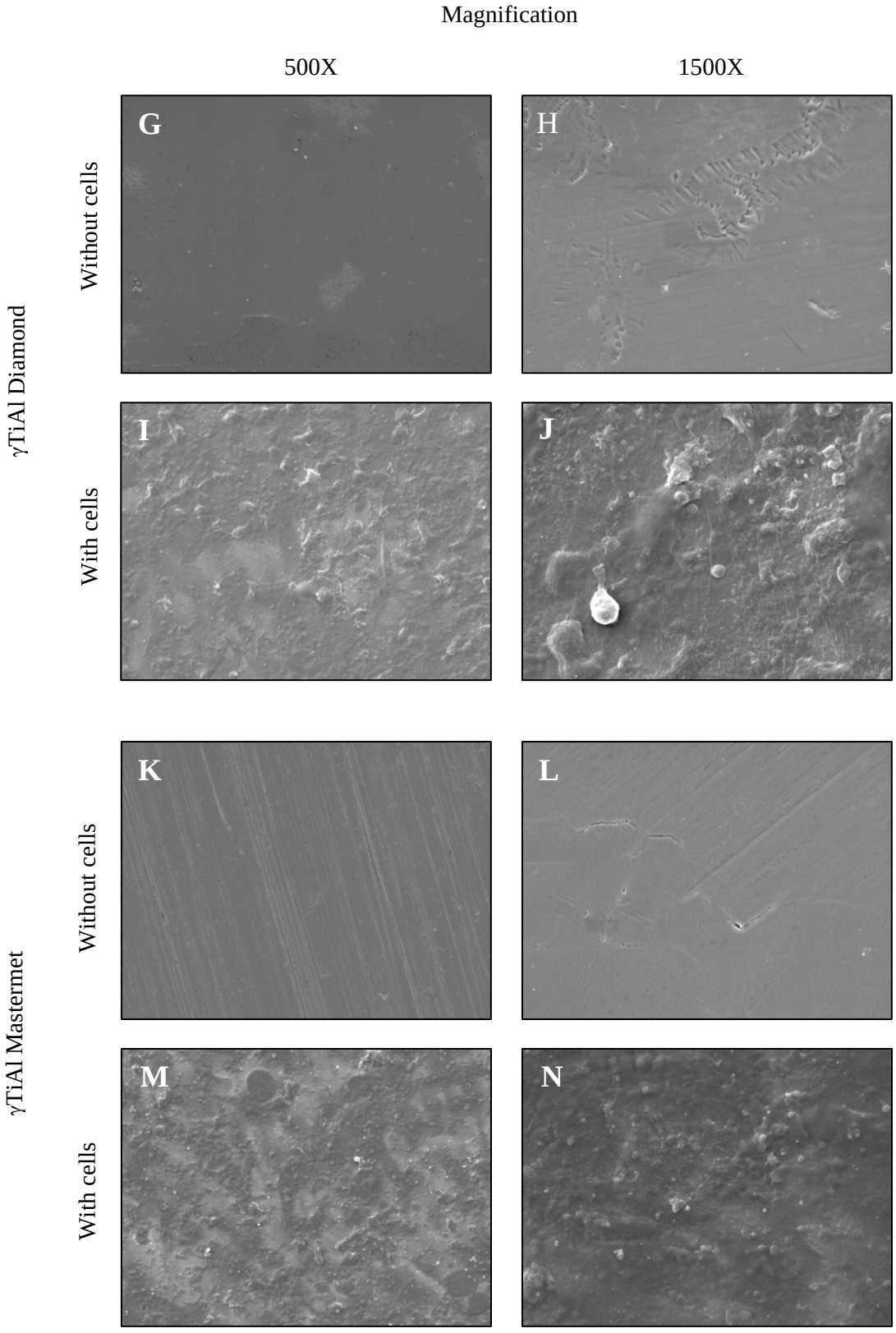


Figure 3. Continuation

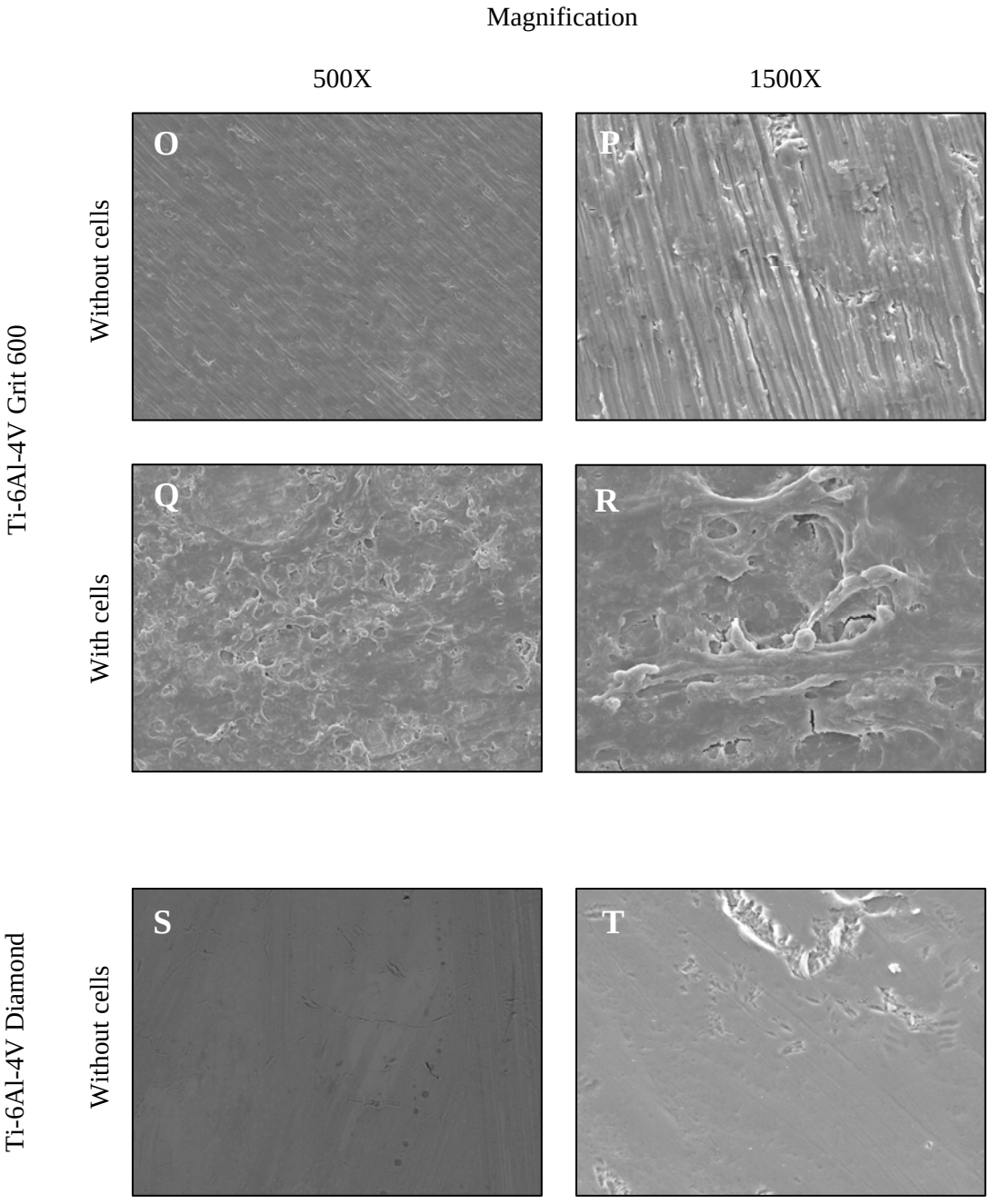


Figure 3. Continuation

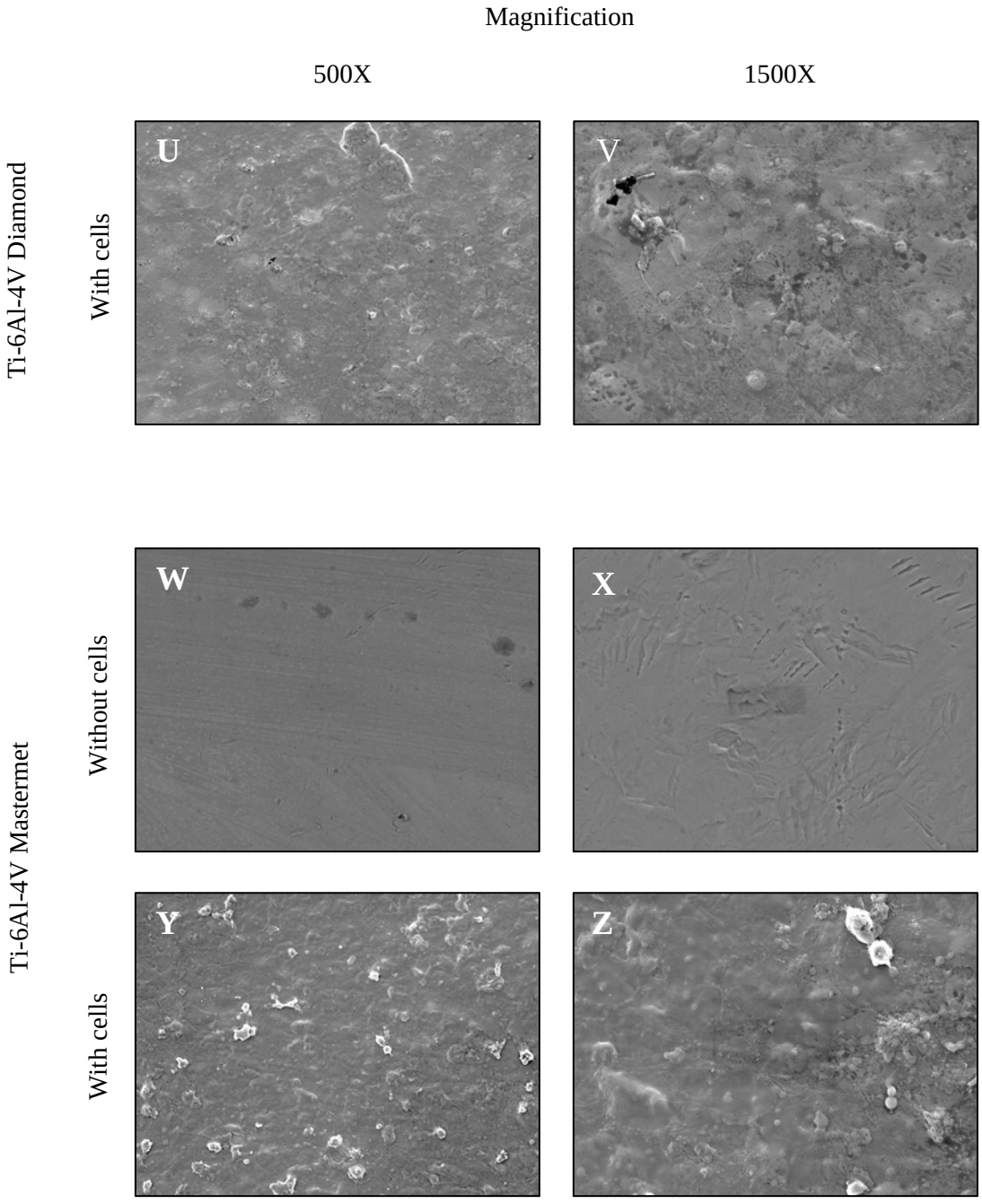


Figure 3. Continuation

Cell growth, attachment and proliferaton are major events that need to occur in order to repair and maintain a tissue. Since cells are sensitive to the physical properties of the materials with which they interact (7), the effects of the material on the surrounding tissue will determine its ability to be used as an implant in the human body. Bone remodeling and mineralization processes at the interface are fundamental elements of a good implant-bone fixation and therefore of the success of a prosthesis (18).

The effect of γTiAl on human fetal osteoblast cells was studied in order to determine its possible applications as an implant material. Cell growth on γTiAl and Ti-6Al-4V was also observed for different surface roughness. A direct comparison of the cells cultured on γTiAl and on Ti-6Al-4V was performed (Figures 4-8). Cell attachment and proliferation was similar between both metals. In these samples the number of cells attached to each surface was small, allowing us to study the direct interaction of the metal-cell interface. At lower magnification cells appeared to spread and anchored on both metal surfaces, independently of their roughness. On smoother surfaces cells had a flattened appearance, this likely a direct result of the smooth surface of the metal, while on the grit polished surface cell topography is similar to that of the underlying metal. Various investigators have found that grooved surfaces were found to stimulate a linearly oriented, cellular topography also known as “contact guidance” where cells align themselves in an end-to-end fashion parallel to the direction of the grooves (23, 57), while growth on smooth surfaces were found to result in flat cells that were randomly oriented.

In this study, although cell topography was different on rough and smooth surfaces, cells were able to attach, grow and proliferate on both surfaces with no

significant difference in cell number, in accordance with previous studies (65). For both γTiAl and Ti-6Al-4V, cells are in direct contact with the metal surface, and, at higher magnifications, the cells appeared to have cellular extensions for anchorage. The presence of microspikes is evident in both metals when cells were observed under higher magnifications (Figures 7 and 8), in accordance with Lauer (46) and Degasne (55). Also, single cells had a flat appearance and a prominent nucleus area [Figure 7 (A and C)], which is the typical appearance of osteoblast cells reported in the literature and references of osteoblast cells observed under SEM (53). In a γTiAl sample a mitosis-like structure could be observed [Figure 7 (B), arrow], with cells in the process of separating. This is also a clear indication that cells can grow and proliferate on γTiAl.

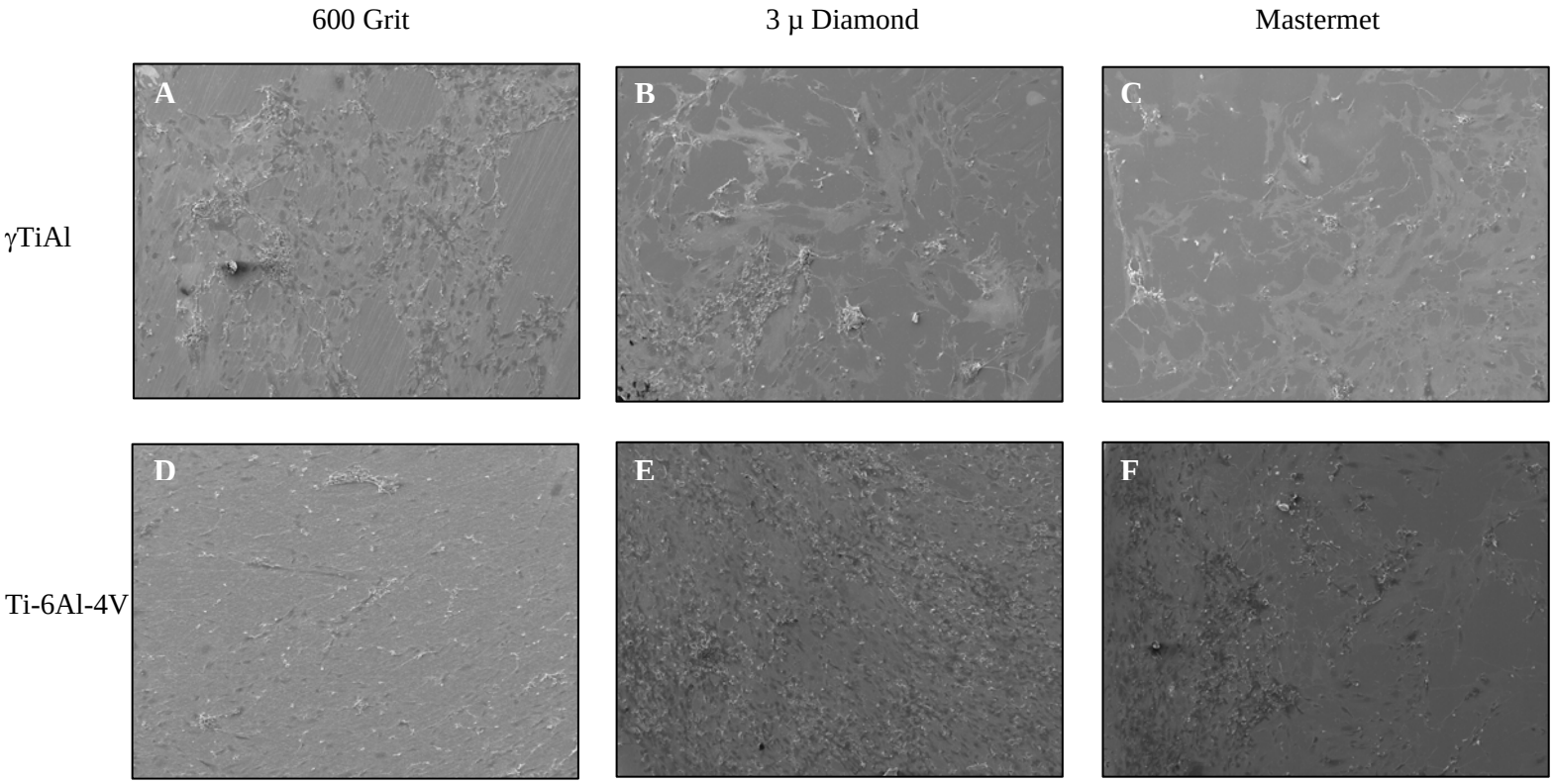


Figure 4. Scanning electron micrographs of hFOB 1.19 cells cultured for 21 days at 37 ºC on γTiAl and Ti-6Al-4V with different surface roughness (A-F) observed at 75X magnification.

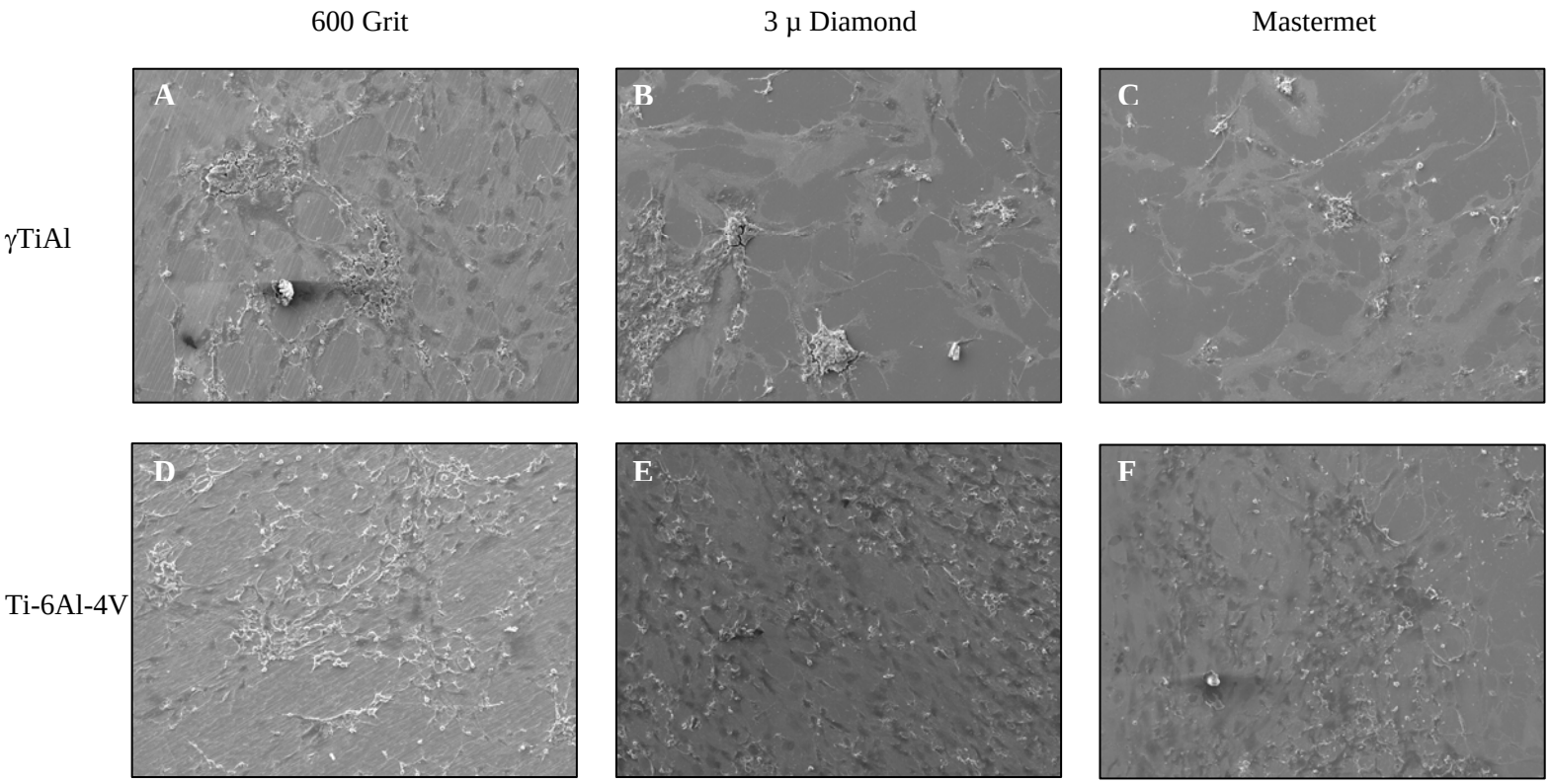


Figure 5. Scanning electron micrographs of hFOB 1.19 cells cultured for 21 days at 37 ºC on γTiAl and Ti-6Al-4V with different surface roughness (A-F) observed at 150X magnification.

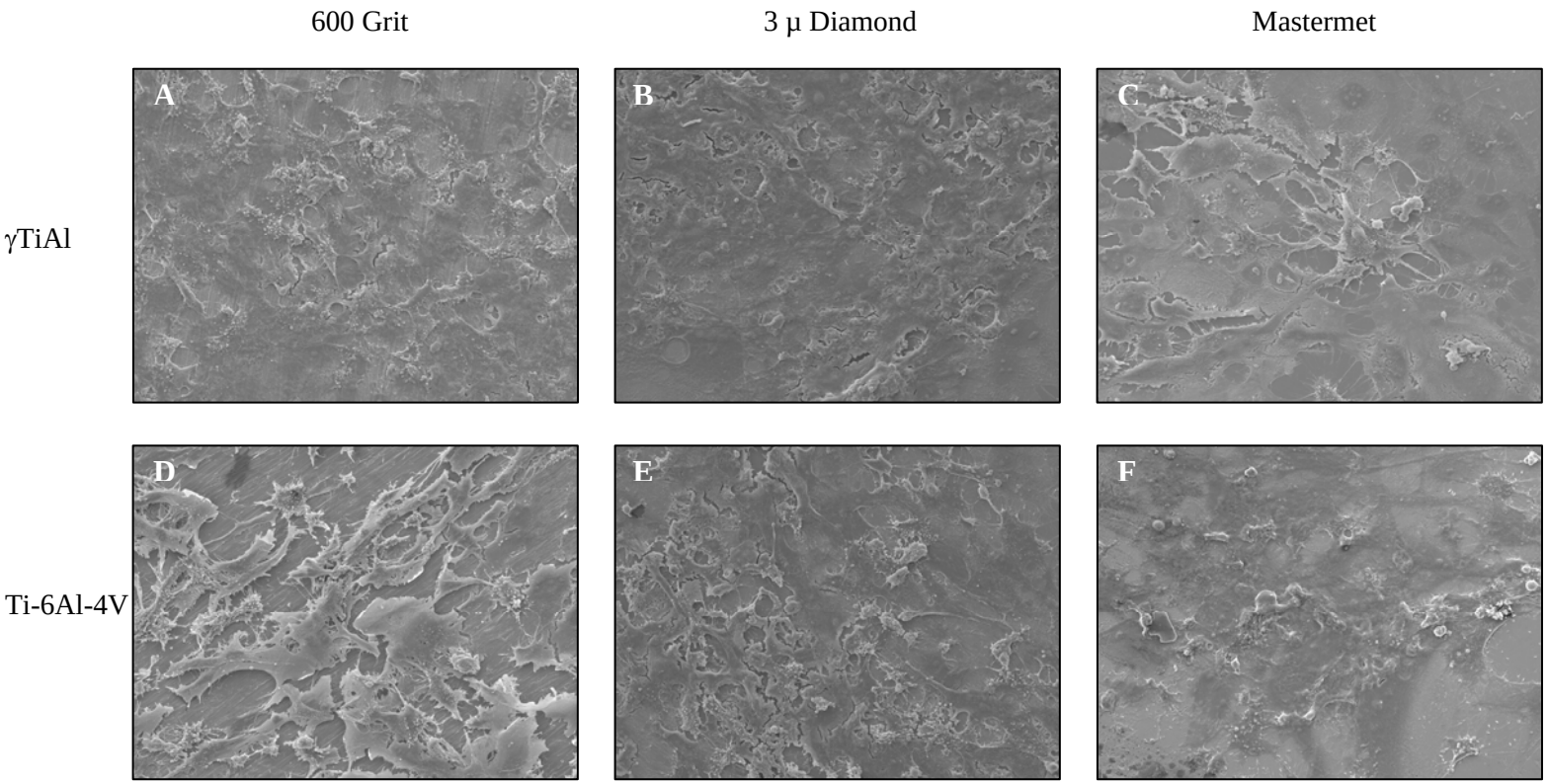


Figure 6. Scanning electron micrographs of hFOB 1.19 cells cultured for 21 days at 37 ºC on γTiAl and Ti-6Al-4V with different surface roughness (A-F) observed at 500X magnification. Cells appear more flattened on the smoother surfaces (B, C, E and F) than on the roughest surface (A and D) for both metal samples.

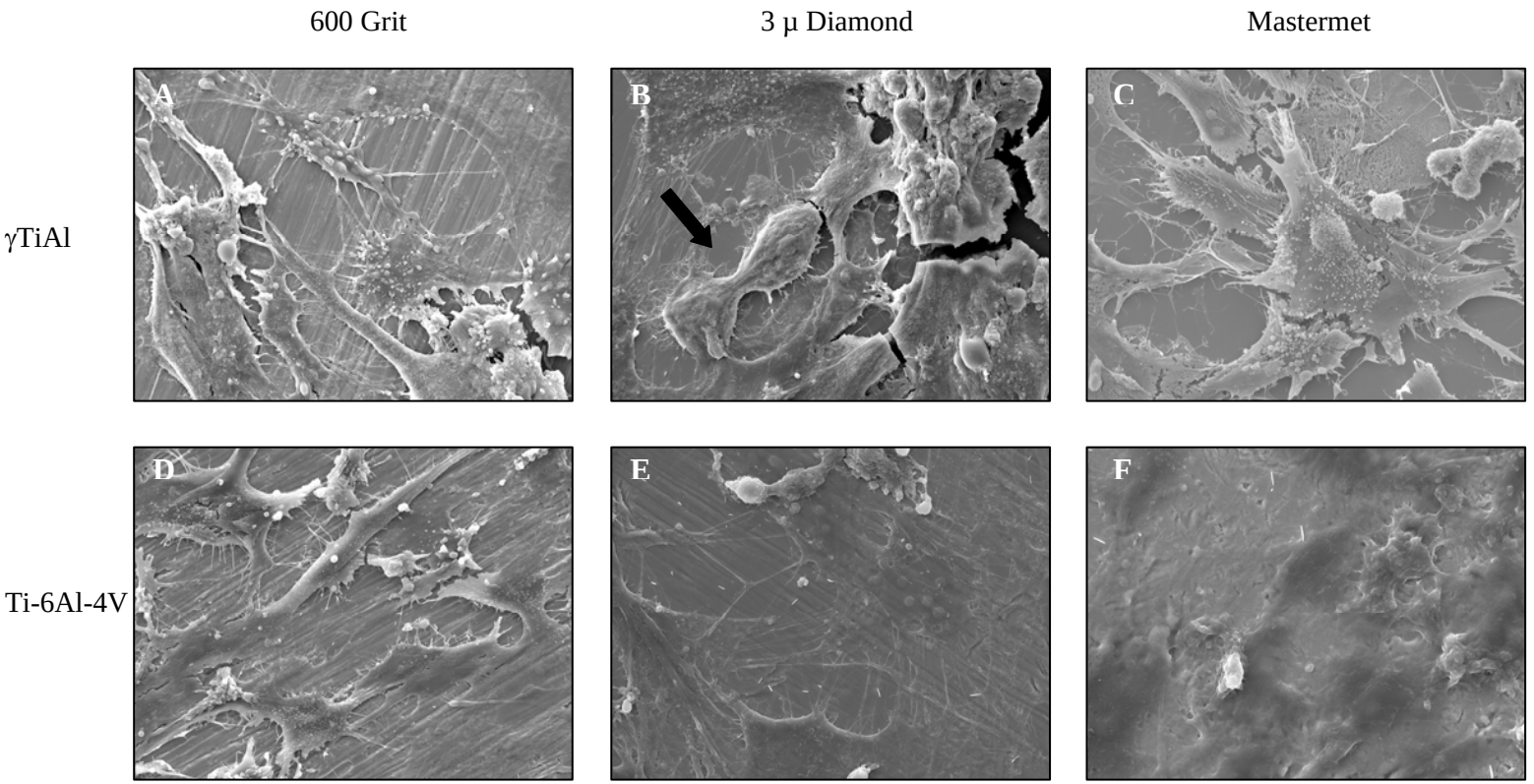


Figure 7. Scanning electron micrographs of hFOB 1.19 cells cultured for 21 days at 37 ºC on γTiAl and Ti-6Al-4V with different surface roughness (A-F) observed at 1500X magnification. Arrow points to a mitosis-like structure in a γTiAl sample.

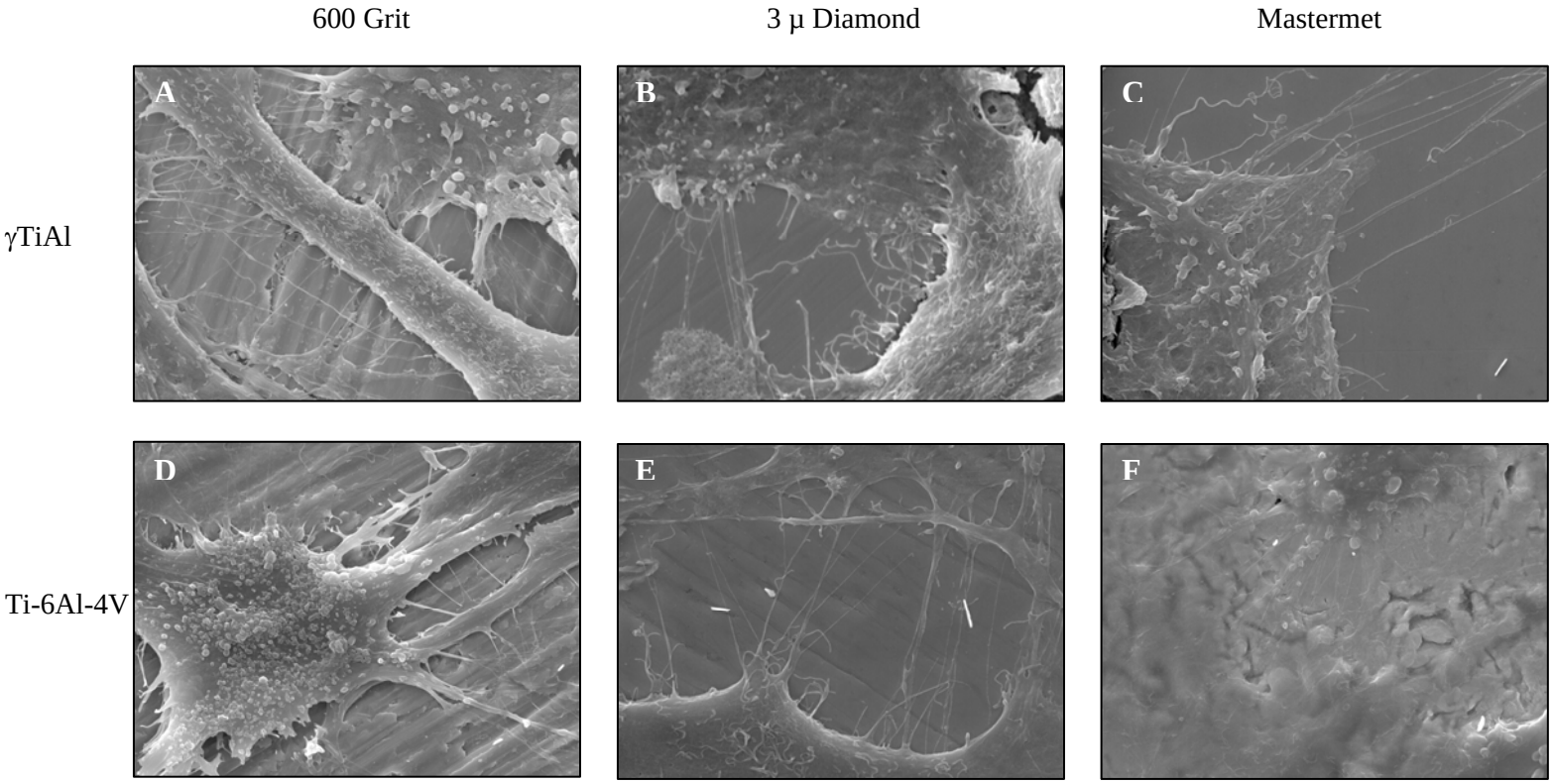


Figure 8. Scanning electron micrographs of hFOB 1.19 cells cultured for 21 days at 37 ºC on γTiAl and Ti-6Al-4V with different surface roughness (A-F) observed at 5000X magnification.

Our results showed that osteoblast cells could be successfully cultivated in γTiAl with no difference observed between various surface roughness. A similar growth of hFOB 1.19 osteoblast cells was observed for smooth and rough surfaces, showing the cell ability to attach, grow and proliferate on both surfaces with no significant difference in cell number between different surface roughness (65). The appearance of the cell layer varied with different surface roughness; cells grown on grit polished surface exhibited ruffling and looked similar to the surface with multiple cell layers, while cells grown on smoother surfaces were well spread and appeared to form a continuous layer, although the presence of multiple layers can not be discarded.

Metal surface topography had a direct effect on cell shape (55), showing that cells are capable of distinguishing between changes in surface topography and respond differently to surface stimuli. Our results concur with observations reported in the literature about the effects of surface topography on cell behavior (26, 51, 72). This indicates that surface roughness plays an important role in the success or failure of a metal implant (44, 58). It must be emphasized that although material properties, such as roughness, are believed to be critical to at bone-implant interface, the subsequent steps in bone healing around an implant may depend more on cells at the surface than on the surface itself (22, 23), since bone formation and regulation involves hormones, cytokines and growth factors which are produced by the osteoblasts.

Immunofluorescent labeling

I. Fluorescein and Rhodamine labeling

Fluorescent labeling has been used to investigate new bone formation around titanium implants or bone remodeling (43). The organic component of the bone extracellular matrix is 90% collagen, while the other 10% is composed of glucosaminoglycans, small glycoproteins (osteocalcin, osteonectin and osteopontin), and sialoproteins (3). Cell line hFOB 1.19 is expected to produce the proteins of the extracellular matrix, which are collagen type I, osteonectin and osteocalcin, among others (40). Confocal microscopy analysis of human osteoblast cells cultured on γTiAl and Ti-6Al-4V with different surface roughness and labeled with Fluorescein (FITC) and Rhodamine clearly revealed the presence of both fluorescent labels on the cell layer and the surface of the metal disc. For all the samples with cell culture (cell growth control and cells cultured on both Ti alloys) the signal corresponding to osteonectin (FITC labeled, green) was higher than the signal corresponding to collagen type I (Rhodamine labeled, red) (Figure 9), which was not expected. We expected the signal for collagen to be higher than the signal for osteonectin.

A possible explanation for these results is a phenomenon called cross-talk. This is a common problem in labeling for multiple proteins, where the interaction between the excitation and emission of the fluorochromes leads to cross-excitation and cross-emission (73). Cross excitation occurs when a fluorochrome is not just excited by wavelength at its peak value, but also by wavelength at certain range around the peak, which can extend into the area used by other fluorochromes. Also, cross emission is the overlapping of the emission spectra of different fluorochromes.

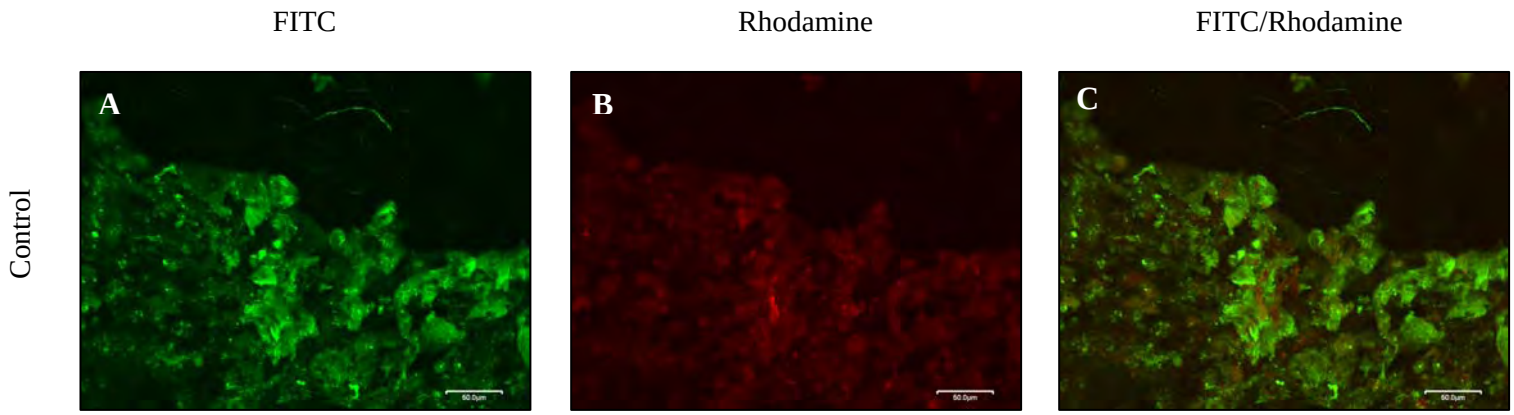


Figure 9. Confocal Laser Scanning Microscopy of the expression of human collagen type I (Rhodamine labeled, red) and osteonectin (FITC labeled, green) in surfaces with or without hFOB 1.19 cells (A- MM) cultured for 21 days at 37 ºC on glass coverslips, and on γTiAl and Ti-6Al-4V with different surface roughness (40X Magnification, scale bar= 50 μm).

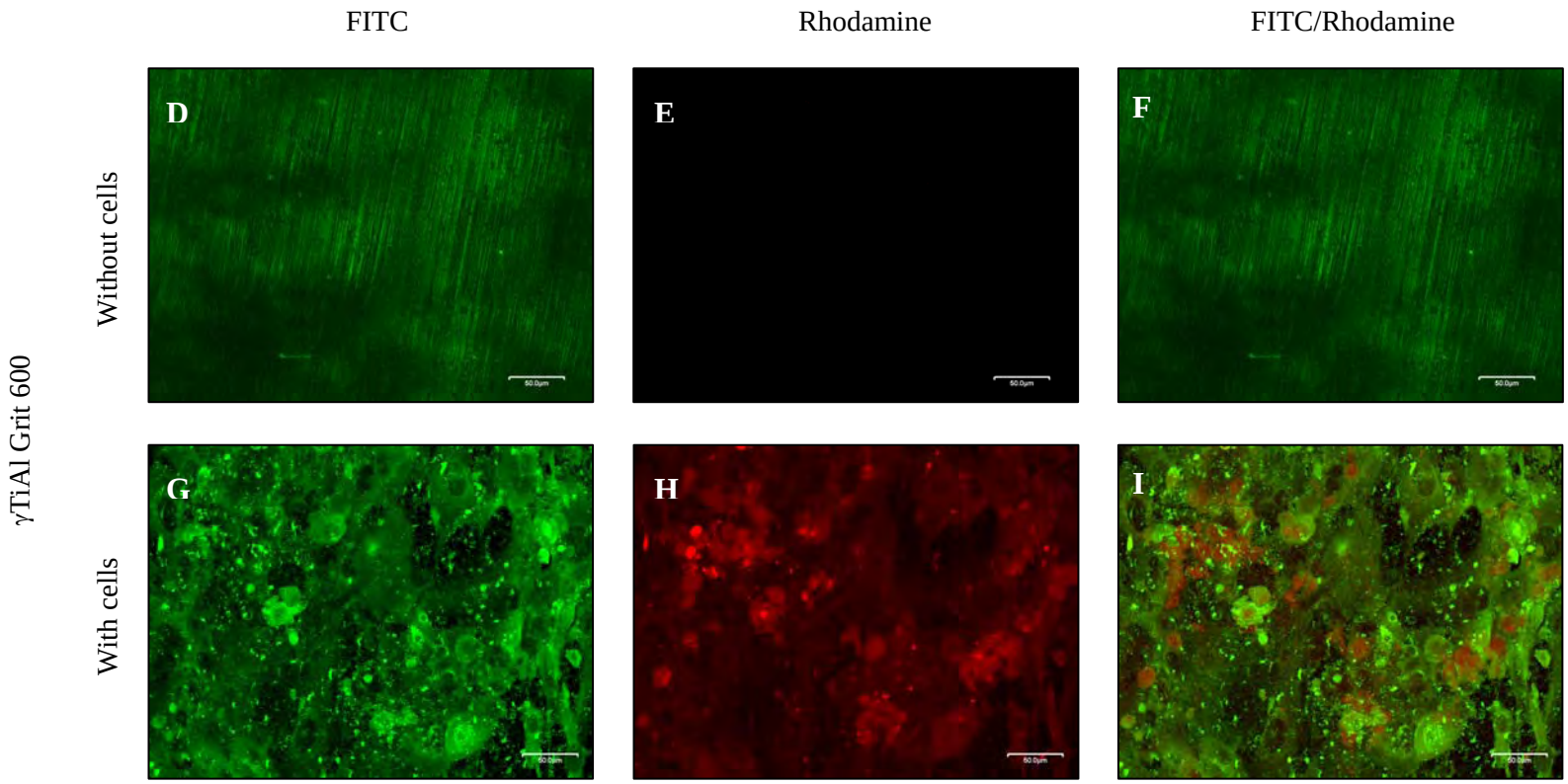


Figure 9. Continuation.

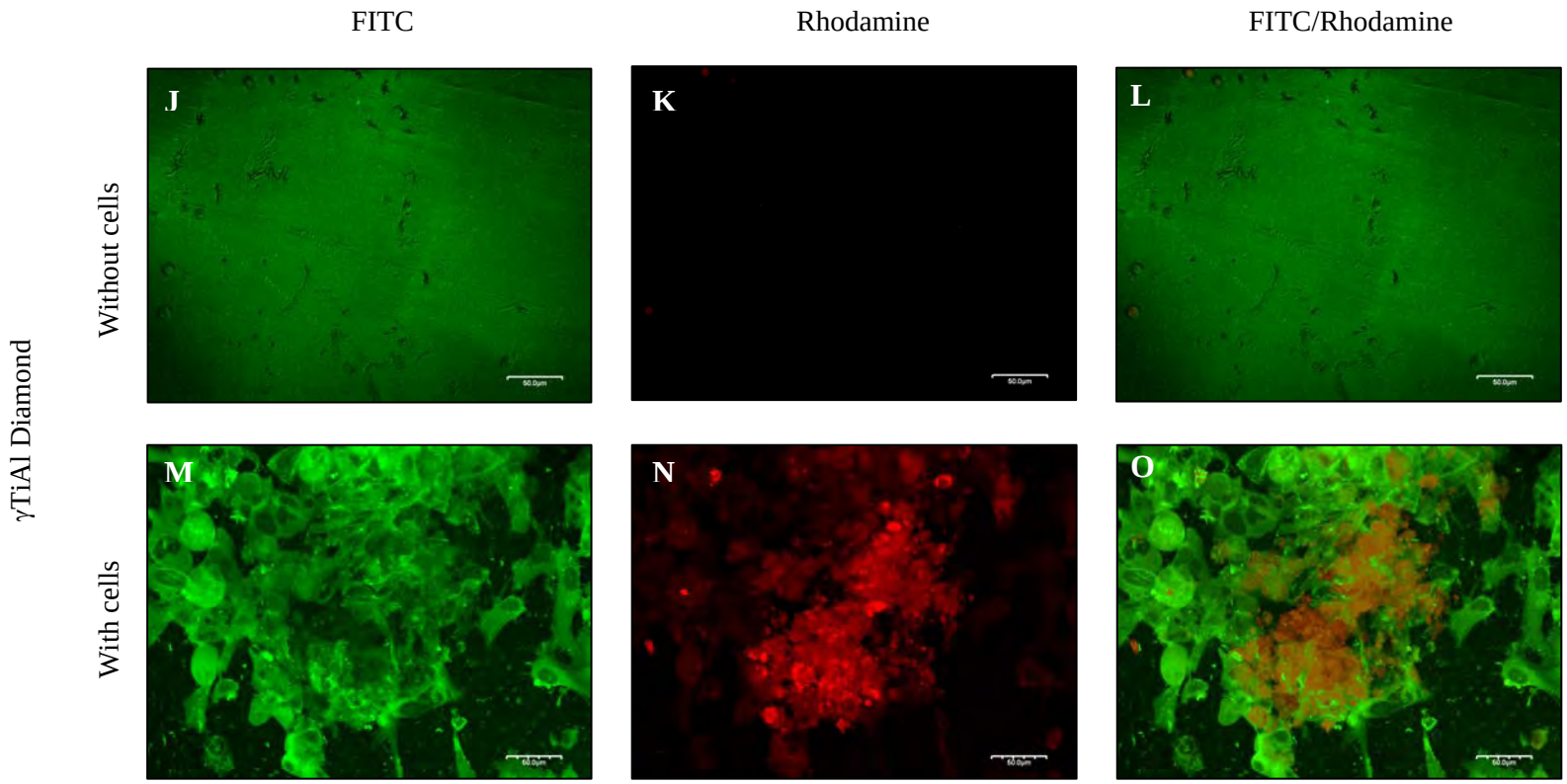


Figure 9. Continuation.

γTiAl Mastermet

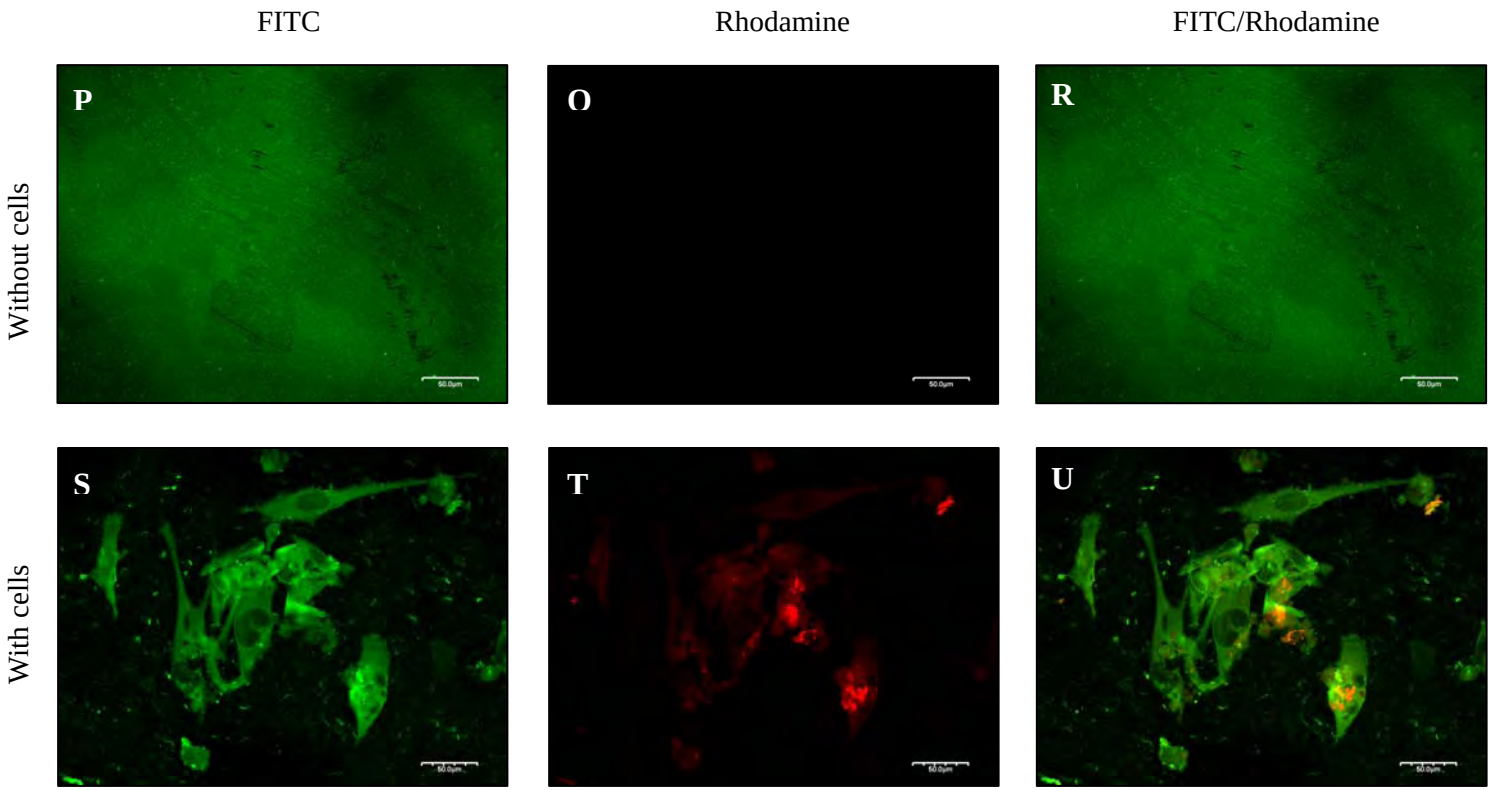


Figure 9. Continuation.

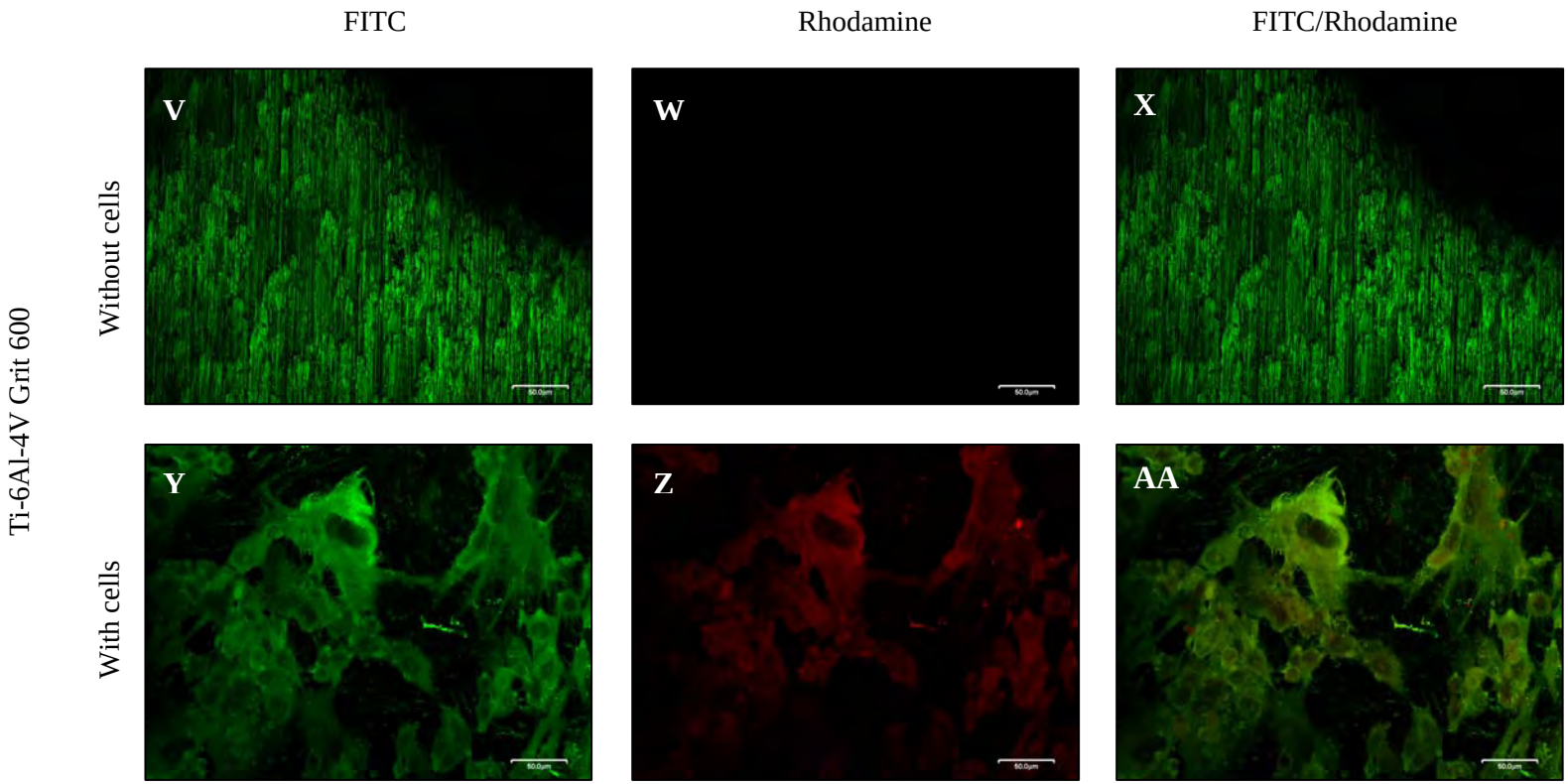


Figure 9. Continuation.

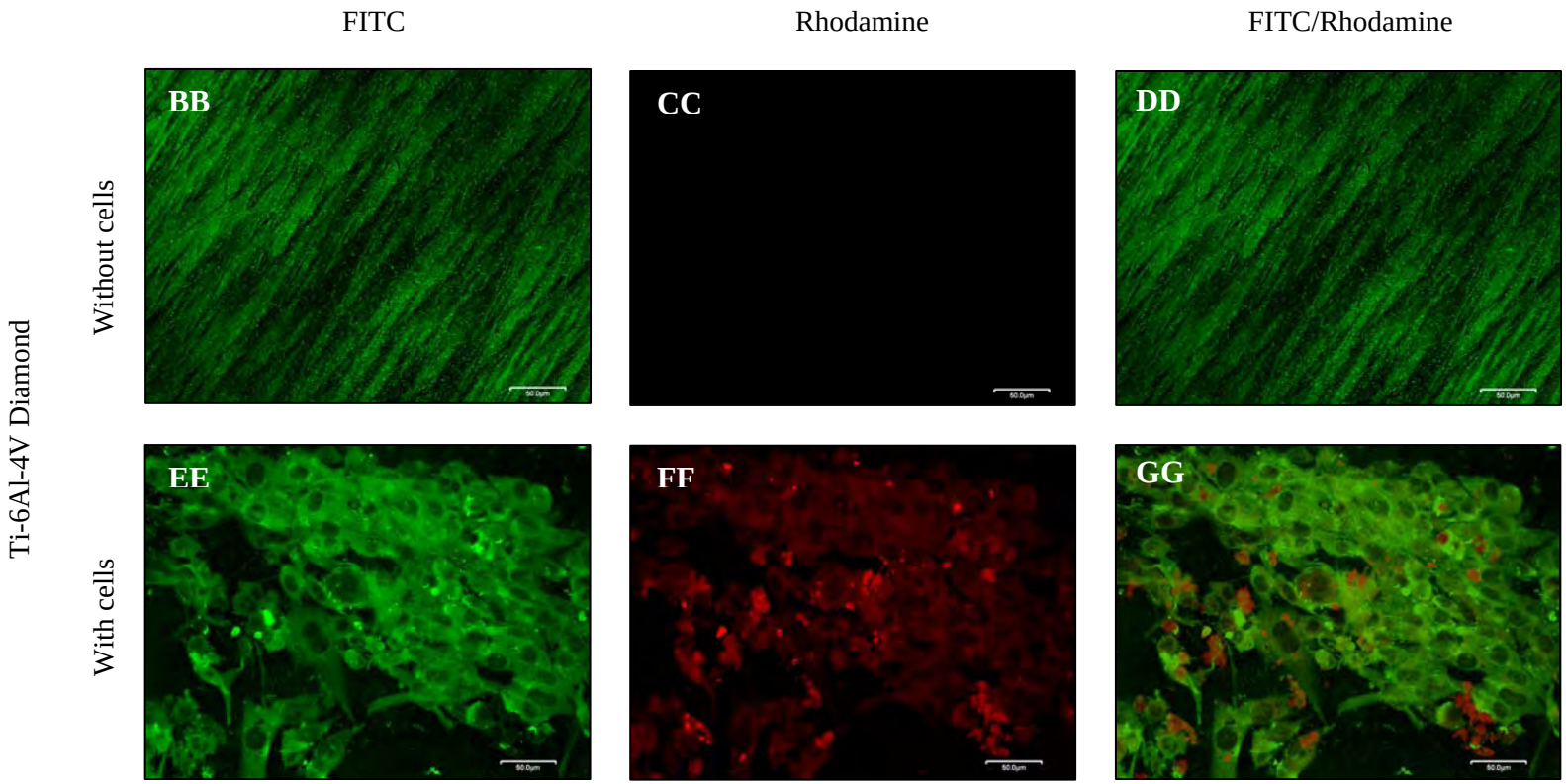


Figure 9. Continuation.

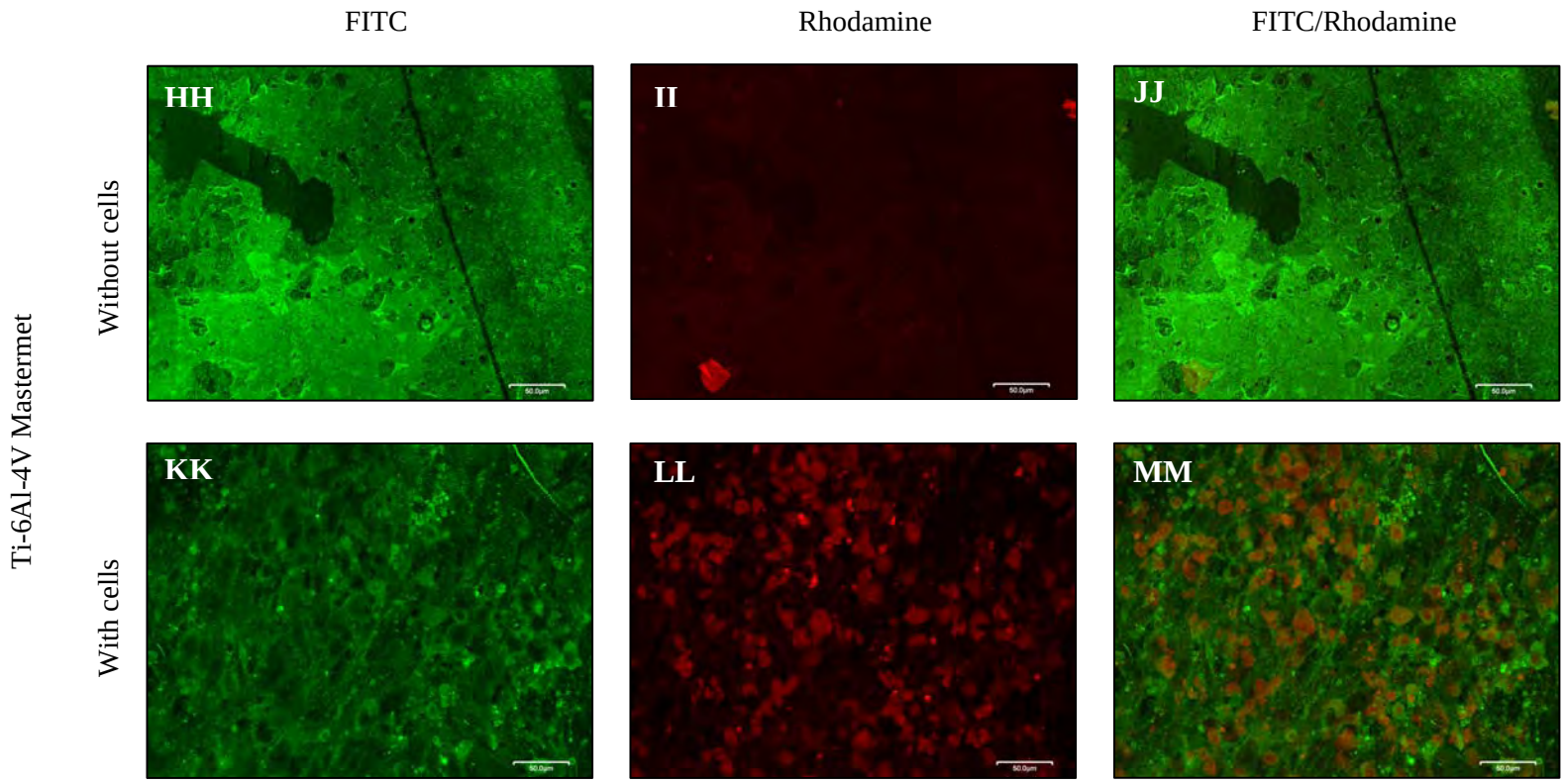


Figure 9. Continuation.

The FITC fluorochrome has a peak excitation at 492nm, and peak emission at 520 nm, while rhodamine has a peak excitation at 550 and peak emission at 573. The use of these fluorochromes together may lead to cross-emission, since they share a range of the emission spectra of the other fluorochrome. It is possible that the FITC fluorochrome is emitting a signal that overlaps with the emission of rhodamine, which will lead to the higher amount of osteonectin signal than that of collagen type I. To avoid cross-emission a different combination of fluorochromes was used.

Another problem observed in the samples observed by confocal microscopy is the ability of the metal surface to be excited by the lasers used. This is evident for all the samples that were cultured without osteoblast cells (Figure 9) of both γTiAl and Ti-6Al-4V. This background signal can interfere with our results. The use of a different fluorochrome should eliminate the background signal. A simple assay was performed to determine which fluorochrome would be most suitable to minimize cross-emission and background.

A clean and sterile metal disc of γTiAl was mounted on a 20 x 40 mm coverslip with Fluorescent Mounting Media (Oncogene, San Diego, California), just as the regular samples, and observed in the confocal microscope using different lasers. The surface of the metal emitted a signal with the same laser that was used for the FITC fluorochrome, the laser used for the rhodamine fluorochrome resulted in no signal (Figure 10). We also found no emission signal from the metal when using the laser for cyanide 5 (Cy5) fluorochrome.

FITC Rhodamine Cy5

Figure 10. γTiAl disks (sterile clean) excited with FITC, Rhodamine and Cy5 wavelength with no Immunofluorescent labeling.

Cy5 fluorochrome has a peak excitation at 649 and a peak emission at 666 nm. This means that its emission spectra is to the right, further away from the emission spectra of rhodamine. These results suggest the use of rhodamine and Cy5 fluorochromes in our immunofluorescent analysis, minimizes the risks of cross-emission or a false signal from the metal.

II. Cy5 and Rhodamine labeling

Anti-mouse IgG with Cy5 conjugate (Zymed Laboratories, San Francisco, California) and anti-mouse IgG (goat) with Rhodamine (Calbiochem, San Diego, California) were used to detect the presence of osteonectin and collagen type I, respectively, on human osteoblast cells. Given that both secondary antibodies were obtained from the same source animal (mouse) detection of collagen type I was performed, followed by the detection of osteonectin. By separating the detection of these two proteins non-specific binding was avoided.

Immunofluorescent analysis of hFOB 1.19 cells cultured on γTiAl and Ti-6Al-4V with different surface roughness, and labeled with Cy5 and Rhodamine clearly revealed the presence of both fluorescent labels, therewith, the collagen type I and osteonectin were present in the cells. As was expected, hFOB 1.19 osteoblast cells cultured on γTiAl and Ti-6Al-4V had a higher concentration of collagen type I than that of osteonectin.

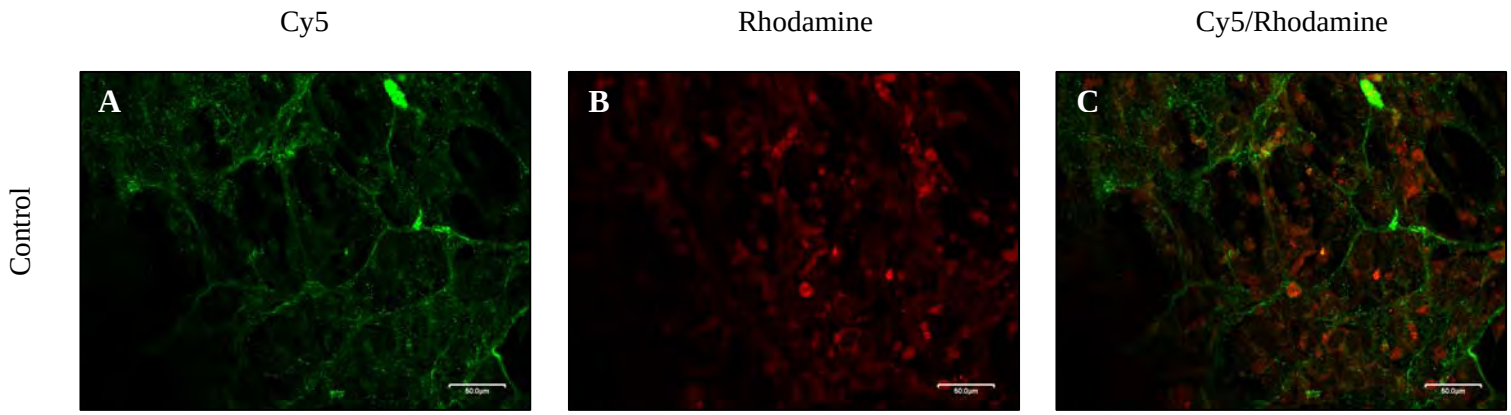


Figure 11. Confocal Laser Scanning Microscopy of the expression of human collagen type I (Rhodamine labeled, red) and osteonectin (Cy5 labeled, green) in surfaces with and without hFOB 1.19 cells (A- MM) cultured for 21 days at 37 ºC on glass coverslip, and on γTiAl and Ti-6Al-4V with different surface roughness (40 X magnification, scale bar= 50 μm).

γTiAl 600 Grit

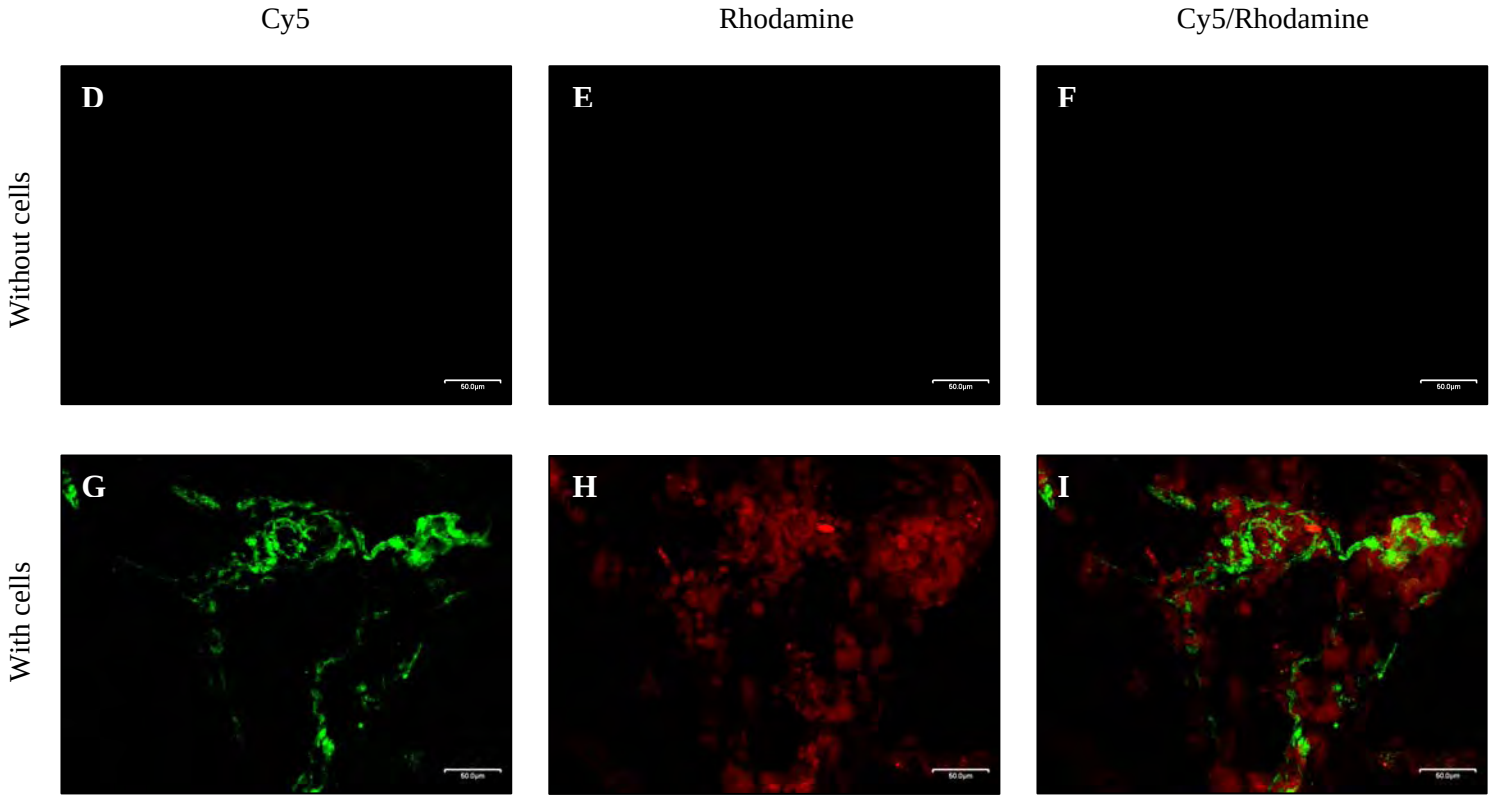


Figure 11. Continuation.

γTiAl Diamond

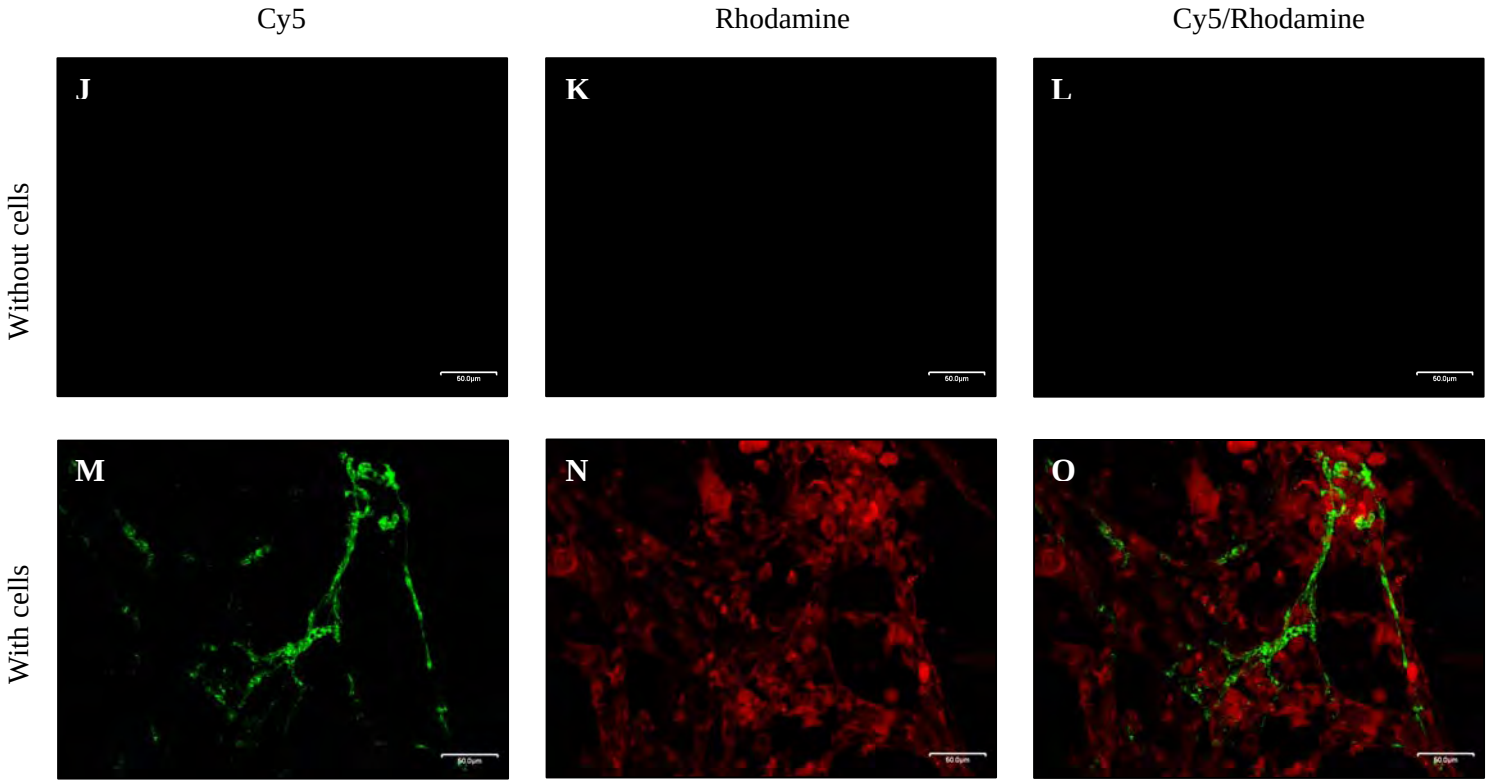


Figure 11. Continuation.

γTiAl Mastermet

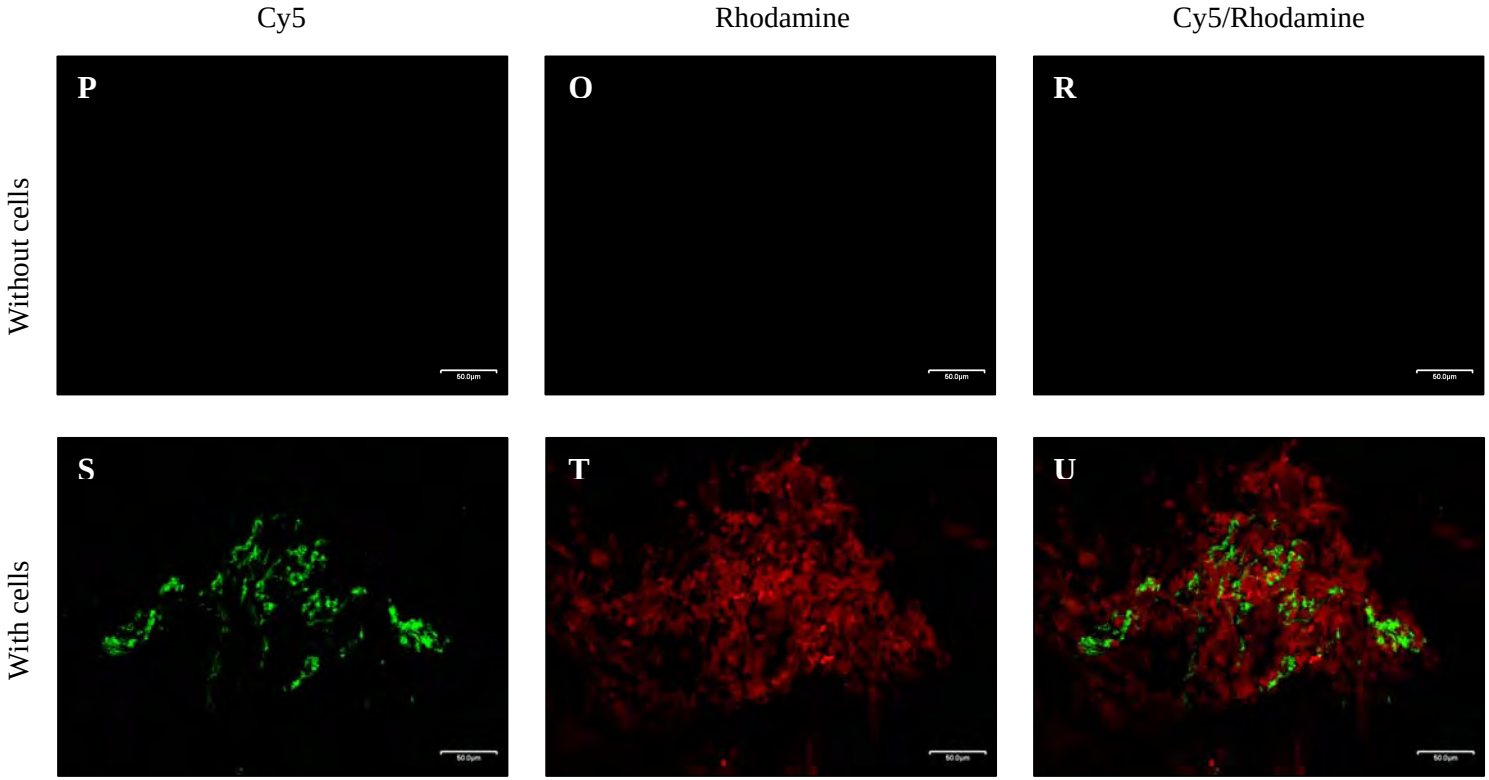


Figure 11. Continuation.

Ti-6Al-4V 600 Grit

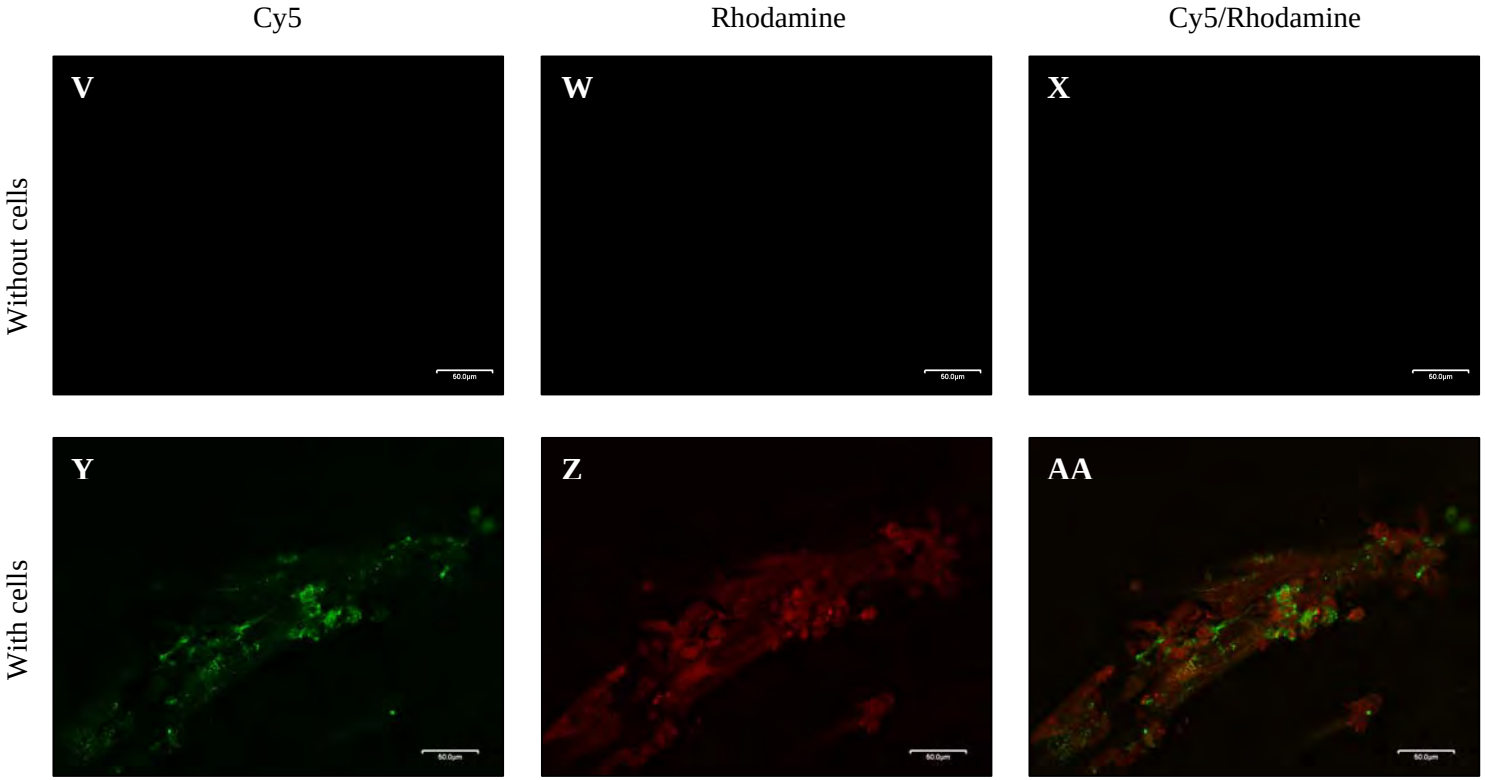


Figure 11. Continuation.

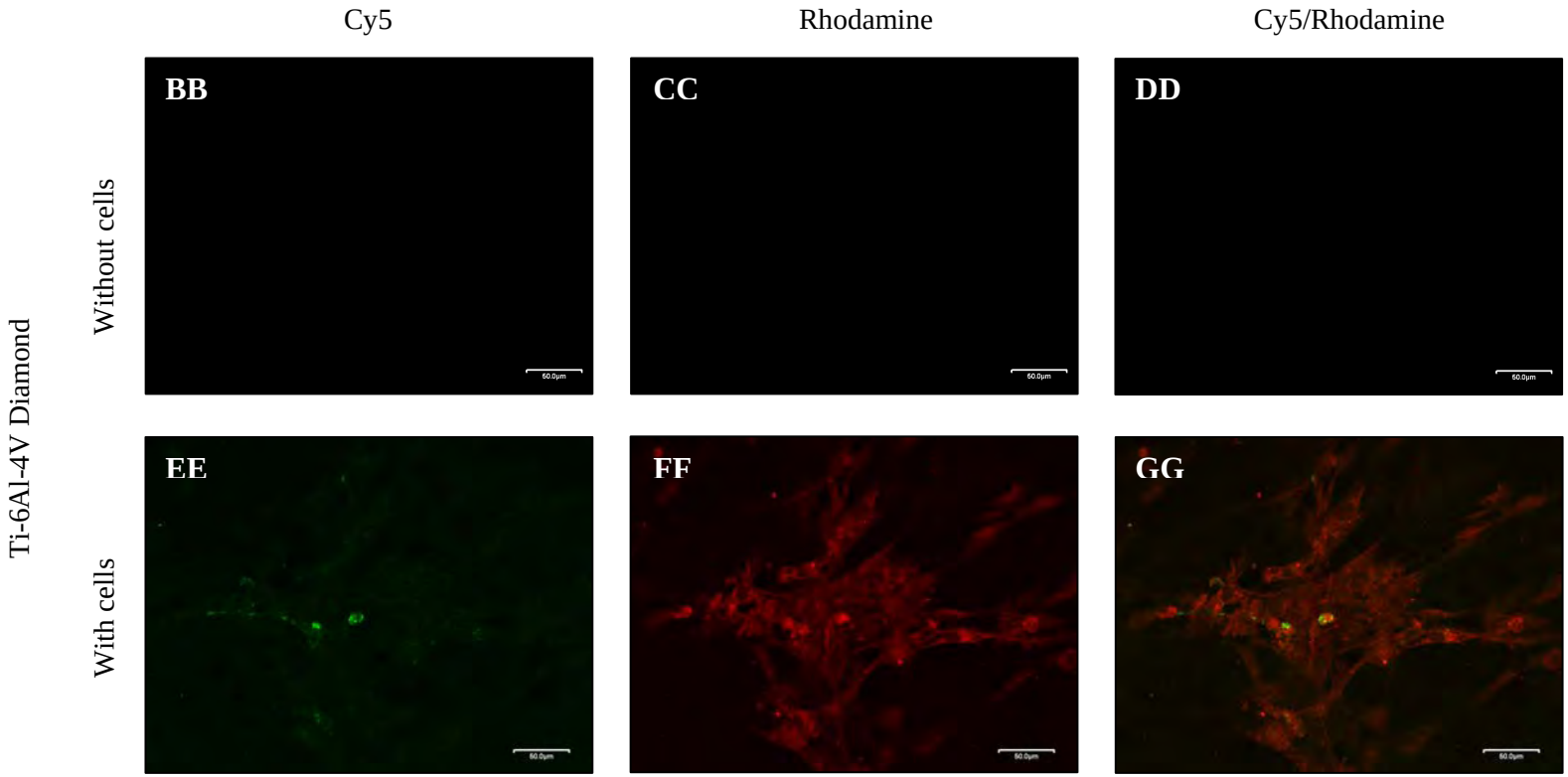


Figure 11. Continuation.

Ti-6Al-4V Mastermet

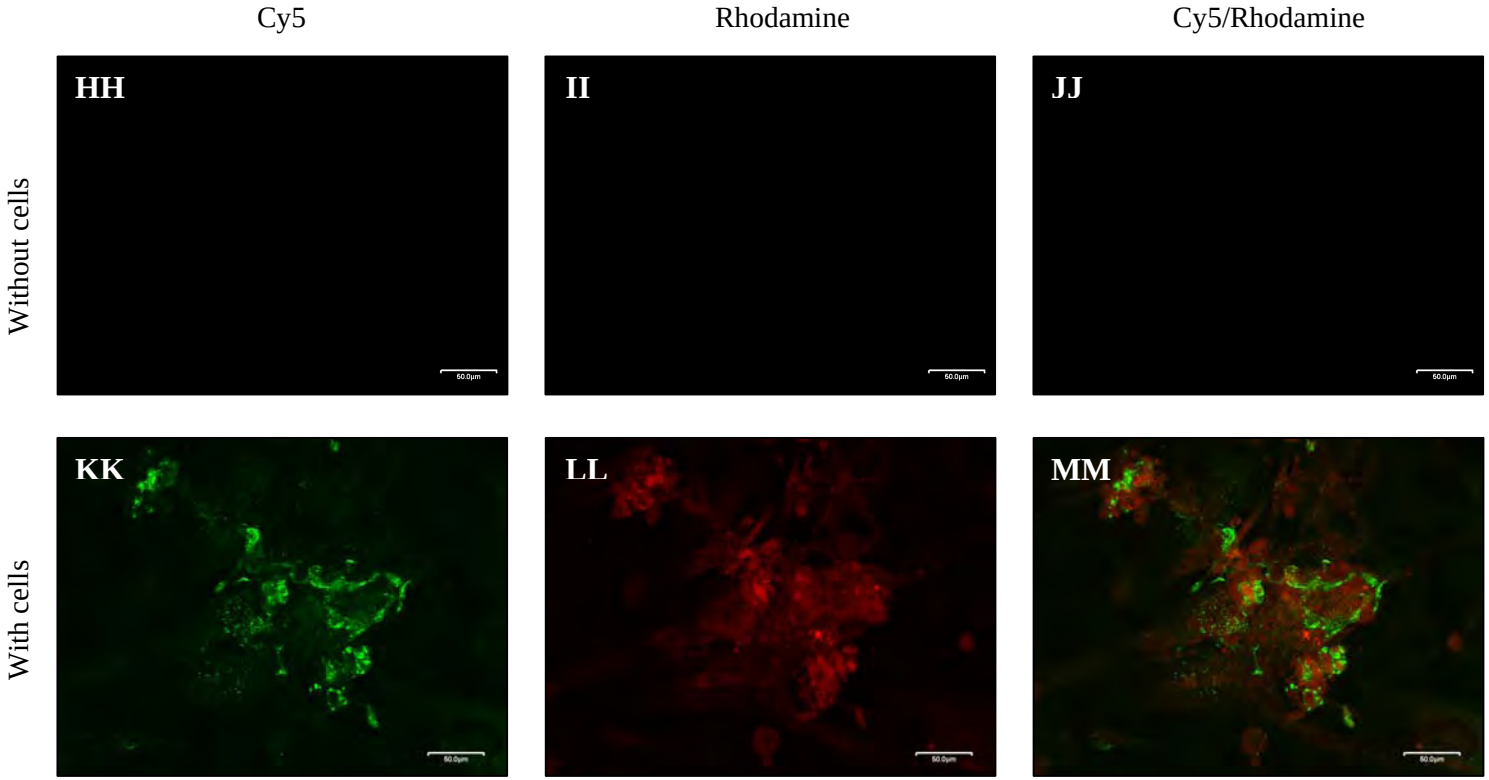


Figure 11. Continuation.

As observed in Figure 11 (C), cells on glass coverslips grew to confluency, the presence of both collagen type I and osteonectin was also detected and the formation of an extracellular matrix is evident. Negative controls showed the expected results, no signal was observed when the lasers that excite both Cy5 and Rhodamine were used. Cells cultured on both γTiAl and Ti-6Al-4V for 21 and 28 days were not confluent and formed clumps or cell conglomerates, with cells growing on top of one another (Figures 14-15). The presence of collagen and osteonectin appeared to be restricted to specific parts of the cells, collagen appeared to form circles around the nucleus of the cells. A difference in the extracellular matrix organization was not apparent for rough and smooth surfaces, which is in contradiction with other studies in which the extracellular matrix was organized and oriented differently on smooth surfaces compared to rough surfaces (43, 53). Others studies have demonstrated that synthesis and mineralization of the bone extracellular matrix are both enhanced when cultured on rough-textured and porous-coated (but not on smooth) titanium surfaces (36). Our data does not agree with these results, since a similar organization of the extracellular matrix was observed for hFOB 1.19 osteoblast cells grown on both rough and smooth surfaces.

Also, this study demonstrated that the expression of collagen type I and osteonectin increased as a function of time (Figures 12-15) for cells cultured on both γTiAl and Ti-6Al-4V, independently of surface topography. This increase in the synthesis of proteins found during the formation of the bone is in accord with previous studies (53). There was no difference observed in the level of protein expression between cells cultured on rough or smooth surfaces of γTiAl or Ti-6Al-4V. Cells attached and grew on all test substrates in a time-dependent manner without signs of disturbing influence from

any materials (51). Nevertheless, when the level of protein expression of cells cultured on γTiAl and Ti-6Al-4V are compared to each other a slight difference was observed. After 14 days of cell growth the number of cells observed on γTiAl appeared to be greater than that of cells cultured on Ti-6Al-4V, with higher expression levels of collagen type I and osteonectin.

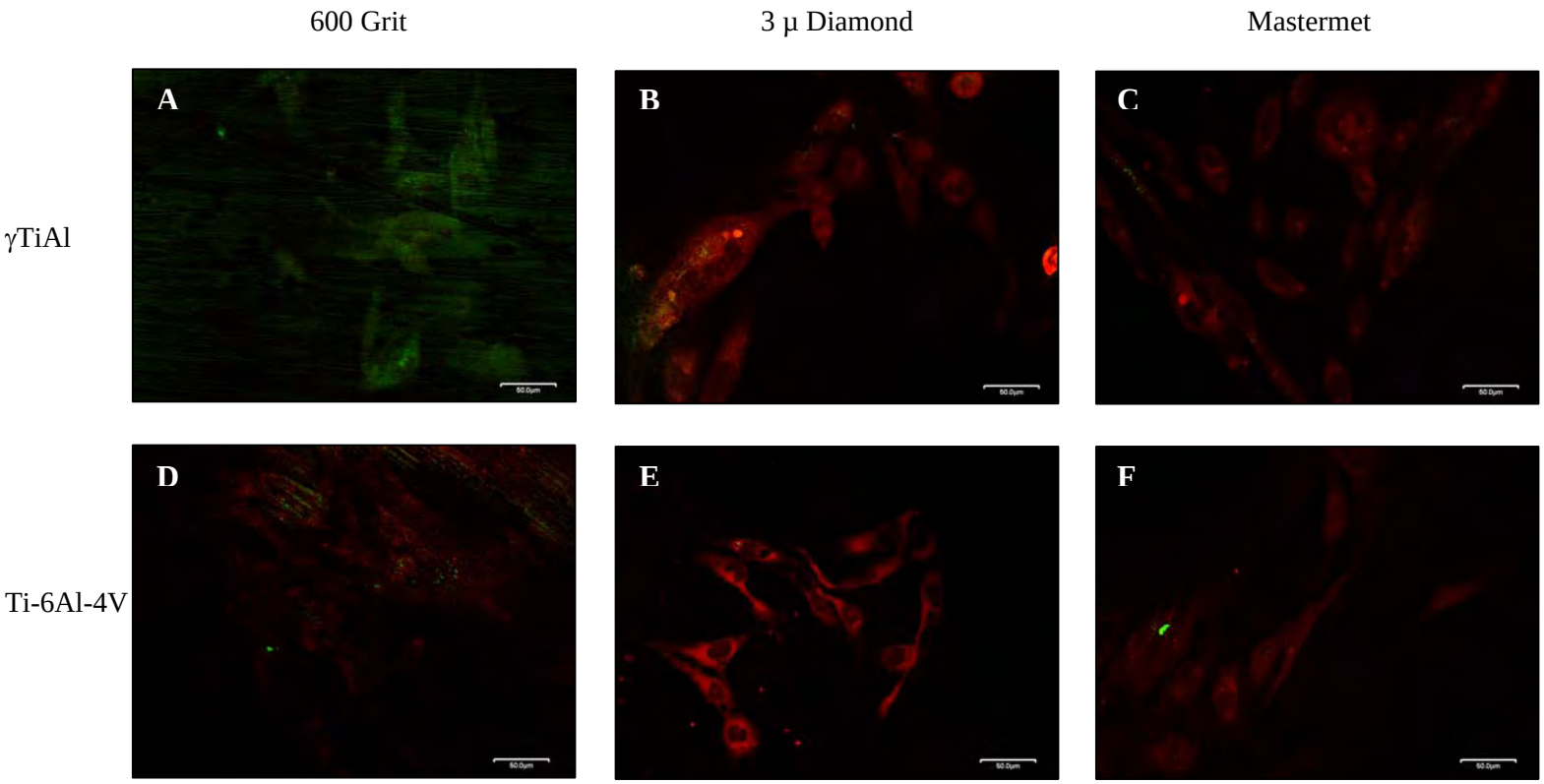


Figure 12. Confocal Laser Scanning Microscopy of the expression of human collagen type I (Rhodamine labeled, red) and osteonectin (Cy5 labeled, green) in hFOB 1.19 cells (A- F) cultured for 7 days at 37 ºC on γTiAl and Ti-6Al-4V with different surface roughness (40 X magnification, scale bar= 50 µm).

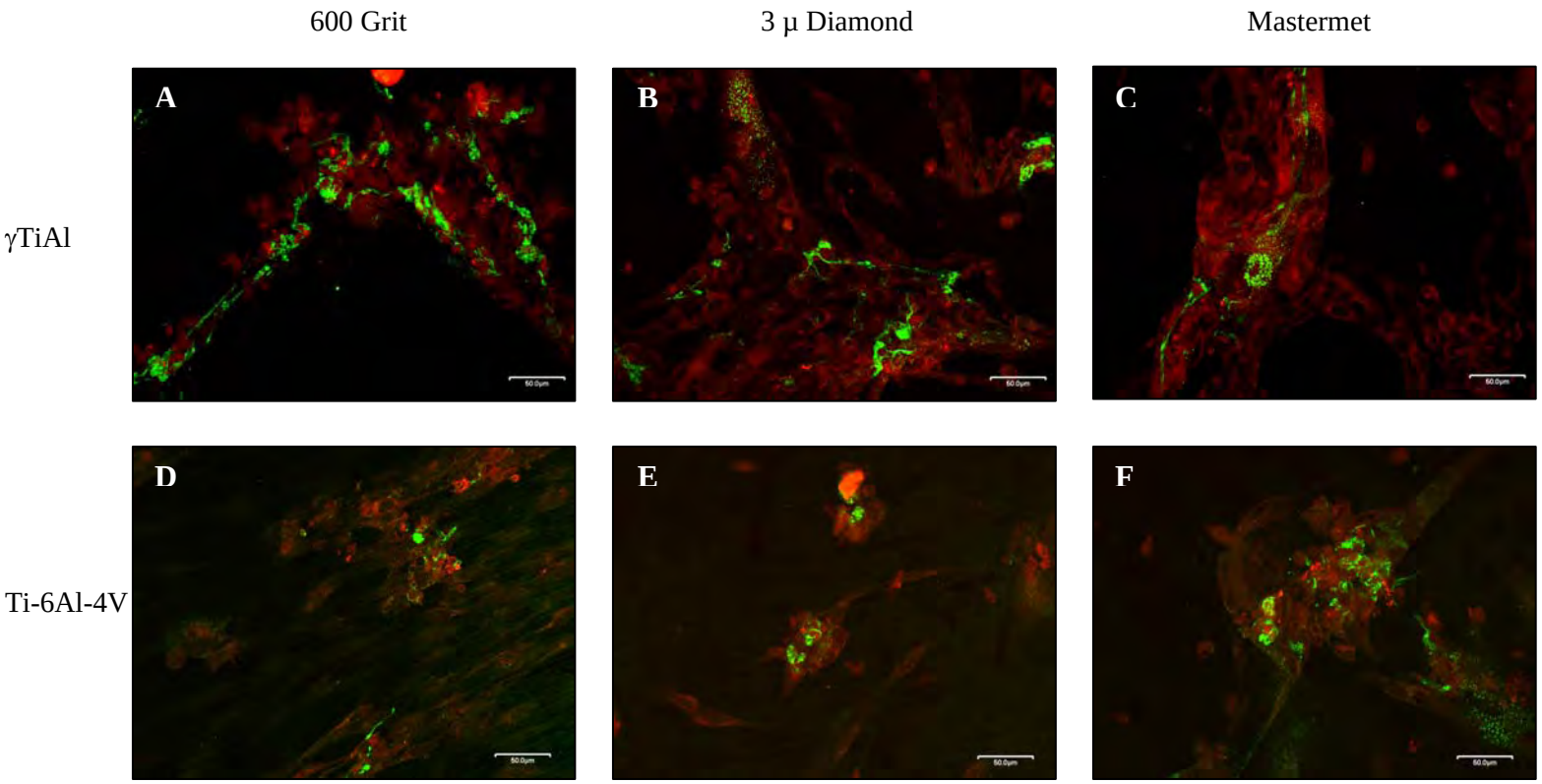


Figure 13. Confocal Laser Scanning Microscopy of the expression of human collagen type I (Rhodamine labeled, red) and osteonectin (Cy5 labeled, green) in hFOB 1.19 cells (A- F) cultured for 14 days at 37 ºC on γTiAl and Ti-6Al-4V with different surface roughness (40 X magnification, scale bar= 50 µm).

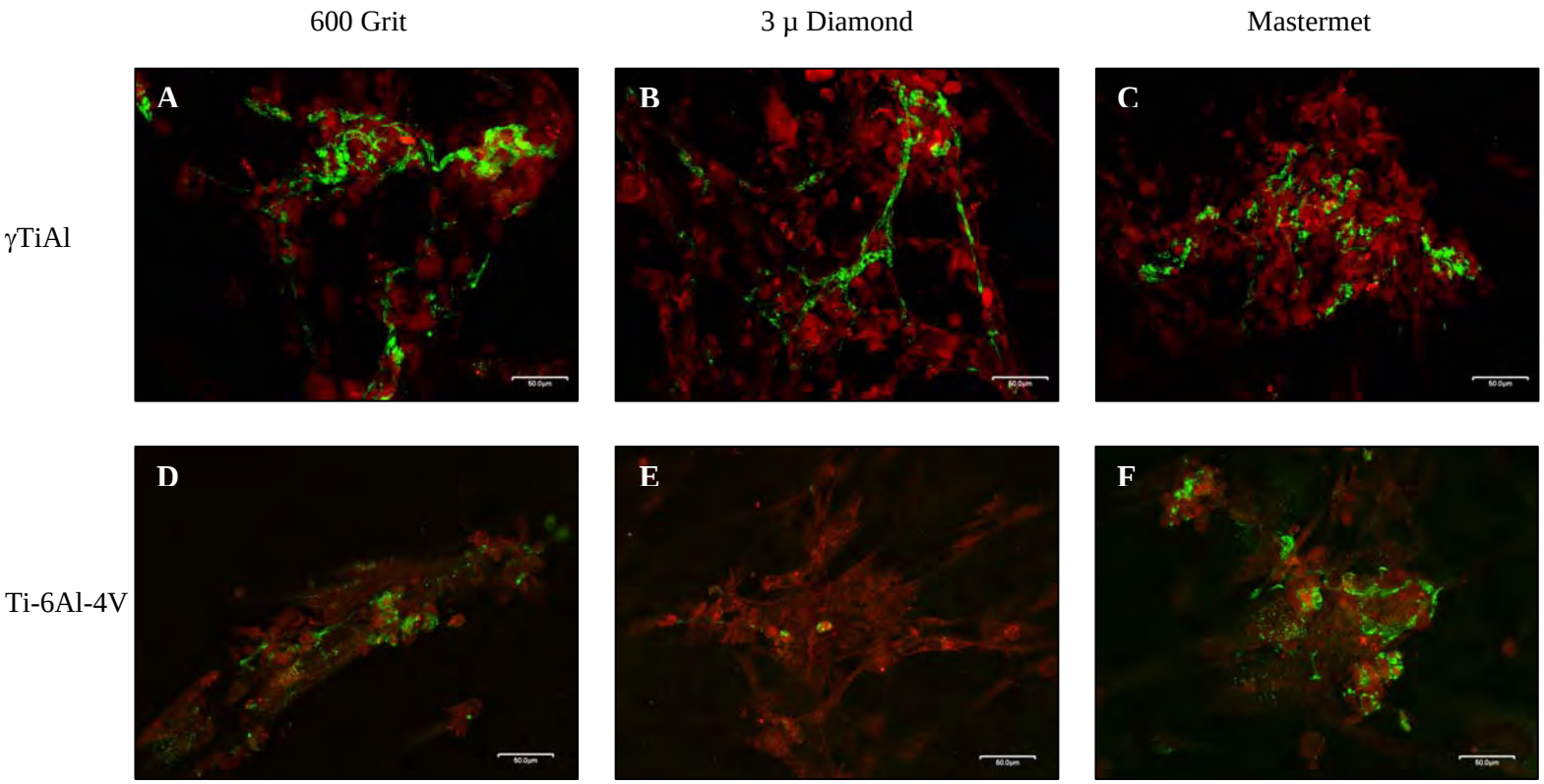


Figure 14. Confocal Laser Scanning Microscopy of the expression of human collagen type I (Rhodamine labeled, red) and osteonectin (Cy5 labeled, green) in hFOB 1.19 cells (A- F) cultured for 21 days at 37 ºC on γTiAl and Ti-6Al-4V with different surface roughness (40 X magnification, scale bar= 50 µm).

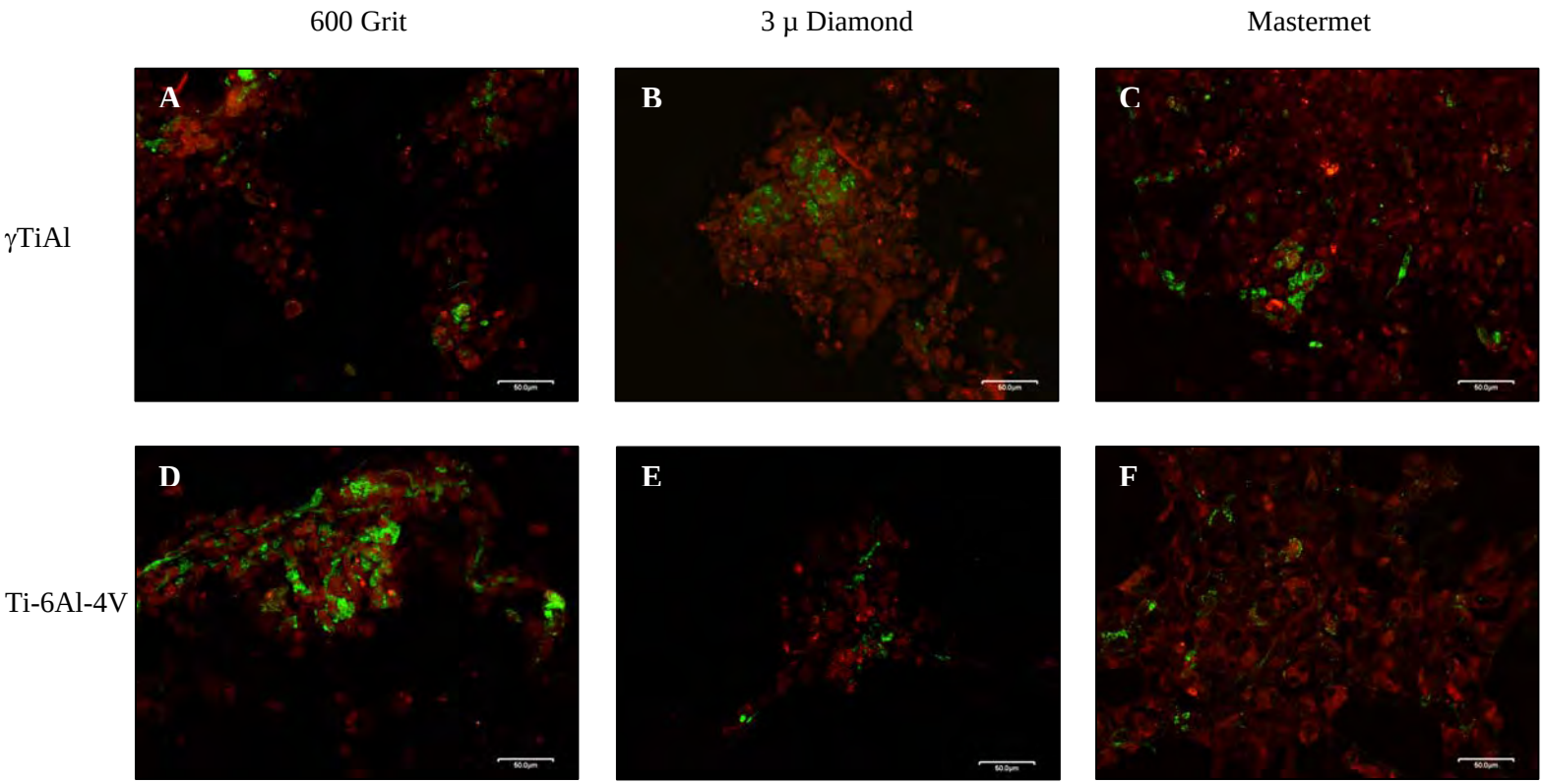


Figure 15. Confocal Laser Scanning Microscopy of the expression of human collagen type I (Rhodamine labeled, red) and osteonectin (Cy5 labeled, green) in hFOB 1.19 cells (A- F) cultured for 28 days at 37 ºC on γTiAl and Ti-6Al-4V with different surface roughness (40 X magnification, scale bar= 50 µm).

Human fetal osteoblast cell (hFOB 1.19) growth assayed by immunofluorescent labeling detected slight differences of cell growth and osteonectin/collagen type I expression when cultured on γTiAl and Ti-6Al-4V. These differences were not evident from SEM analysis, independent of surface roughness.

Based on the results of this study, γTiAl appears to be a biocompatible material for possible medical applications. It has no toxic effects on hFOB 1.19 cells, and cell attachment and proliferation is similar to that obtained for Ti-6Al-4V, the current implant material. Osteoblast cells grew and expressed their normal phenotype when cultured on γTiAl. The synthesis of collagen type I and osteonectin is a clear indication that the presence of γTiAl does not interfere with normal cell function, allowing cells to produce the extracellular matrix. As observed by other investigators (46-48), a significant difference in cell growth, attachment and proliferation could not be observed for samples cultured on γTiAl and Ti-6Al-4V with varying surface roughness: polished with 600 grit, 3μ diamond and Mastermet. These results are very encouraging since a bone-like tissue was formed on all γTiAl samples. The use of γTiAl does not appear to affect the biological activity of hFOB 1.19 osteoblast cells, suggesting that this titanium alloy has the potential to be used as implant material.

CHAPTER V

CONCLUSIONS

- Osteoblast cells hFOB 1.19 growth and attachment on γTiAl was similar to cells grown on Ti-6Al-4V, suggesting that it is not toxic.

- Immunofluorescent labeling assays demonstrated the presence of collagen type I and osteonectin in hFOB 1.19 osteoblast cells cultured on both γTiAl and Ti-6Al-4V surface.

- A difference in hFOB 1.19 osteoblast cell growth, attachment and proliferation was not observed for samples cultured on γTiAl and Ti-6Al-4V with varying surface roughness: polished with 600 grit, 3μ diamond and Mastermet.

- The use of γTiAl does not appear to affect the biological activity of hFOB 1.19 osteoblast cells, suggesting that this titanium alloy has the potential to be used as implant material.

CHAPTER VI

RECOMMENDATIONS

- Separate hFOB 1.19 osteoblast cells from the γTiAl metal surface to study the surface of the tissue layer that is in direct contact with the metal surface to see topography using the SEM.

- Measure the gap between cell layer and metal surface, and cell layer thickness using the SEM.

- Study calcium composition of hFOB 1.19 osteoblast cells cultured on γTiAl using Cameca or EDAX line- scan analysis. The presence of calcium in the samples can serve as a marker for normal bone formation.

- Study cell orientation with regard to the surface topography using shorter periods of incubation in order to determine the effects of γTiAl surface on the cell culture.

- Study alkaline phosphatase (AP) activity of hFOB 1.19 osteoblast cells cultured on γTiAl. Osteoblastic alkaline phosphatase activity is a recognized parameter of cellular differentiation on the implant surface.

- *In vivo* studies should be performed in order to establish the use of γTiAl for bone implants.

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