An *In Vitro* Comparison of Implant Materials Cell Attachment, Cytokine and Osteocalcin Production

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ABSTRACT

Bone deposition, for any implant system, is the deciding factor for the success. The biochemical signals at the cellular level will help elucidate the direction of host response. In this report, intercellular messenger, cytokines, that are regulatory for osteoblast and osteoclast function, were measured. Production of osteocalcin, a marker for osteoblast maturation was also estimated. Human osteoblast-like cells from osteosarcoma cell line MG 63 were grown in wells in the presence of titanium (Ti), titanium alloy (Ti6A14V) and stainless steel implant materials incubated at 37^{0}C. Interleukin- 1α (IL-1α), IL-6, IL-8, IL-11 and osteocalcin were quantitated using standard enzyme linked immunosorbant assay (ELISA) kits from the growth media extracted at specific intervals over the critical ten day period. In all dishes, cells were seen adhering to the base after 24 hours and to confluence at 96 hours. Both IL-1α and IL-11 were not produced in sufficient quantities to be measured in the assay (< pg/ml). Interleukin-6 production was significantly higher for stainless steel than for titanium and the alloy. There was a progressive rise in osteocalcin production for titanium contrasted to a basal rate for stainless steel and alloy. Interleukin-8 levels for all metals and controls increased markedly after two days implicating inherent cellular characteristics. A relatively high constant range for macrophage colony stimulating factor from the first day was seen for all metals, including the controls. In conclusion, it appears that titanium implants activate osteocalcin production while stainless steel activates IL-6.

INTRODUCTION

The definition of implant success is evolving as more knowledge is gained about their performance and the biological reactions to them (1). The first formal assessments conducted in Sweden were based on what was believed as relevant standards in dentistry. That is, gingival and plaque indices, probing depths and aesthetics (2) were considered. Further definitions of success evolved with more emphasis on the amount of bone loss in relation to the height of the implant (3). The widely cited criteria (4), later modified for specific areas in the dental arches, stated that vertical bone loss should be not more than 0.2mm annually following the implant's first year of service (5). A more specific definition of osseo-integration has been defined as a process whereby clinically asymptomatic rigid fixation of alloplastic materials is achieved and maintained in bone during functional loading (6).

The criteria have thus evolved from a clinical to a histological basis. Implant designs have been correlated with a range of success rates (7) and with treatment modalities for failing units (8). Cancellous bone has a limited capacity for carrying load. Provided that overloading is reduced, it will remodel into a more compact form (9). Surgical sites must be individually assessed and adjunct with bone grafts may be necessary to improve outcome (10-13).

Biocompatibility of an implanted device or biomaterial is determined by the response of the host. This involves acute and chronic inflammation as well as the development of granulation tissue. The monocyte macrophage is derived from blood stem cells and is pivotal in direct and indirect mediated inflammatory reactions. In the elucidation of what makes an implant successful, investigations in terms of the biomolecular signals that are transferred between many cell types and which ultimately direct the development of different cell lines must be defined. Some of these biomolecules have been identified and are referred to as cytokines. The term cytokine is applied to water-soluble glycoproteins, which act as chemical communicators between cells, but not effector molecules themselves. The biosynthesis of cytokines depends ultimately on the structure and expression of the genes that encode them. There is evidence that some are presynthesized and stored either in cytoplasmic granules (14), as membrane proteins (15), complexed with cell surface binding proteins and extracellular matrix (16). These pools of cytokine proteins are available for rapid release in response to stimulation. Most cytokines are not continuously expressed in adult animals but are rapidly produced as needed. The functions of

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cytokines are diverse. Along with hormones, they co-ordinate the activities of different tissues and cell types to maintain homeostasis.

Osteoblasts are derived from a common mesenchymal stem cell that can also differentiate into reticular, fibroblastic, adipocytic and osteogenic cells (17). Cytokines have been found to have effects in all the phases of initiation and differentiation of osteoblast precursors. Specific cytokines found at bone remodelling sites may be used as indicators for bone development. These include IL-6, IL-11, granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (MCSF) (18). The list of cytokines and colony stimulating factors implicated in the development of osteoclasts include IL-3, IL-6, IL-11, tissue necrosis factor (TNF), GM-CSF, MCSF 18). It may appear confusing that the same cytokine that performs a constructive role also promotes bone resorption. The same cytokine that stimulate bone cell proliferation may have an inhibitory effect on mature osteoblast function. These agents are generally potent stimulators of bone resorption. An explanation for this is that increased levels of certain cytokines lead to the activation of bone resorption. This stops local bone formation to allow unopposed osteoclastic action and osteoblast precursor proliferation for a subsequent phase of bone formation. In this study, cytokines IL-6, IL-8, and MCSF and osteocalcin produced in the presence of commercially pure titanium (cpTi), Ti6Al4V and stainless steel were measured.

METHODS

Osteosarcoma stem cells MG 63 were cultivated under sterile condition in culture flasks containing growth media in a controlled climate. This osteoblast-like cell line has been well characterized and was used as they produce uniform cultures (19). Equal amounts of these cells were then introduced into a template containing separate vials individually containing cpTi, Ti6Al4V, stainless steel and controls. The controls contained only growth media. Small aliquots of the culture media from each vial was removed at two, four, six, eight and ten-day periods and an equal amount of fresh culture media added. The extracted portion was stored at –20^{0}C.

The Preparation of the Growth Media and Implant Materials

The culture media consisted of a mixture of 0.5% fetal calf serum (GIBCO) in Minimum Essential Media (SIGMA) and 0.2% antibiotics. The antibiotics comprised of 100 units/ml penicillin, 100 µg/ml of streptomycin, 0.25 µg/ml of amphotericin B. The implant materials consisted of commercially pure titanium (cpTi), and titanium alloy (Ti6Al4V) discs 3.5 mm in diameter, weight 124.0 mg and stainless steel mesh (3.5 x 3.5 mm) of weight 14.0 mg. All were treated with 1.0% hydro-phosphoric acid, rinsed with distilled water, autoclaved and then dried in an oven.

Preparation of the Culture Cells: Human Osteoblast-like Stem Cells MG 63

Cells were grown in 12-15 ml culture flasks with a potential growth field area of 75 cm^{2}. They were incubated in a controlled climate at 37^{0}C in humidified atmosphere of 5% CO_2/95% air (LEEC incubator). Cell growth was monitored using a phase contrast microscope and viewed under x10 magnification. When confluent, the culture media was pipetted out and washed with 5 ml Minimum Essential Media, then 5 ml of a 0.25% trypsin in Hanks Balanced Salt Solution (SIGMA) was pipetted into the culture flasks and removed after 20 seconds. This was replaced by 2.5 ml of the same trypsin solution for a further three minutes with slight agitation. This detached the cells effectively from their proteinaceous matrix. Five millilitres of the growth media was pipetted into this suspension and the fetal calf serum in this deactivated any residual trypsin. This suspension was transferred to a centrifuge tube. The cells were centrifuged for 10 minutes at 1300 rpms at room temperature. The media was then aspirated leaving the cells at the bottom.

Cell Count and Plate Set-up

These centrifuged cells were re-suspended in 10 ml of fresh growth media and the cell density determined using a haemocytometer chamber slide stained with 0.4% Trypan Blue Exclusion (Flow Laboratories, IRVINE, Scotland KA12 8NB). Forty microlitres of this suspension containing approximately 40 000 cells were added to each experimental well, in a sterile 24-well experimental plate, containing cpTi, Ti6Al4V and stainless steel. An additional 2 ml of growth media was added to each well. Controls were designed using the identical procedure but leaving out the metals. This experimental template was incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Sample Preparation

Two hundred microlitres were pipetted from each well over a ten-day period every 48 hours and 200 µl of the growth media added at the same time. One control was left untouched throughout the duration of the experiment, after which, samples were taken. The 200 µl aliquot samples were pipetted and frozen at -20°C. In preparation for the quantitation analysis, all 200 µl samples were thawed to room temperature then centrifuged for five minutes at 5000 rpms at room temperature and were divided into four 50 µl portions for analysis by the Enzyme Linked Immuno Assay (ELISA) procedure, (R&D Systems, 4-10 The Quadrant, Barton Lane, Abingdon, OX14 3FA, UK). The results were analyzed by standard ‘t’ test.

Electron Microscope Studies

Scanning electron microscope as well as elemental analysis was undertaken on the implant materials prior to performing the cytokine assay experiments. The elemental analysis of the three metals was carried out under the following conditions. Scan duration – 100 seconds. Beam acceleration potential –

15 KV, beam current – 500 nanoamps. For the growth pattern studies, the experimental procedures were repeated in an identical manner earlier except that a sterile glass cover slip, 13 mm in diameter was introduced in the wells on top of which the various test metals were then placed. The implant materials with their respective cover slips were removed over the identical ten-day period at the same two, four, six, eight, and ten-day intervals. They were then placed in a sterile 5 ml beaker and fixed with 2.5% gluteraldehyde in 0.5% phosphate buffer solution, pH 7.3 and stored at 4°C in preparation for examination in the electron microscope (Hatachi S 520. Japan). All samples were treated in the following manner: washed in 10% ethanol for 10 minutes, washed in 70% acetone for 20 minutes, washed in 90% acetone for 15 minutes and washed three times in 100% acetone for 15 minutes. The samples were then dried to critical point (Critical point dryer CD 750 ENSCOPE, England) at 900lbs/in^2 for 1.5 hours. Increased pressure to 1200 lbs/in^2 then slowly reduced pressure to normal atmospheric. Gold coated with a sputter coater (Sputter coater SC %) ENDSCOPE, England).

At the end of the experiments, all cells and disposable equipment were soaked in 15% clorax solution. Pipettes, reagent bottles and other plastic containers were placed in a toxic disposal bag for appropriate treatment.

RESULTS

Quantitative analysis of IL-6, IL-8, MCSF and osteocalcin are shown in the Table. Interleukin-1α and IL-11 were not found in any samples within the sensitivity range (pg/ml) of the ELISA kits. IL-6 was produced mainly by titanium and stainless steel. For titanium, IL-6 values ranged from 131.7 to 217.8 pg/ml and for stainless steel from 125.0 to 452.2 pg/ml. Thus, a much higher production of IL-6 was found for stainless steel. The alloy Ti6Al4V produced 112.9 pg/ml on the eighth day (Fig. 1). Interleukin-8 production rose significantly for all samples including the controls after two days. After two days IL-8 peaked to 5671.8 pg/ml and maintained a constant high for titanium (5671.8-5567.0 pg/ml). Smaller values were found for the stainless steel (4563.0-3789.0 pg/ml) and the alloy (3456.7-2167.0 pg/ml). The controls also produced IL-8 but profiles showed the smallest quantities (1980.0-2145.0 pg/ml) (Fig. 2). All wells produced high levels of MCSF from the first day, titanium (6782.0-7823.0 pg/ml) stainless steel (6912-7342.0 pg/ml) alloy (6945.0-7891.0 pg/ml) and controls (6914.0-7312.0 pg/ml) (Fig. 3). Osteocalcin was not found in any of the controls. There was a progressive production of this glycoprotein from 4.2 ng/ml to 24.6 ng/ml in wells containing titanium. A basal rate of osteocalcin production was seen for stainless steel (3.2-3.7 ng/ml) over the ten-day period and even less, 1.4 ng/ml and 2.9 ng/ml on the second and eighth days respectfully for the alloy (Fig. 4). Elemental

Table: Quantitative analysis

Cytokine

Interleukin-6 (pg/ml)

	Titanium	STDEV(+/-)	Stainless Steel	STDEV(+/-)	Alloy	STDEV(+/-)	Control	STDEV(+/-)
2 days	131.7	17.4	125.7	16.2		0		0
4 days	156.7	23.4	143.6	21.7		0		0
6 days	146.8	21.6	452.2	27.8		0		0
8 days	217.8	19.8	356.7	34.6		112.9	32.9	0
10 days	143.6	12.8	289.4	19.8		0		0

Interleukin-8 (pg/ml)

	Titanium	STDEV(+/-)	Stainless Steel	STDEV(+/-)	Alloy	STDEV(+/-)	Control	STDEV(+/-)
2 days	256.8	45.1	234.9	98.1	121.9	55.9	290.1	58.9
4 days	5671.8	234.8	4563	198.1	3456.7	234.1	1980	312.1
6 days	5123	215.9	4106	209.7	3178	176.8	2145	165.8
8 days	5423	256.9	3137	242.8	2167	199.3	1784	167.8
10 days	5567	267	3789	195.9	3126	215.9	2081	189.2

Macrophage Colony – Stimulating Factor (pg/ml)

	Titanium	STDEV(+/-)	Stainless Steel	STDEV(+/-)	Alloy	STDEV(+/-)	Control	STDEV(+/-)
2 days	7049	123.8	6998	128	7431	178	6914	218
4 days	7823	109.8	7189	151.8	7534	128.9	7312	170
6 days	6782	112.8	6912	98.7	6945	143.7	7431	191
8 days	7213	132.1	7342	80.3	7110	153.5	7191	182
10 days	7321	142.3	7103	121.8	7891	187.5	7214	197

Osteocalcin (ng/ml)

	Titanium	STDEV(+/-)	Stainless Steel	STDEV(+/-)	Alloy	STDEV(+/-)	Control	STDEV(+/-)
2 days	4.2	1.1	3.7	1.1		2.9	1.7	0
4 days	7.8	2.8	3.4	1.9		0		0
6 days	12.8	4.2	3.2	2.1		0		0
8 days	18.8	5.3	3.5	2.4		1.4	0.9	0
10 days	24.6	3.7	3.7	1.7		0		0

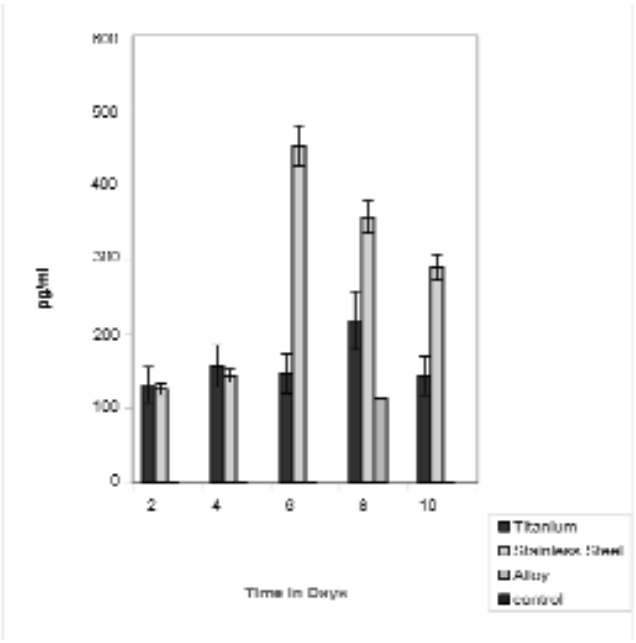

Fig. 1: The production of interleukin–6 from MG 63 cells

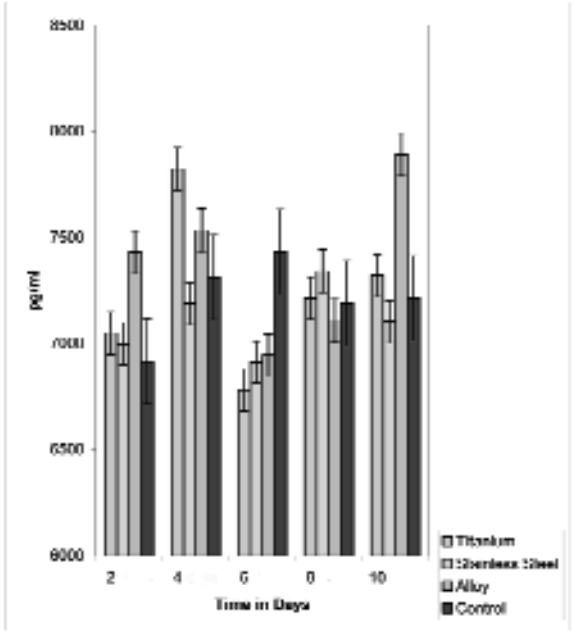

Fig. 3: Production of macrophage colony-stimulating factor

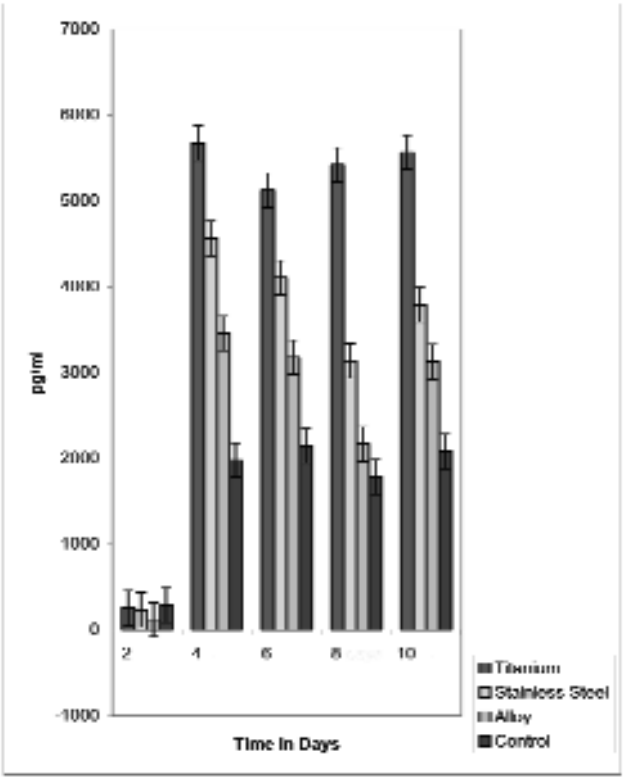

Fig. 2: Production of Interleukin-8

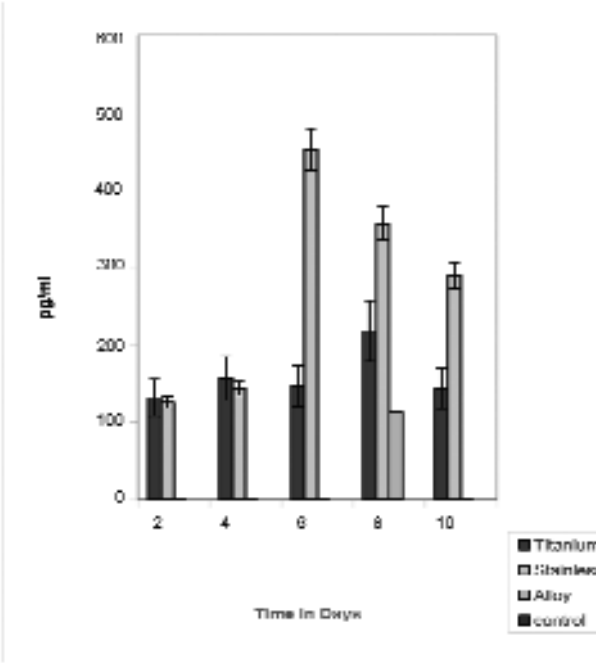

Fig. 4: Production of osteocalcin

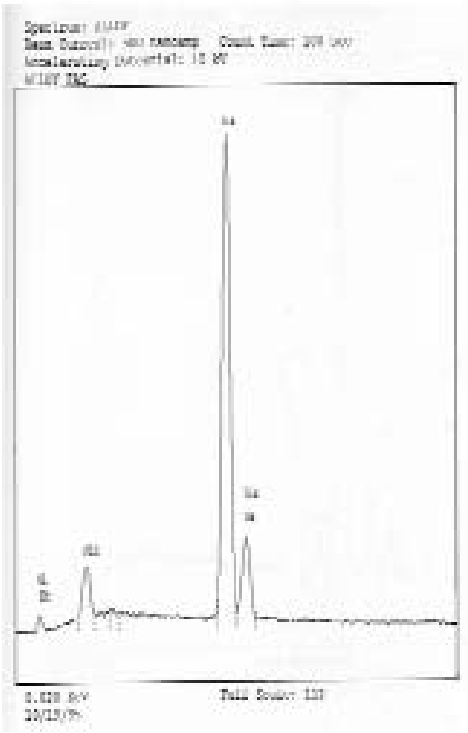

Fig. 5: Spectral analysis of titanium alloy

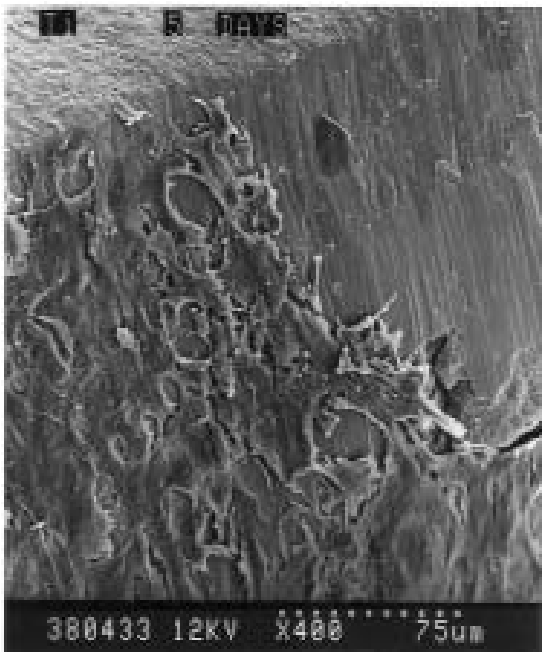

Fig. 6: Cell MG 63 Osteoblast cell line showing growth pattern

analyses for the three metals show six peaks for stainless steel. These correspond to the presence of chromium, iron, and nickel. There were two peaks for titanium and four peaks for the alloy (Fig. 5). Different surface topography was seen between the metals. The least irregular surface was seen for the stainless steel mesh at a magnification of x350 when compared to the other metals at the same magnification. Titanium surface at x350 magnification revealed debris that was removed after cleaning. At x350 magnification, the alloy appeared similar to titanium except that striation can be seen on the alloy. This may be due to the machine preparation of the disc. Cells were seen adherent on all metals within five days. At ten days, an increase in cell growth was seen for all metals (Fig. 6).

DISCUSSION

The use of primary bone cells has been hampered by the consistency of the cell population. Current *in vitro* model systems include transformed cell lines. These exhibit osteoblast behaviour at a specific stage of maturity (17). Transformed human osteosarcoma cell lines, in this case, MG63 may contribute to the understanding of osteoblast function because they represent initial clonal population derived from a specific osteoblast lineage (19). It has been argued that *in vitro* systems cannot replicate the intact mediator or intracellular coregulatory mechanisms that can be found *in vivo* (20). Widespread use of all these culture systems has resulted frequently in divergent responses to the same osteotropic agents being observed. It is not always possible to extrapolate effects of osteosarcoma cell culture with those of bone cell cultures because osteosarcoma cells possess abnormal growth characteristics. Primary bone cell cultures contain heterogeneous cell populations comprised of cells at a different stage of maturation.

Interleukin-1α levels were not detected and if present, were below the sensitivity range. It is synthesized primarily by the monocyte macrophage lineage including osteoclast. In degenerative conditions, it is produced primarily by activated macrophages. Its production by osteoblast thereby occurs in less significant quantities. Studies conducted on quantitative IL-1α secreting assay around bone implants have shown that it is a reflection of the number of macrophages around the failing implants (21). Interleukin-1 is centrally involved in the effector phase of inflammatory response. It may be found in fluids around failing implants in patients who exhibit T-lymphocyte mediated hypersensitivity to metal prostheses (21). It has been commonly mentioned as a marker for bone resorption and periodontal disease (22). In a review of the cause of implant failures (22), an increased response from T-lymphocytes implies that failure may not be due to simple mechanical failure or giant cell reaction to wear debris. The presence of T-lymphocytes and the absence of accompanying B-lymphocytes or plasma cells suggest immunological reactions in the tissues adjacent to the prosthesis. Such a response indicates type IV sensitivity. Implant failure can thus occur after primary osseo-integration. Elemental analysis of metals adjacent to failing implants revealed the complete absence of aluminium and vanadium. Animal studies show that vanadium is very soluble and is cleared from the circulation quickly through the kidneys while titanium is insoluble and remains in the

adjacent tissues (21). It is interesting to note that patients who had negative patch test to titanium salt solution reacted positively to metitanium ointment (23). These results are of course contradictory. There is no standardized procedure for testing titanium sensitivity and no data on the incidence of sensitivity in the general population. Several reports call into question the suitability of titanium alloy as a material for prosthetic implants (21). It is susceptible to fretting corrosion and its metal debris can cause cellular reaction and osteolysis (24–26).

Interluekin-11 was not found in any of the samples including controls in this study whereas MCSF was found in all wells. MCSF is important for the survival, proliferation and differentiation of mononuclear phagocytes, including osteoclast. It is an important mediator of the inflammatory response and can regulate the release of other pro-inflammatory modulators from macrophages. The high and relatively constant value for the MCSF is notable. However, no factor can be correlated with the inhibition or stimulation of this cytokine.

From the experimental results, it can be seen that under the conditions specified, both titanium and stainless steel stimulate IL-6 production. A relatively constant lower rate for titanium and a three-fold increase for stainless steel by day six was observed. An increase in osteoclast activity may also infer a coupling mechanism thereby releasing growth factors for osteoblast precursors. Interleukin-6 is a multifunctional cytokine. Osteoblastic cells in bone have been reported to produce IL-6 (27). This activity is also stimulated by factors that enhance bone resorption (27). Elevated levels of IL-6 are involved in bone destruction and induce hypercalcaemia (27). In some instances, IL-6 failed to demonstrate bone resorbing activity and low levels may act as a local inhibitor of bone resorption (27).

Significant production of IL-8 was found in all wells by day four. While the levels decreased for stainless steel, alloy and the controls, titanium maintained a fairly constant high range from day four to termination of the experiment. The various activities of IL-8 implicate this cytokine as having a major role in mediating inflammatory responses. A correlation exists between the expression of IL-8 and altered cell shape (28, 29). Early studies indicate that cell attachment is better on a roughened surface (30), although the surface morphology does not appear to affect cell spreading (31). Titanium may be considered, in light of the kenetropic effects of IL-8 on cells, to enhance initial cell spreading over the implant surface.

In this study, osteocalcin remained at a steady low for stainless steel. There was a progressive increase for titanium. This may suggest titanium as a factor for faster bone mineralization around titanium implants. A low basal rate with stainless steel may possibly cause a slower response. The deficiency of osteocalcin production seen for the titanium alloy is difficult to interpret since comparable osseointegrative properties of titanium and its alloy have been reported (17). The role of osteocalcin as a marker for late maturation and mineralization of bone reveals favourable support for titanium implants. It has been used as an indicator for osteoblast activity in several studies (32). A low basal rate for stainless steel may indicate a slower response for osteoblast maturation. The relative steady state production of osteocalcin by MG 63 cells exposed to stainless steel in these experiments correlates with earlier reports with human differentiated osteoblast cells (33). One of the main limitations of stainless steel for clinical use is the tendency to corrode when implanted. The release of metallic ions of iron, chromium and nickel into human tissue and fluids must be regarded as a likely source of long-term problems owing to their known toxicity. Chromium has been reported to concentrate in the nucleus and mitochondria, interact with DNA and RNA, inhibit oxidative metabolism and induce neoplastic cell formation (34). Nickel has been proven to induce significant inhibition of mitosis (35). Measurements of osteocalcin directly reflect the metabolic activity of osteoblastic bone cells.

The usefulness of cytokine measurements solely to monitor disease activity is controversial. Assays of bioactivity, notorious for their lack of specificity, have long given way to sensitive immunochemical assays with improved specificity. The ELISA kits, as well as other immunological assay systems, have been facilitated by the rapid development of recombinant cytokine technology. These, however, have been plagued with problems; for example, the lack of cytokine inhibitors, soluble receptors, autoantibodies and complement components (36). Different assay systems may therefore account for some of the discrepancies in the literature regarding the presence or absence of certain cytokines. It remains to be determined how these autoantibodies alter the measurement of serum cytokine by ELISA kits.

Controlled experiments performed *in vitro* undoubtedly provide valuable information of primary functioning systems. *In vivo* testing may find more factors directly and indirectly involved with bone metabolism and possible reveal new variables. In the asymptomatic patient, it is not always clear if the clinical or radiographic findings represent active disease or evidence of past disease. Analyses, which may be more sensitive and highly specific, must be developed to eliminate the false positive results that occur with various proteins that react with assay reagents.

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