A novel spectrofluorometric technique for specific biocompatibility testing of implantable materials by cell culture

Report on use for multiparameter analysis of human osteoblasts cultured on commercially pure titanium and hydroxyapatite

Pierre Doglioli¹, Gérard Scortecchi^{2*} & Mahdocht Falatouni¹

¹ Centre de Formation aux Biotechnologies, 06402 Cannes, France; ² Faculté d'Odontologie, Université de Nice-Sophia Antipolis, Pôle Universitaire St. Jean d'Angély, 24, Avenue des Diablos Bleus, 06357 Nice Cedex 4, France

(* Author for correspondence; 19, Rue Rossini, 06000 Nice, France)

Received 7 December 1998; accepted 7 July 2000

Key words: fluorochromes, osteoblasts, specific biocompatibility, spectrofluorimetry

Abstract

The authors describe a novel spectrofluorometric technique based on double-labelled fluorescence imaging using immunoconjugates labelled with fluorochromes. Following isolation and characterization, cells are seeded on the surface of disks of the material(s) to be tested. After application of a primary antibody and an antibody bearing a fluorochrome, the signal emitted by the molecules in the extracellular matrix on the surface of the test disks is measured by spectrofluorimetry. Measurement is thus independent of the surface characteristics of the test material. Measured values are compared with pre-established standard curves. This technique facilitates determination of the characteristic molecules expressed by a given cell type, thus allowing accurate evaluation of the response of pertinent biological samples to implantable biomaterials.

Introduction

Cell cultures are used extensively to evaluate the biocompatibility of potentially implantable biomaterials. The novel spectrofluorometric technique we have developed measures the intensity of the signal emitted by target molecules in the extracellular matrix following application of appropriate immunoconjugates labelled with the fluorochromes fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) (Begg et al., 1989; Rat et al., 1993). This allows accurate determination of the characteristic molecules expressed by various cell types.

To illustrate the interest of this double-labelled fluorescence imaging technique, osteocalcin and type I collagen expression by osteoblasts cultured in the absence and presence of commercially pure titanium (T40) or hydroxyapatite disks were determined. The cell growth curve reflected the quantitative relation between the test material and the number of cells de-

veloping over a period of time. Cells cultured on a support giving a positive response served as negative controls. Study design simulated the clinical situation encountered in implant dentistry when titanium or hydroxyapatite-coated implants are placed in the jaw.

Materials and methods

Osteoblast isolation and culture

Human osteoblasts were selected as the target cell in this study. Enzymatic isolation of human osteoblasts by collagenase digestion has been described extensively (Escarrot-Charrier et al., 1983; Maurizi et al., 1983; Nefussi et al., 1985; Peck et al., 1964). Characterization is based on determination of membrane alkaline phosphatase activity (Aston et al., 1985; Manduca et al., 1993; Smith et al., 1973; Williams et al., 1980), expression of type I collagen (Beresford et al.,

Table 1. Collagen Type I Standard Curve

	Well number			
	1	2	3	4
1-5 Collagen Type I (ng)	50	100	200	300
UF (mean)	38	80	171	235
SD	0.1	0.2	0.4	0.6

Figure 1. Collagen Type I Standard Curve.

1986; Kasperk et al., 1995; Scott et al., 1980) and osteocalcin (Beresford et al., 1984; Majeska et al., 1985; Price et al., 1976; Rodan et al., 1984; Stenner et al., 1984), and reactivity to 1,34-PTH (Chavassieux et al., 1990).

As described previously (Doglioli and Scortecchi, 1991), fragments of bone from human mandibles and maxillae obtained during preparation of dental implant sites were immediately placed in Dulbecco's minimum essential medium (DMEM) supplemented with antibiotics (1% penicillin-streptomycin). After washing in PBS, the bone tissue was cut up and digested enzymatically using 1 mg ml⁻¹ collagenase in calcium- and magnesium-free phosphate-buffered saline (PBS).

The supernatant fractions were removed 6 times, at 30 min intervals. The first 3 fractions were discarded. The 4th, 5th and 6th fractions, which essentially contain preosteoblasts and osteoblasts (Wong and Cohn, 1975), were filtered on a nylon membrane (pore size 40 μ), centrifuged, and counted by the trypan blue viability test. The cells were then placed in 4-well plastic culture dishes in DMEM supplemented with 10% FCS. The dishes were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air.

In vitro test design

Test disks (dia. 13.5 mm) of commercially pure (CP) titanium (T40) or CP titanium spray-coated with hydroxyapatite were autoclaved for 30 min, then placed in 4-well plastic dishes (2 test wells and 2 control wells per dish). Each well had a diameter of 14 mm.

After a first detachment procedure using trypsin-EDTA, the viability of the osteoblasts destined for biocompatibility studies was determined by cell counts in the presence of trypan blue. The cells were then seeded onto a test disk (titanium or hydroxyapatite) or a plastic culture dish (negative control) (Buser et al., 1991; Doglioli and Scortecchi, 1991; Harmand et al., 1984; Hensten-Patterson, 1977; Taylor, 1970). All of the wells were seeded with 20 000 cells cm⁻² in 0.5 ml DMEM plus 10% fetal calf serum (FCS). The cells were seeded on the surface of the disks. No attempt was made to prevent cells from penetrating under the disks because conditions were such that any such cells would quickly die.

All tests were performed in triplicate, and in sufficient quantity for assays on days 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20, i.e. 30 dishes for determinations on titanium, 30 dishes for hydroxyapatite, and 30 dishes for cell counts. The culture medium was renewed every 4 days.

A range of standards prepared with osteocalcin and type I collagen solutions were used for internal

Table 2. Osteocalcin Standard Curve

	Well number			
	1	2	3	4
Osteocalcin (ng)	50	100	150	200
UF	40	88	118	170
SD	0.1	0.2	0.1	0.4

Figure 2. Osteocalcin Standard Curve.

calibration of the spectrofluorimeter (Fluoroskan II) (Tables 1 and 2; Figures 1 and 2).

Immunolabelling

In order to dose the type I collagen and osteocalcin typically secreted by osteoblasts in the extracellular matrix (zone between the surface of the test disk and the cytoplasmic membrane of the cells), specific antibodies (i.e. anticollagen or antiosteocalcin) and antibodies bearing a fluorochrome (excited molecule that emits a measurable light signal) were applied. The signal emitted at the level of each molecule of collagen or osteocalcin was measured by the spectrofluorimeter (Figure 3).

Type I collagen

At the indicated times, the culture medium was drained from 3 dishes and the wells were rinsed twice with PBS. The cells for immunolabelling were fixed for 1 h with 0.5 ml of 1% glutaraldehyde in PBS. After two rinses with PBS, the antibodies were applied to the cells. The primary antibody consisted in 100 μ l rabbit anti-human type I collagen at a dilution of 1:50 in PBS. After application for 2 h at room temperature, the cells were rinsed three times with PBS. The secondary antibody consisted in 100 μ l FITC-conjugated goat anti-rabbit IgG at a dilution of 1:50 in PBS. This

antibody was applied for 2 h in a humidified atmosphere at room temperature in the dark. The cells were then rinsed three times with PBS.

Three readings were made per well (excitation 495 nm, emission detection 525 nm, gain 60). For internal calibration, the 4 wells of a dish were filled respectively with 150, 100, 50 and 25 μ l of type I collagen solution at a concentration of 2 μ g ml⁻¹ PBS. The dish was then incubated for 2 h at 37 °C, drained, and rinsed twice with PBS. To each well were then added 200 μ l of a solution of 1% bovine serum albumin (BSA) in PBS; the dishes were further incubated for 2 h at 37 °C. After 2 rinses with PBS, each of the wells prepared in this manner was immunolabelled, and results were read in the spectrofluorometer.

Osteocalcin

After fixation of the cells, in the same manner as described for type I collagen immunolabelling, the two antibodies were applied. The primary antibody consisted in 100 μ l mouse anti-human osteocalcin at a dilution of 1:100 in PBS. After application for 2 h at room temperature, the cells were rinsed twice with PBS. The secondary antibody consisted in 100 μ l TRITC-conjugated goat anti-mouse IgG at a dilution of 1:50 in PBS. After application for 2 h in a humidified atmosphere in the dark, the cells were rinsed three times with PBS.

Three readings were made per well (excitation 552 nm, emission detection 570 nm, gain 60). For

Figure 3. Principle of spectrofluorimetry.

Table 3. Cell counts on the test disks and negative controls (1000 cells/cm²)

	Days											
	0	2	4	6	8	10	12	14	16	18	20	
Negative Control	20	15	20	28	39	48	60	71	78	80	78	
SD	1	0.8	1	1.2	1.8	2	2.8	3.1	3.2	3.1	3.1	
Titanium	20	10	12	18	27	38	48	59	66	72	74	
SD	1	0.5	0.5	0.6	1.1	1.3	1.6	2	2.1	2.2	2.2	
Hydroxyapatite	20	24	26	31	34	36	42	49	54	58	62	
SD	1	1	1.2	1.3	1.3	1.4	1.5	1.6	1.8	1.8	1.9

Figure 4. Growth curve of osteoblasts on pure titanium, hydroxyapatite coating and control.

Table 4. Collagen type I in the extracellular matrix (ng/cm²)

	Days											
	0	2	4	6	8	10	12	14	16	18	20	
Negative Control	0.5	5	11	21	37	53	59	60	61	62	62	
SD	0.01	0.1	0.2	0.3	0.4	0.5	0.5	0.5	0.5	0.5	0.5	
Titanium	0	5	17	31	48	71	97	124	133	147	154	
SD	0.001	0.1	0.25	0.4	0.5	0.6	0.8	1	1	1.1	1.2	
Hydroxyapatite	0	0	3	9	15	27	47	64	82	88	92	
SD	0.01	0	0.08	0.15	0.2	0.3	0.5	0.6	0.7	0.7	0.75

Figure 5. Collagen Type I in the extracellular matrix.

Table 5. Osteocalcin in the extracellular matrix (ng cm⁻²)

	Days											
	0	2	4	6	8	10	12	14	16	18	20	
Negative Control	0	0	0	0,1	0,15	0,18	0,21	0,21	0,22	0,22	0,22	
SD	0.01	0,01	0,01	0,1	0,1	0,1	0,20	0,2	0,2	0,2	0,2	
Titanium	0	0	0,05	0,08	0,2	0,32	0,4	0,53	0,72	0,83	0,97	
SD	0.01	0,01	0	0,1	0,2	0,3	0,40	0,5	0,7	0,8	0,9	
Hydrpxyapatite	0	0	0	0,08	0,12	0,3	0,42	0,6	0,72	0,72	0,72	
SD	0.01	0,01	0,01	0,01	0,1	0,3	0,40	0,6	0,7	0,7	0,7

Figure 6. Osteocalcin in the extracellular matrix.

internal calibration, the 4 wells of a dish were filled respectively with 300, 150, 100 and 50 μl of an osteocalcin solution at a concentration of $10^{-2} \mu\text{g ml}^{-1}$ PBS. The dish was then incubated for 2 h at 37 °C, drained, and rinsed twice with PBS. To each well were then added 200 μl of a solution of 1% bovine serum albumin (BSA) in PBS; the dishes were further incubated for 2 h at 37 °C. After 2 rinses with PBS, each of the wells prepared in this manner was immunolabelled, and results were read in the spectrofluorometer.

Cell counts

The disks and the control wells were treated with 0.4 ml trypsin-EDTA for 4 to 5 min. The reaction was stopped by adding 0.1 ml DMEM plus 10% FCS. The suspension was centrifuged for 5 min at 1000 rpm, and the cells were harvested in 1 ml PBS. A trypan blue viability test was performed for each count.

Results

Cell growth (Table 3, Figure 4) over the 20-day study period under identical culture conditions was equivalent on the CP titanium and the negative control (plastic dish). In contrast, cell growth on the hydroxyapatite was 20% lower.

Results

Elevated type I collagen synthesis was observed on both biomaterials (Table 4, Figure 5). After 8 days, spectrofluorometry revealed a 130% increase on the titanium and a 28% increase on the hydroxyapatite compared to the negative controls. At completion of the study (day 20), the ratios were 2.4 for the titanium and 1.48 for the hydroxyapatite compared to the negative control. This intense and very rapid synthesis of type I collagen, especially on the CP titanium, led to production of an extensive extracellular matrix on the biomaterials.

Results

Similar observations were made concerning osteocalcin (Table 5, Figure 6). After 8 days, a 10% increase was observed in the osteoblasts grown on the titanium

while a 20% increase was seen on the hydroxyapatite. At completion of the experiment (day 20), the ratio between the control (0.22) and the titanium (0.97) was 4.4 versus 3.4 with the hydroxyapatite (0.75).

Discussion

Widely used in oral implantology, numerous *in vitro* studies on the biocompatibility of CP titanium have confirmed the positive clinical results reported for over 20 yr. Use of hydroxyapatite coatings has been advocated based on the hypothesis that this substance might improve biointegration by providing the host bone cells with a number of mineral elements involved in the metabolism of osteoblasts (phosphate and calcium ions) (Kent, 1986; Krauser, 1990; Rey, 1991).

Under our experimental conditions, alkaline phosphatase activity and osteocalcin expression on the hydroxyapatite disks were always lower than on the CP titanium, but constantly higher than on the negative control. However, both of these biomaterials were well tolerated by the osteoblast model derived and characterized *in vitro*.

This spectrofluorometric technique facilitated determination of two specific parameters of bone tissue: expression of type I collagen and osteocalcin, the major constituents of the extracellular matrix. Furthermore, these molecules are directly involved in the bone mineralization process, a factor that conditions the long term success of dental implants.

The differences observed between the two biomaterials might be due to the fact that the CP titanium disks had a smooth surface finish whereas the hydroxyapatite was tested in the form of a 50 μ thick coating with a rough surface. Changes in the physicochemical properties of the hydroxyapatite (surface finish, coating thickness, chemical composition, etc.) might thus modify results. However, our study was not designed to compare various surface states, i.e. we were not trying to compare smooth versus rough surfaces.

Spectrofluorometry facilitates biocompatibility studies owing to its reproducibility, reliability and specificity. By improving analysis of specific cell types in direct contact with a biomaterial, this novel technique should contribute to the development of investigations on specific biocompatibility, which up until now have been hindered by technical problems for quantification of the biological elements involved in

cell-biomaterial relationships (alkaline phosphatase, collagen, osteocalcin, proteoglycans).

Furthermore, this technique obviates the need for time-consuming and costly chemical treatments. The test conditions closely reproduce actual conditions in the extracellular matrix secreted by the specific cell type under consideration. Additional studies on other biomaterials are warranted to corroborate these initial findings.

Acknowledgement

The authors wish to thank Nancy Reed-Rameau for translation and preparation of the manuscript.

References

- Begg AC and Mooren E (1989) Rapid fluorescence-based assay for radiosensitivity and chemosensitivity testing in mammalian cells *in vitro*. *Cancer Res* 49: 565.
- Beresford JN, Gallagher JA, Posen JW and Russel RG (1984) Production of osteocalcin by human bone cells *in vitro*. Effects of 1,25 (OH)₂D₃, 24,25 (OH)₂D₃, parathyroid hormone and glucocorticoids. *Metab Bone Dis Res* 5: 229–234.
- Bornstein P and Sage H (1980) Structurally distinct collagen types. *Ann Rev Biochem* 49: 957–1003.
- Boskey AL (1985) Overview of cellular elements and macromolecules implicated in the initiation of mineralization. In: WT Butler (ed.) *The Chemistry and Biology of Mineralized Tissues* (pp. 335–343). EBSCO Media, Birmingham.
- Buser B, Schenk RK, Steinemann S, Fiorellini JP, Fox CH and Stich H (1991) Influence of surface characteristics on bone integration of implants. A histomorphometric study in miniature pigs. *J Biomed Mater Res* 25: 889–902.
- Butler WT (1984) Matrix macromolecules of bone and dentin. *Coll Relat Res* 4: 297–307.
- Camarda AJ, Butler WT, Finkelman RD and Nanci A (1987) Immunocytochemical localization of γ -carboxyglutamic acid-containing proteins osteocalcin in rat bone and dentin. *Calcif Tissue Int* 40: 349–355.
- Chavassieux P, Chenu C, Delmas P and Meunier JP (1990) Influence of experimental conditions on osteoblast activity in human primary bone cell cultures. *J Bone Min Res* 5: 337–343.
- Cook SD, Kay JF, Thomas KA and Jarcho M (1987) Interface mechanics and histology of titanium and hydroxylapatite-coated titanium for dental implant applications. *Int J Oral Maxillofac Implants* 2: 15–22.
- De Groot K, Geesink R, Klein C and Serekian P (1987) Plasma sprayed coatings of hydroxylapatite. *J Biomed Mat Res* 21: 1375–1381.
- Dietrich JW, Canalis EM, Maina DM and Raisz LG (1976) Hormonal control of bone collagen synthesis *in vitro*: Effects of parathyroid hormone and calcitonin. *Endocrinology* 98: 943–949.
- Doglioli P and Scortecchi G (1991) Characterization of endosteal osteoblasts isolated from human maxilla and mandible: An experimental system for biocompatibility tests. *Cytotechnology* 7: 39–48.
- Ducheyne P (1988) Titanium and calcium phosphate ceramic dental implants, surfaces, coatings and interfaces. *J Oral Implant* 14: 325–340.
- Escarot-Charrier B, Glorieux FH, Van der Rest M and Pereira G (1983) Osteoblasts isolated from mouse calvaria initiate matrix mineralization in culture. *J Cell Biol* 96: 639–643.
- Gottlander M, Albrektsson T and Carlsson LV (1992) Histomorphometric study of hydroxylapatite-coated and uncoated CP titanium threaded implants in bone. *Int J Oral Maxillofac Implants* 6: 399–404.
- Harmand MF, Bordenave L, Duplul R, Jeandot R and Ducassou D (1986) Human differentiated cell cultures: *In vitro* models for characterization of cells/biomaterial interface. In: Cristel P et al. (eds) *Biological and Biochemical Performances of Biomaterials*. Elsevier Science Publishers, Amsterdam.
- Hensten-Patterson A and Lieglund K (1977) Evaluation of biologic effects of dental materials using four different cell culture techniques. *Scand J Dent Res* 85: 291–296.
- Kasperk C, Wergedal J, Farley J, Ziegler L et al. (1995) Human bone cell phenotypes differ depending on their skeletal site of origin. *J Clin Endocrin Metab* 80: 2511–2517.
- Kay JF, Jarcho M and Logan G (1986) The structure and properties of HA-coatings on metal. *Biomaterials* 13: 9–15.
- Kent JN, Block MS and Kay JF (1986) Hydroxylapatite coated and noncoated dental implants in dogs. *Biomaterials* 16: 172–176.
- Krauser JT, Boner C and Boner N (1990) Implants dentaires recouverts d'une couche d'hydroxyapatite. *Cah Prothèse* 71: 57–75.
- Lowenberg JT, Pillar RM, Aubin JE, Sodek J and Melcher AH (1989) Cell attachment of human gingival fibroblasts *in vitro* to porous-surfaced titanium alloy discs coated with collagen and platelet-derived growth factor. *Biomaterials* 9: 423–432.
- Lum LB, Beirne OR and Curtis DA (1991) Histologic evaluation of hydroxylapatite-coated versus uncoated titanium blade implants in delayed and immediately loaded applications. *Int J Oral Maxillofac Implants* 6(4): 456–462.
- Majeska RJ, Rodan SB and Rodan GA (1985) Culture and activity of osteoblasts and osteoblast-like cells. In: Butler WT (ed.) *The Chemistry and Biology of Mineralized Tissues*. Birmingham, Alabama, EBSCO Media Inc, 279–285.
- Manduca P, Sanguineti C and Santolini F et al. (1993) Differential expression of alkaline phosphatase in clones of human osteoblast-like cells. *J Bone Mineral Res* 8: 291–300.
- Maurizi M, Binaglia L and Venti Denti G (1983) Morphological and functional characteristics of human temporal bone cell cultures. *Cell Tissue Res* 229: 505–513.
- Peck WA, Birge SJ and Fedak SA (1964) Bone cells: Biochemical and biological studies after enzymatic isolation. *Science* 146: 1476–1477.
- Price PA, Otsuka AS, Poser JW, Krislaponis J and Raman N (1976) Characterization of a β -carboxyglutamic acid-containing protein from bone. *Proc Natl Acad Sci USA* 43: 1447–1451.
- Rat P (1993) New microtitration fluorometric technology: Applications to dermatotoxicity. *Nouv Dermatol* 12: 471.
- Rey C, Precher M, Lacout JL and Vignoles M (1991) Apatite chemistry in biomaterial preparation, shaping and biological behaviour. In: Bonfield W, Hastings GW and Tannei KE (eds) *Bioceramics*, Vol. 4, London.
- Rivero DP, Fox J, Skipor AK, Urban R and Galante JO (1988) Calcium phosphate-coated porous titanium implants for enhanced skeletal fixation. *J Biomed Mater Res* 22: 191–202.
- Rodan GA and Rodan SB (1984) Expression of the osteoblastic phenotype. In: Peck WA (ed.) *Bone and Mineral Research* 2 (pp. 244–285). Elsevier Science Publishers, Amsterdam.

- Schroeder A, Van der Zypen E, Stich H and Sutter F (1981) The reactions of bone connective tissue and epithelium to endosteal implants with titanium sprayed surfaces. *J Maxillofac Surg* 9: 15–25.
- Scott D, Kent GN and Cohn DV (1980) Collagen synthesis in cultured osteoblast-like cells. *Arch Biochem Biophys* 201: 384–391.
- Sennerby L (1991) On the Bone Tissue Response to Titanium Implants. Thesis, University of Göteborg, Sweden.
- Smith DM, Johnston CC and Severson AA (1973) Studies of the metabolism of separated bone cells. *Calcif Tissue Res* 11: 56–59.
- Stenner DD, Romberg RW, Tracy RP and Mann KG (1984) Monoclonal antibodies to native noncollagenous bone-specific proteins. *Proc Nat Acad Sci USA* 81: 2868–2872.
- Taylor AC (1970) Adhesion of cells to surfaces. In: RS Manly (ed.) *Adhesion in Biological Systems*. New York, Academic Press.
- Taylor DL, Amato PA and Tanasugarn T (1986) In: Talor DL and Birge RR (eds) *Applications of Fluorescence in the Biomedical Sciences* (pp. 347–376). Liss, New York.
- Termine JD, Belcourt AB, Conn KM and Kleinman HK (1981) Mineral and collagen-binding proteins of fetal calf bone. *J Biol Chem* 256: 10403–10408.
- The TH and Feltkamp TEW (1970) *Immunology* 18: 865–873.
- Triffitt JT (1987) The special proteins of bone tissue. *Clin Sci* 72: 399–408.
- Wergedal JE and Baylink DJ (1984) Characterization of cells isolated and cultured from human bones. *Proc Soc Exp Biol Med* 176: 27–31.
- Williams DC, Boder GB, Toomey RE, Paul DC and Hillman Jr, CJ (1980) Mineralization and metabolic response in serial passaged adult rat bone cells. *Calcif Tissue Int* 30: 233–246.
- Wong GL and Cohn DV (1975) Target cells in bone for parathormone and calcitonin are different: Enrichment for each cell type by sequential digestion of mouse calvaria and selective adhesion to polymeric surfaces. *Proc Natl Acad Sci USA* 72: 3167–3171.
- Ziats NP, Miller KM and Anderson JM (1988) *In vitro* and *in vivo* interactions of cells with biomaterials, *Biomaterials* 9: 5–13.