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The Separation of Proteins from media of Rat Osteoblast grown in the presence of biomaterials using Polyacrylamide Gel Electrophoresis

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Abstract
Osteoblasts harvested from calvaria from newly born Sprague-Dawley rats by enzyme digestion were cultured in a 24 well plate with the require media along with Co-Cr, PMMA, titanium implant fixtures, suture material and heat-treated wood. Growth media was sequentially removed at three, seven and ten days and subjected to one dimensional gel electrophoresis. At day three, there as a difference in protein expression between the materials. At day seven, there is greater uniformity in protein distribution seen for all materials. At day ten the media taken from wells containing cobalt-chrome, wood, and implant fixtures did not show a protein band corresponding to 24-25 kDa molecular weight. The experiments performed is sensitive enough to separate proteins consistent with a typical *in vitro* pre-clinical biocompatibility test. The results found here clearly indicate that the osteoblasts cells behave differently when grown with different materials.

Keywords: Biomaterials, Osteoblasts, Cell cultures, Biocompatible Wood, Implants, Medical Devices.

1. Introduction

Cell culture experiments are an integral step for *in vitro* testing of chemicals and biomaterials that are used for human. They are an invaluable technique and represents the closest model for investigating the physiological and biochemical properties of normal cells. Primary cell cultures, although more difficult to culture, have been proven to be far more representative of the animal than immortalised cell lines [1-4]. The culture media where cells are grown represents a form of extracellular matrix. This media has a wealth of information from which the activity of the cells can be observed. Osteoblast are specialized cells produced from mesenchymal cell lineage [5]. In the skull, the process of the direct transformation of these mesenchymal cells to bone occurs as intramembranous ossification. Another pathway occurs where the mesenchymal cells first differentiate into cartilage which later becomes ossified is referred to as endochondral ossification and occurs in the long bones. There are collagenous as well as non-collagenous proteins secreted from the osteoblast. Collagen type I, III and V have been found in extracellular matrix of rat calvaria cultures [6]. The same study found alkaline phosphatase as one band at 116 kDa at confluence and two bands 116, 140 kDa in older cultures. Similar finding of 160 kDa was found from 8–12-day old calvaria harvested from Wister rats [7]. It is generally accepted that the alkaline phosphatase found in rat osteoblast has an average molecular weight of 125-130 kDa [8]. Osteoblasts synthesize collagen which is subsequently mineralized [9]. Collagen type I is the predominant protein found in the extracellular matrix of osteoblast. It consists of a triple alpha chain, 110 kDa and a peptide of approximately 1 kDa [10]. There are many other proteins produced by rat osteoblast cells including osteocalcin which has a molecular weight of 5.6 kDa [11]. There is a phosphorylated glycoprotein which shows up as a band at 45 kDa using gel electrophoresis [12] and a large protein termed osteoactivin which is closely related to osteoblast activity and has a molecular weight of 63.8 kDa [13]. There are still many more proteins secreted by osteoblasts. After proliferation, there is a deposition of extracellular matrix, following which the bone becomes mineralised [14]. During these changes it would be expected that the proteins expressed would vary based on the cellular function.

Amongst the proteins secreted there is osteonectin 43 kDa, osteopontin 27 kDa, although these weights do not refer to the rat [15]. There are several smaller proteins that work in the signalling pathway as part of the metabolic processes of the cell changes. These include factors Osterix approximately 45 kDa, ATF4, 39 kDa, SATB2, 81 kDa, TGF-beta 44 kDa, FGF, 23 kDa and ephrin, 24 kDa [16]. These and more are secreted into the extracellular matrix of bone in the process of metabolism.

2. Aim

The aim of this experiment was to determine if one dimensional gel electrophoresis could detect any difference in proteins expression by osteoblast grown in the presence of various biomaterials.

3. Materials and Methods

This research proposal for the use of animals was approved by the Ethics Committee, Faculty of Medical Sciences, The University of the West Indies, Trinidad (Ref number: EC65:21/12-06/07).

3.1 Sample materials tested

The materials tested in this experiment were cobalt-chromium obtained from Nobilium®. The polymethyl methacrylate (PMMA) purchased from SuperDental Co® was prepared according to the manufacturer's instructions. Titanium was purchased from American Elements® Merelex Corp. The implant fixtures were obtained from Astra Tech®. They were made from Ti, micro-threaded with roughened surface and 6.0 mm in length, plus 3-0 Coated Vicryl™ 16.0 mm in length manufactured by Ethicon® and supplied by Johnson and Johnson Intl. Vicryl™ is an absorbable synthetic suture material made of a polymer of 90% glycolide and 10% L-lactide. The entire length was rolled into a circle 8.0 mm in diameter, so it fitted into the vial of the experimental template. The wood discs (*pinus)*were pre-treated by placing them in an oven for 12 hours at 200 ºC.

3.2 Stock reagents preparation and cell harvest procedure

One litre of phosphate buffered (PBS) saline was prepared 1x PBS using sterile distilled water containing 8.0 g NaCl, 1.44 g Na_2HPO_4, 0.24 g KH_2 PO_4, and 0.2 g KCl (10 mM PO_4^{3-} 137 mM NaCl, and 2.7 mM KCl). This solution was adjusted to pH 7.4 using 10 M NaOH and concentrated HCl then cold filtered into sterile bottles under vacuum using the Nalgene® 20 µM bottle top filter and stored at room temperature. Hank's balanced salt solutions (HBSS) were made up with and without calcium and magnesium as 1 x HBSS using sterile distilled water containing 8.0 g NaCl, 0.358 g Na_2HPO_4, 0.6 g KH_2 PO_4, 0.4 g KCl, 1.0 g glucose, 0.185 g $CaCl_2$ and 0.0978 g of anhydrous $MgSO_4$. The pH was adjusted to 7.4 using 10 M NaOH and concentrated HCl then cold filtered and stored in the refrigerator at 4 ºC in dark bottles. Both solutions were tested for fungal/bacterial growth by incubating small aliquots in the humidified CO_2/Air incubator for 2 weeks. Complete growth media consisted of a cocktail of the following chemicals: basal cell media 199, or Minimum Essential Media (MEM) purchased from Sigma-Aldrich, 10% foetal calf serum; and 0.2% antibiotic (100 U penicillin/100 µg/mL streptomycin and 0.25 µg/mL amphotericin B) also purchased from Sigma-Aldrich. Collagenase enzyme solution was prepared fresh using HBSS. Collagenase dry powder was purchased from Fisher Bioreagents. It was made into solution that contained, 0.25% collagenase, 0.5 mM $CaCl_2$ and 0.5 mM $MgSO_4$ freshly made up to 5.0 mL. The collagenase used to make this solution appeared cloudy and particulate in solution. This was then cold filtered through Nalgene® bottle-top filters non-surfactant cellulose acetate membrane 20 µM pore size, under vacuum and collected into sterile glass bottles. This was used immediately on preparation. Trypsin-EDTA solution without calcium and magnesium solution was purchased from Sigma-Aldrich. This was stored in aliquots of 5 mL between 4 ºC and 6 ºC in a refrigerator. The solution consisted of 1.0 mg/mL trypsin in PBS pH 7.4. Vials were quickly equilibrated to 37 ºC prior to use. Tyrode's solution, Ca^{2+} and Mg^{2+} free, containing 5 mM EDTA was also purchased from Sigma-Aldrich and stored between 4 ºC and 6 ºC in a freezer. This reagent was thawed and equilibrated to 37 ºC prior to use.

3.3 Cell harvest and cell count

Sprague Dawley rats used in these experiments was obtained from Charles Rivers Laboratories® USA. Three to four-day old rats were used. In this instance 5 rats, 3 - 4 days old were used. A description of the rats used is given in Table 1. Osteoblast cells was harvested by the sequential enzymatic digestion of the calvaria. They were decapitated using a sharp, clean guillotine. Each head was dipped in a solution of 70% ethanol and place on a sterile paper towel in the laminar fume cabinet. The skull was exposed by cutting through the skin. The calvaria was scraped free of the periosteum and cut out using a small scissors. The exposed tissue was kept moist by dropping small amounts of sterile Ca^{2+}-free and Mg^{2+}-free Tyrode's solution onto the surface then placed in a petri dish containing 10.0 mL of Tyrode's solution. They were cut up using a small scissors into ~ 0.5 mm pieces and transferred to a sterile bottle containing 2.5 mL of 1.0 mg/mL trypsin in PBS, pH 7.4 and incubated for 10 minutes at 37 ºC. After 10 minutes the trypsin reaction was stopped by the addition of 5 mL of Medium 199, with glutamine and Earle's salts (MEM) with 10% foetal calf serum (FCS) (Sigma-Aldrich). The trypsin digest was decanted, and the bone fragments washed with 5 mL of PBS, this liquid was also discarded. The bone fragments were then immediately transferred to a sterile 150 mL glass bottle containing 5.0 mL of 0.25% collagenase (Fisher Bioreagents) in Hank's balanced salt solution with 0.5 mM calcium chloride and 0.5 mM magnesium chloride without EDTA and incubated for one hour with slight agitation in a water bath at 37 ºC. After one hour the container was removed from the water bath and placed on the bench top for 5 minutes. The liquid was gently aspirated and transferred to a sterile centrifuge tube and spun at 400 g for 5 minutes. The cells that made up the pellet were dispersed by the addition of 10.0 mL of growth medium followed by gentle vortex. The growth media consisted of MEM containing 10% FCS and 0.2% antibiotics (100 units penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B). Freshly re-suspended cells were then distributed equally into 24 wells containing titanium, cobalt-chromium, PMMA, suture material, implant fixtures and wood. These materials were in the form of a disc that fitted into the vials. The dimension of all the discs used for the experiments were approximately 14.5

mm in diameter and 0.2 - 0.5 mm in thickness. The exception being suture material and the implant fixtures. Two 24-well experimental templates were used and 250 µL of the cell suspension was added to each of 24 wells. A blank with media only was included. All materials including the blank were done in replicates of 4. A glass slide haemocytometer was used to count live cells that did not take up stain with Trypan Blue solution. To count the cells, entire suspension was aspirated using a 25 mL pipette and gently dispensed back into the same container. From this, 100 µL was pipetted and placed in an Eppendorf tube and 400 µL of 4% Trypan Blue added. A micropipette was used to transfer quickly and smoothly 10 µL of this suspension into the haemocytometer slide. The count was repeated 4 times and the average used to obtain the average amount of cells in the stock solution using the formula: Average number of cells/ml = Average number of cells in 5 squares x 2 x10^4 x dilution factor. Average amount of cells in 5 squares = 25.6 in 0.1 µL which amounted to 25.6 x 10^4 cells in diluted sample 1 mL. Multiply by 5 for the dilution = 128 x 10^4 cells/mL of stock solution. In each well, 250 µL of cell suspension was dispensed. This provided an average of 32 x 10^4 osteoblast cells per well.

3.4 Polyacrylamide gel preparation and sample preparation
Acrylamide/Bis (Sigma/Aldrich) (30%), 100 mL was prepared using distilled water. This was filtered through Nalgene® bottle-top filters non-surfactant cellulose acetate membrane 0.2 µm pore size, under vacuum and collected into sterile dark glass bottles. This solution was kept at 4 ºC in the dark. Tris-HCl (Sigma/Aldrich), 300 mL, concentration of 1.5 M adjusted to pH 8.8 was prepared using distilled water, also stored at 4 ºC. Tris-HCl (Sigma/Aldrich), 100 mL, concentration of 0.5 M was prepared and stored in a similar manner. SDS was prepared at a concentration of 10% using de-ionized water dIH_2O and made up to 100 mL. The sample buffer was made up to 8 mL using the following reagents: 3.8 mL distilled water, 1.0 mL of 0.5 M Tris-HCl, pH 6.8, 0.8 mL glycerol, 1.6 mL of 10% SDS, 0.4 mL 2 β-mercaptoethanol and 0.4 ml of 0.05% bromophenol blue. The electrode SDS running buffer was prepared 5X by combining Tris base (15 g/L), Glycine (72 g/L) and SDS (5 g/L). This was made up to 300 mL of dH_2O and the pH adjusted to 8.3. This was stored at 4 ºC. Before using it was warmed to 37 ºC in the water bath and diluted 300 mL in 1200 mL dIH_2O. The separating gel was made using a 12% concentration of acrylamide. It was prepared by combining the following reagents. Distilled water 33.5 mL, 1.5 M Tris-HCL, pH 8.8, 25.0 mL, 1.0 mL of 10% SDS, 40.0 mL of 30% acrylamide stock. It was de-gassed for approximately 15 minutes before use. Freshly prepared 10% ammonium persulfate, 500 µL and 50 µL TEMED added as the last reagent and this liquid was poured into 7.3 x 8 cm gel electrophoresis plates separated by a spacer of 0.5 mm. The stacking gel that formed the comb or wells to hold the samples on top the separating gel contained a lower concentration of acrylamide. The concentration of acrylamide used in the preparation of this gel was 4%. It was prepared using the following reagents: Distilled water 6.1 mL, 2.5 mL of 0.5 M Tris-HCl, pH 6.8, 100 µL of 10% SDS and 1.3 mL of 30% acrylamide/Bis which was again degassed for 15 minutes. TEMED 10 µL and 10% ammonium persulfate, 50 µL was added as the last reagents and this liquid was poured on the top of the separating gel in the glass slabs. A 10-well spacer 0.5 mm thickness was inserted immediately after the stacking gel was introduced. This effectively formed the wells or sample chamber.
The electrophoresis chamber used was the PROTEAN® 3 MINI-GELS. The gels used here were 7.3 x 8 cm. The staining solution used was Coomassie Blue R-250. The powder was purchased from Sigma-Aldrich. The staining solution used consisted of a 0.1% Coomassie Blue R-250 solution made up in 40% methanol and 10% acetic acid, both of which was also purchased from Sigma-Aldrich. The destaining solution was made up with 40% methanol and 10% acetic acid. The running potential was set at 120 V and the running time was kept at 1 1/2 hours.

3.5 Sample Preparation and Running the Gel
This procedure was performed in a sterile fume hood over a period of 10 days with samples collected at 3-, 7- and 10-day intervals. The experimental cell culture plate was removed from the incubator and 1.0 mL of the culture solution pipetted out of each well and placed into sterile Nunc® CryoTubes® cryogenic vials, 1.8 mL, internal thread, and stored at liquid nitrogen. Then 1.0 mL of fresh culture media was added to all the wells to replace the volume removed and culture plate returned to the incubator. The samples were removed from the freezer and thawed rapidly by inserting the vials in a water bath set at room temperature. Each vial was spun at 1000 g for 20 mins to settle any dead cells, membranes, fragments and debris. The supernatant was decanted and 20 µL of this sample was pipetted and added to 80 µL of the sample buffer containing SDS and β-mercaptoethanol in 2 mL Eppendorf tubes. These samples were then heated at 95 ºC in a water bath for 4 minutes. The wide molecular weight standard (Sigma) was reconstituted using 100 µL dIH_2O, also heated at 95 ºC for 4 minutes. Each 25 µL of diluted sample was carefully transferred using a micropipette into the wells of the stacking gels. Twenty-five microliters of the molecular weight standard were placed in the last well. Sample consisted of media collected from wells containing each of the various test materials. Each sample was run in duplicates at the same time. The exact protocol was repeated in triplicate for each of day 3, 7 and 10 respectfully totalling 6 plates. Additionally, samples of media from wells containing one material were run on separate gels, also collected at 3, 7 and 10 days. This was tried for 5 different materials making a total of 10 gel plates. The images of the various gels were photographed, after which the images uploaded onto the computer. A software program SynGene® was used to identify the presence of bands and relative molecular weight based on the standard wide molecular weight range Sigma® (No: 4036) molecular weight ladder. The amount of protein was not estimated in this instance.

4. Results and Discussion
Graphs were plotted of relative molecular weight of the protein bands from the gels. The values were calculated using the molecular weight of the standard and the distances the bands travelled. When all materials were matched on one gel at day three, for the implant fixture, wood, titanium and Vicryl® there was an absence of a protein band in the 51-59 kDa molecular weight range. The media that contained heat-cured PMMA, cobalt-chrome

and cells only produced similar number of protein bands with equally matching molecular weight. At day 7, there is greater uniformity in protein distribution seen for all materials. However, the number of bands appearing in the gel for each material dropped from 5-6 bands at day 3 to 4 bands for each material tested. At day 10 the pattern remained essentially consistent with respect to the media only, PMMA, titanium and Vicryl®. Four bands were seen at approximate molecular weights range 70-126. kDa. On day seven, the media taken from well containing cobalt-chrome, wood, and implant fixtures did not show a protein band corresponding to 24-25 kDa molecular weight. Similar observations were found in the gels ran for samples of media for individual test material collected on different days. This was true for all the materials. In the gels where samples from the well containing the implant fixture, wood, titanium and Vicryl®, there was an absence of protein bands in the 51-59 kDa range on day three. At day 7, and 10, the protein bands produced here are comparable to those found in gels when all materials were matched on one gel.

The results found here clearly indicate that the osteoblasts cells behave differently when grown with different materials. This was reflected in the difference in protein bands seen in the gels. There is a difference in bands after day 7. ALP isoenzyme exists as high molecular weight and low molecular weight forms as a result of genetic and post-translational modifications. The molecular weight is generally quoted within the range 19-97 kDa [17-20]. Variations in the molecular weight of ALP found in the gels may reflect the presence of various isoenzymes as well as heterogeneity between samples.

Another marker is called 'bone gla protein', BGP, osteocalcin. As with the ALP test, samples that are used comprise serum. Serum BGA is a commonly used marker for bone formation [19]. This small protein of 49 amino acids with an estimated molecular weight of 5.8-6 kDa is produced by osteoblasts [21]. Early past experiments have isolated and purified osteocalcin from rat teeth again using the identical protocol of 5-15 % SDS-PAGE method [22]. They found the osteocalcin to weigh 18 kDa. Collagen based markers for bone formation include C-terminus and N-terminus propeptide of type I procollagen (PICP and PINP). The larger procollagen type I polypeptide has an amino acid chain attached on each end of a collagen chain and is the precursor for collagen type I, the major component of bone matrix. During the process of collagen assembly these additional amino acids chains are cleaved off. The PICP is a globular glycoprotein with mannose, 115 kDa molecular weight located at the C- terminus end of the procollagen. PINP is an extended phosphorylated shorter chain with a molecular weight of 70 kDa attached at the N-terminus end. These are used as markers of bone formation, and in the evaluation during the treatment for osteoporosis [23].

These experiments found that electrophoresis of the various culture media in which osteoblast were grown produce gels that show a definite inconsistency of proteins. It is not possible to say if some proteins are consumed by cellular function, if they are de-regulated or produced as a result of the material tested. Although the analysis done here is qualitative the inference that different implant material might alter osteoblast physiology and that this pattern maybe reproducible warrants further calibrated analysis and validation.

The experiments performed so far have proven that the method is sensitive enough to separate proteins and observe differences according to the requirements of a typical *in vitro* pre-clinical biocompatibility test. Results of duplicate experiments from the same laboratory as well as in other laboratories will need to be compared and matched validate this technique.

5. Conclusion

Biocompatibility experiments should evolve to use simpler and more relevant models for testing devices that are used for human consumption.

Table 1. The Weights and Sex of the 5 rats used in the One-Dimension (PAGE) Experiment.

Weight of rat	Sex (M or F)
4.0	M
3.8	F
4.0	F
3.9	F
4.1	F

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