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Protein adsorption to titania surfaces

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Titanium and its alloys are used widely in the manufacture of orthopaedic and dental implants. Their popularity is encouraged by the excellent biocompatibility of the surface oxide layer and the phenomenon of osseointegration that occurs following surgical implantation into bone. This term describes the fixation of a medical device through the formation of a direct interface with bone tissue. However, the processes that lead to osseointegration are not fully understood and, in particular, the contribution of selective protein adsorption to clinical success is the subject of debate. The aim of this study was, therefore, to investigate the adsorption of serum proteins to titanium oxide (titania) and to non-integrating surfaces as controls using a number of methods. *In situ* labelling of proteins with biotin and subsequent detection with avidin peroxidase on electroblots and an ELISA method gave the best results. Differences were observed between protein adsorption to the two classes of surfaces, with selective adsorption of vitronectin to the osseointegrating surfaces. It is suggested that this may influence the subsequent behaviour of cells and the process of osseointegration.

1. Introduction

Titanium implants were first introduced by Branemark in the early 1970s [1] and have been widely used in the medical and dental fields with excellent clinical results. They owe this success to their good biocompatibility with living tissues. In addition, bone appears to bond with the titanium oxide layer following implantation, a phenomenon termed osseointegration [2]. Whilst this concept of osseointegration is generally accepted, the series of events leading up to it is poorly understood.

It is well known that serum proteins adsorb to inorganic surfaces almost immediately on contact with blood following surgical implantation and that the original surface, therefore, is no longer in contact with the host tissue [3]. However, little is known about the specificity of this process, and still less is known about the proteins that adsorb on to titania surfaces. In terms of the host tissue–biomaterial interaction, the role of adsorbed proteins is likely to be an important one because subsequent events may depend on the composition and conformation of this protein layer and its tendency to change over time. The final protein film may trigger the activation of complementary coagulation cascades and other inflammatory responses [4, 5].

There is extensive literature on protein binding to polymeric materials, with some speculation on the possible mechanisms [6–11], but there has been little attempt at identifying the specific proteins present. This is, in part, due to the limitations of the techniques available for such a study. This paper outlines two methods for evaluating the proteins that adsorb from complex mixtures to material surfaces. For the purpose of this study, particular attention was paid to some of the adhesive proteins, namely fibrinogen, fibronectin and vitronectin, because of their known ability to interact with collagen and influence cellular adhesion.

2. Materials and methods

2.1. Materials

Titania surfaces designed to mimic the surface of a commercially pure titanium implant (cpTi) were produced using a sol–gel technique. Plain glass pieces (5 × 15 mm) were dip-coated with a sol of titanium (IV) propoxide and isopropanol. Dipping was carried out in a laminar flow cabinet at a controlled withdrawal rate of 0.1 mm/s. Gel formation was initiated by the addition of a controlled amount of distilled water before dipping. The process was completed by air-drying and densification was achieved by firing at a temperature of 600 °C for one hour in an air furnace. The coatings thus produced were characterized using X-ray Photoelectron Spectroscopy (XPS) and compared to the surface of a cpTi implant. These sol–gel surfaces offer some advantages over titanium metal because they are relatively inexpensive, easy to produce and reproducible. They may confer

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osseointegrating properties to inert substrates, and ultimately, this route could offer truly novel or designer surfaces with which to control biological responses. Details of preparation and surface chemistry have been given in a previous paper [12].

2.2. Protein adsorption studies

2.2.1. Electrophoresis and immunoblotting

Adsorption of serum proteins to various test materials was investigated. The test materials represented osseointegrating (sol–gel titania and cpTi) and non-integrating (plain glass and polytetrafluorethylene-PTFE) surfaces. These materials were incubated in human citrated plasma for one hour at 37 °C, removed and washed three times with 50 mM phosphate buffer (pH 6.5). Adsorbed proteins were then extracted with 0.5% (w/v) sodium dodecyl sulphate (SDS) for at least 4 h at room temperature, concentrated by freeze drying and evaluated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed using 10% acrylamide and the gels were either stained with coomasie blue or electroblotted on to nitrocellulose (Schleicher and Schuell). Blots were blocked with 5% haemoglobin in Tris-saline (pH 7.5), probed with primary antibody followed by a suitable second antibody conjugated to horseradish peroxidase. The blots were then developed in 4-chloro-1-naphthol in methanol and 50 µl of 6% hydrogen peroxide. The primary antibodies used were rabbit anti-IgG, IgA, IgM (Sigma Ltd, UK), sheep anti-fibrinogen, -fibronectin and -vitronectin (The Binding Site, Birmingham, UK) and rabbit anti-albumin (Sigma Ltd, UK).

In an attempt to amplify protein detection, two approaches were employed. First, proteins were biotinylated *in situ*, on the material surface using biotinamidocaproate N-hydroxysuccinimide ester dissolved in dimethyl formamide at pH 7.7 in PBS, before extraction with SDS as described above. Western blots were then probed with horseradish peroxidase conjugated to avidin D, which only binds to proteins that have been biotin labelled. The protein bands were compared to biotinylated molecular weight standards (Sigma Ltd, UK). Whole plasma was probed with antibodies to the immunoglobulins IgG, IgA, and IgM, fibronectin, fibrinogen and vitronectin, to show their positions for comparison.

2.2.2. ELISA

The second approach was to probe the material surfaces directly using antibodies to the three adhesive proteins, fibrinogen, fibronectin and vitronectin, and albumin, because the latter is known to decrease cellular adhesion to solid surfaces. The ELISA was performed using polyclonal antibodies to the four proteins selected. Once again the test materials were incubated in human citrated plasma for one hour at 37 °C, washed twice with 50 mM phosphate buffer and probed with primary antibody, followed by horseradish peroxidase conjugated second antibody. Samples were developed with 1 mg/ml orthophenylenediamine + 50 µl hydrogen peroxide in phosphate citrate buffer (pH 5.0) and the resultant colour was read at 492 nm.

3. Results

3.1. Electrophoresis and immunoblotting

Gels stained with coomassie blue showed the presence of a band the same size as albumin on all surfaces, with trace amounts of another one or two weak protein bands on the titania surfaces. However, Western blotting and probing with antibody to whole plasma improved sensitivity and confirmed that a major component was albumin (arrowed), but that another major protein was present on all the surfaces tested, which could be an immunoglobulin (Fig. 1a). Further work, probing for the three immunoglobulins, revealed the presence of each of IgG, IgM, and IgA (data not shown). Because adhesive proteins are likely to be important in the interaction of materials with host tissues, blots were also probed with antibody to fibronectin and vitronectin. The latter was found to be present on all surfaces tested, but appeared to be present in higher amounts on the titania surface (Fig. 1c). Trace amounts of fibronectin were seen binding to PTFE (Fig. 1b).

3.2. Biotin labelling

The biotinylated blots had more protein bands visible than detected by antibody to whole plasma, some of which desorbed relatively easily with sodium chloride, but more were eluted with SDS. This highlights the possible problems of removing all protein residues from the material surface. Similar band patterns were noted between the sol–gel titania and the cpTi surfaces, while the glass and PTFE showed different patterns to these as well as to each other (Fig. 2). However, small differences between the titania and cpTi were also noted. Detected protein bands ranged in molecular weight from approximately 17–98 kDa. Whilst the amount of protein was not measured, the intensities of the bands were comparable since they had all been taken up in the same volume.

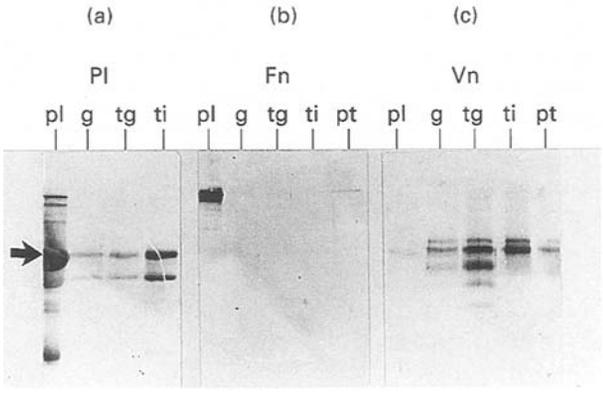

Figure 1 Western blots probed with (a) antibody to whole plasma (b) antibody to fibronectin and (c) antibody to vitronectin. Key: pl = plasma, g = plain glass, tg = sol-gel titania, ti = commercially pure titanium and pt = polytetrafluoroethylene.

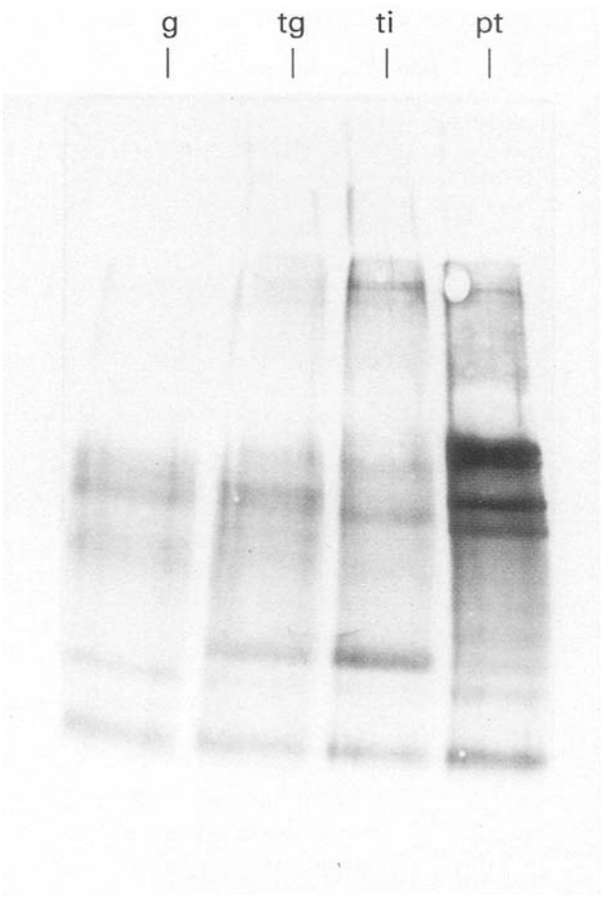

Figure 2 Biotinylated Western blot. Key: g = plain glass, tg = sol-gel titania, ti = commercially pure titanium and pt = polytetrafluoroethylene.

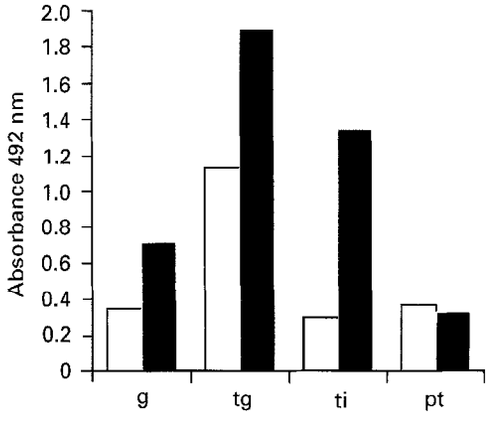

Figure 3 Histogram showing results of *in situ* ELISA for (□) fibrinogen and (■) vitronectin adsorbed to material surfaces. Key: g = plain glass, tg = sol-gel titania, ti = commercially pure titanium and pt = polytetrafluoroethylene.

3.3. ELISA

The ELISA results showed a distinct difference between the proteins which adsorbed to the two classes of materials. Fibrinogen bound more readily to the sol–gel titania (Fig. 3), while vitronectin appeared to adsorb particularly well to both of the integrating surfaces (titania and cpTi). No significant difference in adsorption was observed for the other two proteins.

4. Discussion

It is clear from the above results that selective protein adsorption does take place on all of the surfaces tested, but there were quantitative and qualitative differences between them. Such differences may have the potential to influence subsequent cellular events. One such protein is vitronectin, which is thought to be important in cell adhesion and cell spreading [13–15], and we found bound in greater amounts to the osseointegrating surfaces. In addition we found more fibrinogen on the titania surface than on the cpTi, but why these two physico-chemically similar surfaces showed this difference is unknown. A possible explanation for this discrepancy could be the presence of higher levels of contaminants on the cpTi surface [12] and these may prevent fibrinogen binding.

Various methods have been employed in the detection and identification of proteins adsorbed to material surfaces [16, 17]. However, no single, presently available method is ideally suited to the detection and identification of these surface proteins. One of the main problems is dealing with the very small amount of protein that adsorbs. Western blotting is a sensitive technique for the identification of proteins, but its success depends on the efficient extraction of the adsorbed proteins from material surfaces and assumes that sufficient protein epitopes survive denaturation to be detected by specific antisera. Furthermore, the presence of specific proteins has to be pre-judged in order to select the antisera used and these have to be relatively easy to obtain. A similar concept was used recently by Warkentin *et al.* [17], in which adsorbed proteins were detected by direct transfer from surfaces to nitrocellulose. While this method overcame the problems inherent in extraction (but could not cover for proteins that did not readily desorb), the same limitations of dependence upon the use of specific antibody still applied.

As an alternative approach, we labelled proteins *in situ* by biotinylation. This has the advantage over western blotting that the proteins are labelled before extraction. In addition, the biotin–avidin detection system is more sensitive than antibody based systems and, also, detection is not influenced by the conformational integrity of the desorbed proteins. However, its disadvantage lies in not providing any direct information on the identity of the proteins detected, apart from their molecular weights.

In contrast to both of these methods, ELISA carried out *in situ* overcomes the need to extract proteins from the material surface prior to detection. Proteins are detected in their native state and must have been available on the surface and, therefore, not hidden by other components. In addition, ELISA gives quantitative data on the proteins detected more easily than could be achieved by Western blotting. However, as with Western blotting, the presence of certain proteins still has to be pre-judged in order to select the relevant antibodies.

5. Conclusions

From the methods evaluated for studying protein adsorption to biomaterial surfaces, a combination of biotin labelling and ELISA provided the maximum information on the proteins adsorbing from complex mixtures to material surfaces. Biotinylation is useful as an initial screen to determine which species may be present, thus providing a guide for specific identification and quantification with ELISA.

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