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Surface morphology and adsorbed proteins affect phagocyte responses to nano-porous alumina

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Abstract This study evaluates human neutrophil responses to aluminum oxide membranes with different pore sizes (20 nm and 200 nm in diameter) uncoated and pre-coated with serum, collagen I, or fibrinogen. The effect of released neutrophil granule components on the survival of osteoblastic cells (MG63) bound to the alumina membranes has also been evaluated. Without protein coatings the 20 nm pore-size membranes prompt higher reactive oxygen species (ROS) production as assessed by luminol-amplified chemiluminescence than the 200 nm pore-size membranes. Such pore-size depending responses were also found on membranes pre-coated with fibrinogen, but not with collagen or serum were in fact a much lower ROS production was observed. In addition, uncoated and fibrinogen-coated membranes prompt stronger release of the granule enzymes, myeloperoxidase and elastase, than collagen or serum-coated alumina. Equally important, we found that surface-mediated phagocyte activation and the subsequent release of granule components had a significant affect on the adhesion, viability and proliferation of osteoblasts. This stresses the importance of studying not only cell/surface interactions but also cell/cell interactions in wound healing and tissue regeneration processes.

Introduction

The difference between successful tissue integration and failure partially lies in how the biomaterial surface interacts with the different mechanisms of the normal wound healing process. Complications of medical devices are largely based on both the effects of the implant on the host tissue and the effects of the host on the implant. Intensive research efforts have been made on the development of materials and coatings with better tissue compatibility. Such research has lead to the creation of a number of new materials. Unfortunately, these materials still to some extent trigger inflammatory responses, which in some cases cause failure of the implanted device [1].

In an effort to find a better coating for joint implants we have studied nano-porous aluminium oxide [2, 3]. This anodized form of aluminium has never to the authors knowledge been used for hard tissue replacement. Our previous results have shown excellent biocompatibility between human osteoblasts and this material [4]. In the current study we have evaluated the pro-inflammatory properties of nano-porous alumina since it is generally believed that the extent of biomaterial-mediated inflammatory responses determines the fate of medical implants [5, 6]. Implanted biomaterials often accumulate a large number of inflammatory cells, such as polymorphonuclear granulocytes (PMN) [7–10]. Using an in vitro model we recently showed that the pore size of the alumina membranes affected the degree of PMN activation [11]. Membranes with 20 nm pores (in diameter) seemed more prone to cause neutrophil activation and thus inflammation, than membranes with 200 nm pores. This is not surprising since it has long been known that substrate topography can affect cell morphology and behavior as reviewed by Dunn [12] and Desai [13]. Investigators have demonstrated that shapes such as ridges/grooves, spikes, holes, spirals and their dimension and distribution can have a significant effect on cell behavior [14–17].

Shortly after implantation, the biomaterial will encounter blood. Thus the implant surfaces will almost instantly be

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covered with plasma proteins [5, 8–10, 18]. Because this adsorption of proteins is much more rapid than the migration of cells to a foreign surface, the proteinaceous film is most probably important for the subsequent attraction, adsorption and activation of phagocytes [5, 7, 9, 10]. Many authors have shown that when materials are pre-coated with proteins, the neutrophils will respond differently depending on the type of protein and the material to which it has been adsorbed. Albumin has e.g. been shown to reduce neutrophil adhesion and activation on glass surfaces while proteins such as immunoglobulins give the opposite effect [19, 20]. Adsorbed fibrinogen has been proven responsible for triggering biomaterial-mediated inflammatory responses and stimulating neutrophil activation [5, 21] while Collagen type I on the contrary is known to somewhat reduce neutrophil adhesion and activation [19]. Serum was also included in the study to mimic the diversity of the body fluid in an *in vivo* situation.

To assess the proinflammatory properties and thus be able to improve the tissue compatibility of nanoporous aluminium oxide, a series of studies were carried out were human neutrophil responses to nano-porous alumina (pore diameters of 20 and 200 nm) precoated with human serum, collagen I, or fibrinogen were investigated. Furthermore a study was carried out to assess the influence of neutrophil activation and granule component release on osteoblastic survival.

Materials and methods

Anodisc^{TM} alumina membranes produced by Whatman International Ltd (Maidstone, England) were used. The membranes are 13 mm in diameter and 60 μm thick, with narrow pore size distribution. Membranes with two pore-sizes (200 nm and 20 nm in diameter) were included in this study. The membranes have similar surface roughness and surface chemical characteristics [11]. It should be noted that the 20 nm layer of pores is only about 1 μm thick, the diameter of the pores in the remaining 59 μm of the membrane cross section is 200 nm.

Protein coating

The alumina membranes were pre coated at room temperature for 24 h in 1 ml of 5% human serum (3.5 mg/ml) (from apparently healthy volunteers at the Academic Hospital in Uppsala, Sweden), 0.250 mg/ml of collagen type I (from calf skin, Sigma, Sweden), 0.250 mg/ml of human fibrinogen (Sigma Sweden), 0.250 mg/ml of bovine serum albumin (BSA, Serological proteins inc, Kankakee, Illinois, USA), and 0.250 mg/ml of Immunoglobulin (IgG) (Sigma, Sweden) all diluted in Hanks balanced salt solution (HBSS) (Sigma, Sweden). The concentrations of adsorbed proteins were estimated using both a Micro BCA^{TM} Protein assay kit (Pierce, USA) and nitrogen analysis (Mikro Kemi AB, Uppsala, Sweden). Duplicates of each sample for each method were used.

Neutrophil isolation

Neutrophils, also called polymorphonuclear leukocytes (PMN), were prepared from heparinized blood of apparently healthy blood donors (Academic Hospital, Uppsala, Sweden) following a routine dextran sedimentation method, essentially as described by Håkansson and Venge [22].

To each of ten test tubes containing 5 ml heparinized blood, dextran (Dextran T500, Pharmacia, Uppsala, Sweden) was added to a final concentration of 1% and the sedimentation was then allowed to proceed for 30 min at room temperature. The neutrophil rich supernatants were harvested, pooled and centrifuged at 160 g for 5 min. The pellet was washed twice with 0.9% NaCl. Contaminating erythrocytes were lysed by a 30 s exposure with water (Milli Q) after which 3.6% NaCl was added to reach a final concentration of 0.9%. The obtained suspension was then centrifuged for 5 min at 160 g after which the pellet was once again resuspended in 0.9% NaCl and centrifuged for 5 min at 160 g. Finally the pellet was dispersed in Gey's buffer and the average content of PMN (approximately 90%) was calculated using Türks staining and hemocytometer method. Neutrophils were isolated on three occations from different donors. Experiments were performed within 90 min after isolation.

Scanning electron microscopy (SEM)

Neutrophils were diluted to a concentration of approximately 6×10^5 cells/ml in Hanks Balanced Salt Solution (HBSS) (Sigma-Aldrich, St. Louis, MO). 0.5 ml of this suspension was then added to 24-well ultra low attachment polystyrene plates (Costar 3473), containing collagen, fibrinogen, and serum-coated alumina membranes with two different pore sizes (20 nm and 200 nm in diameter) and non-coated controls. Cells were then allowed to adhere for 30 min at 37°C. Quadruplicates of each type of alumina membrane were used. After 30 min of incubation cells were fixed with 1.5% glutaraldehyde, dehydrated through a series of acetone concentrations (50, 70, 80, 90 and 100%), critically point dried and sputtered with gold and finally studied under a LEO 1530, Gemini SEM.

Chemiluminescence (CL)

To assess the extent of surface-mediated neutrophil activation, the generation of oxygen free radicals over time from neutrophils interacting with the protein-coated alumina membranes and non-coated controls was quantified using a Victor^2 1420 multi label counter (Wallac) in the

luminescence mode. All measurements were performed at 37°C in HBSS using white 24-well optiplates (Perkin Elmer). Approximately 300 K neutrophils followed by, horseradish peroxidase (4 U/mL) and luminol (5-amino-2,3-dihydro-1,4-phtallazinedione) (50 μM), were added to wells containing collagen, fibrinogen and serum-coated alumina membranes and non-coated controls with pore sizes of 20 and 200 nm in diameter. Luminescence intensity was read every 5 min. The measurements were commonly performed for 30–45 min on quadruplicate samples [23].

Elastase activity

To assess the extent of surface-mediated neutrophil degranulation, the activity of elastase released from the neutrophil granules and bound to the alumina membranes was measured using an elastase specific chromogenic peptide substrate [24]. Approximately 300 K neutrophils were added to the collagen, fibrinogen and serum-coated alumina membranes and non-coated controls for either 30 min or 2 h after which the membranes were carefully washed once with 2 ml of HBSS and moved to new 24-well plates. Thereafter 400 μl aliquots of 1 mM MeO-Suc-Ala-Ala-Pro-Val-pNA (Sigma, Sweden) in HBSS containing 10 μg/ml BSA and 10% DMSO (Sigma, Sweden) was added to each membrane. After 1.5 h of incubation in 37°C, released p-nitroaniline was measured against blanks (protein coated alumina membranes not exposed to PMN) at 405 nm using a multiscan MS spectrophotometer (Labsystems). Triplicates of each sample were measured.

Myeloperoxidase detection (Radio immuno assay)

The amount of myeloperoxidase (MPO) released from the neutrophil granules and bound to the alumina membranes was measured using a radiolabelled polyclonal antibody. Approximately 300 K neutrophils were added to the collagen, fibrinogen and serum-coated alumina membranes and non-coated controls for either 30 min or 2 h. After this the membranes were carefully washed once with 2 ml of HBSS and moved to new 24-well plates. 400 μl of radiolabelled rabbit-anti human MPO polyclonal antibody (^{125}I-pab, 80,000 cpm/ml, Pharmacia Diagnostics AB, Uppsala, Sweden) dissolved in 0,1 M PBS, pH 7.4 (containing 1% BSA) was thereafter added. Incubation was then carried out for 6 h (at room temperature on a shaker), after which the alumina membranes were washed 6 times with 1 ml of the previously mentioned buffer solution to remove non-bound antibody. The membranes were then glued to cello tape crushed, folded and transferred to Ellerman tubes. The amount of bound radioactivity was measured using a gamma counter 1260 multigamma II (LKB Wallac). All values were corrected for radiation from antibody incubated with protein-coated alumina without neutrophils. Triplicates of each sample were measured.

Osteoblast viability study

An ideal hard tissue biomaterial should trigger minimal inflammatory responses while stimulating osteoblast adhesion, proliferation and differentiation. Based on this hypothesis, an in vitro cell culture system was developed to study osteoblast compatibility of the nano-porous alumina membranes. Specifically, 300 K neutrophils were added to the collagen, fibrinogen and serum-coated alumina membranes and non-coated controls for either 30 min or 2 h. After which the membranes were carefully washed once with 2 ml of HBSS (removing loosely bound neutrophils and cell debris) and moved to new 24-well plates. Thereafter approximately 100 K osteoblasts (MG63) were added to the membranes and cultured in Dulbeccoís Modified Eagles Medium (nutrient mixture F-12 HAM, Sigma) supplemented with 10% FCS (Sigma) and 0.1 mg/ml gentamycin (Gibco), at 37°C, 5% CO_2 in a humidified atmosphere for 24 h. Some of the membranes were fixed with 1.5% glutaraldehyde and the numbers of adhered osteoblasts were quantified using an optical microscope (870 ×) (Carl Zeiss 51993 light microscope). On the rest of the alumina samples the Almar Blue assay was performed. Alamar Blue [25] is a non-toxic metabolic indicator for viable cells. Upon uptake into cells the dye becomes reduced and changes colour. The colour change correlates approximately with the number of living cells in the sample. The culture medium was removed from the wells after 24 h and replaced by 0.5 ml of Alamar Blue stock solution (Serotec) diluted 1:10 in HBSS. The membranes were then further incubated at 37°C, 5% CO_2, in a humidified atmosphere for 1.5–2 h. Aliquots of 100 μl from each well were transferred to a 96-well plate and the absorbance (570 nm) was read using a multiscan MS spectrophotometer (Labsystems, USA). All values were corrected for background absorbance from reagent incubated with membranes that had not been exposed to cells.

Results

Protein adsorption analysis

The concentrations of adsorbed proteins were estimated using both nitrogen analysis and a Micro BCA™ Protein assay kit. The approximate amount adsorbed to each surface was calculated based on standard curves made from each individual protein and serum. As can be seen in Table 1 less protein was adsorbed onto alumina membranes with pore sizes of 20 nm than to the 200 nm pore-size membranes. This was the case for both the proteins and the serum. Incubation with serum gave the highest protein content. This is most likely

Table 1 Protein concentration on alumina membranes after 24 h of incubation. The values given below are mean values calculated from the Micro BCA measurements and the nitrogen analysis

Protein	200 nm, μg/membrane	20 nm, μg/membrane
Collagen I	42.9 ± 1	27.9 ± 4.1
Fibrinogen	27.1 ± 6.6	18.4 ± 2.3
Serum	115 ± 2.8	95.7 ± 10.8
Albumin	34 ± 1.4	31.2 ± 1.6
IgG	22.7 ± 0.2	19 ± 0.6

due to the much higher protein concentration in 5% serum (3500 μg/ml) compared to the pure protein solutions used (250 μg/ml), and the fact that serum contains a large number of plasma proteins of various molecular weights, which are likely to occupy the alumina surface. On the 200 nm pore-size alumina the largest amount of adsorbed protein was detected for collagen followed by albumin fibrinogen and IgG. On the 20 nm pore-size alumina, the largest amount of adsorbed proteins detected was albumin followed by collagen, IgG and fibrinogen.

Neutrophil morphology

We have compared the morphology of neutrophils cultured on alumina membranes with two different pore sizes, uncoated and precoated with serum, fibrinogen and collagen. After incubation for 30 min. the cellular responses on the different membranes were observed using SEM. As expected, we found that the species of adsorbed proteins had significant effect on cell morphology. Neutrophils on membranes pre coated with either collagen or serum had a similar appearance. They commonly have a round non-activated morphology (Fig. 1a and 1b). On the contrary, neutrophils adsorbed to the fibrinogen-coated surfaces had spread out with protruding filopodia showing typical signs of frustrated phagocytosis (Fig. 1c) [26, 27]. However, no difference in cell morphology was observed when comparing membranes with different pore sizes pre-coated with the same proteins. When comparing the non-coated controls relatively more intact (rounded), non-activated cells were seen on the 200 nm membrane (Fig. 1d) in contrast to quite a large number of cells with a flattened activated morphology on the 20 nm membrane (Fig. 1e). This observation is in agreement with our previous work [11]. Neutrophils thus seem to be sensitive to the porosity when in a protein free environment, however when proteins are present on the surface the nano-porous structure seems to have very little affect on cell morphology.

Chemiluminescence (CL)

The generation of reactive oxygen species (ROS) as a function of time from neutrophils interacting with the different protein-coated alumina membranes and non-coated controls was studied by measuring luminescence. CL-intensity was recorded every 5 min for 40 min.

No difference was seen when comparing collagen and serum coated membranes for either type of alumina (20 or 200 nm) the CL-intensity was very low in both cases. However on the fibrinogen-coated membrane slightly higher luminescence intensity was seen on the 20 nm pore-size alumina compared to the 200 nm pore size membrane. This is clearly demonstrated in Fig. 2.

For the non-coated membranes CL-levels were initially higher and peaked much earlier on the 20 nm pore-size membrane than on the 200 nm membrane, where the activation time was longer and the production of ROS started decreasing later. This observation is in agreement with our previous work [11].

Elastase activity

The activity of the neutrophil granule enzyme elastase was measured using an elastase specific chromogenic peptide substrate. The highest enzymatic activity was seen on the non-coated alumina membranes, followed by the fibrinogen-coated membranes while the lowest elastase activity was detected on the collagen and serum coated membranes (Fig. 3). No significant difference could be seen between the protein and serum coated alumina due to pore size. A slight difference was however detected between the non-coated alumina membranes, with the 20 nm pore size alumina showing slightly higher elastase activity than the 200 nm alumina. When comparing the 30 min neutrophil exposure to the 2 h incubation the same pattern was observed, however with slightly higher elastase activities in all cases. This was expected since the longer exposure gave more time for the neutrophils to become activated and thus release more granules and their components.

Myeloperoxidase detection (Radio immuno assay)

Myeloperoxidase (MPO) released from the neutrophil granules was measured using a radiolabelled polyclonal antibody. The highest amount was seen on the non-coated alumina membranes, followed by the fibrinogen-coated membranes while the lowest amount of MPO was detected on the collagen and serum coated membranes (Fig. 4). We find that pore-size has little or no influence on MPO release among all membranes tested. When comparing the 30 min PMN exposure to the 2 h incubation the same pattern was observed, however with slightly higher MPO amounts in all cases. Overall the same pattern could be seen for both granule proteins i.e. elastase as described above and MPO.

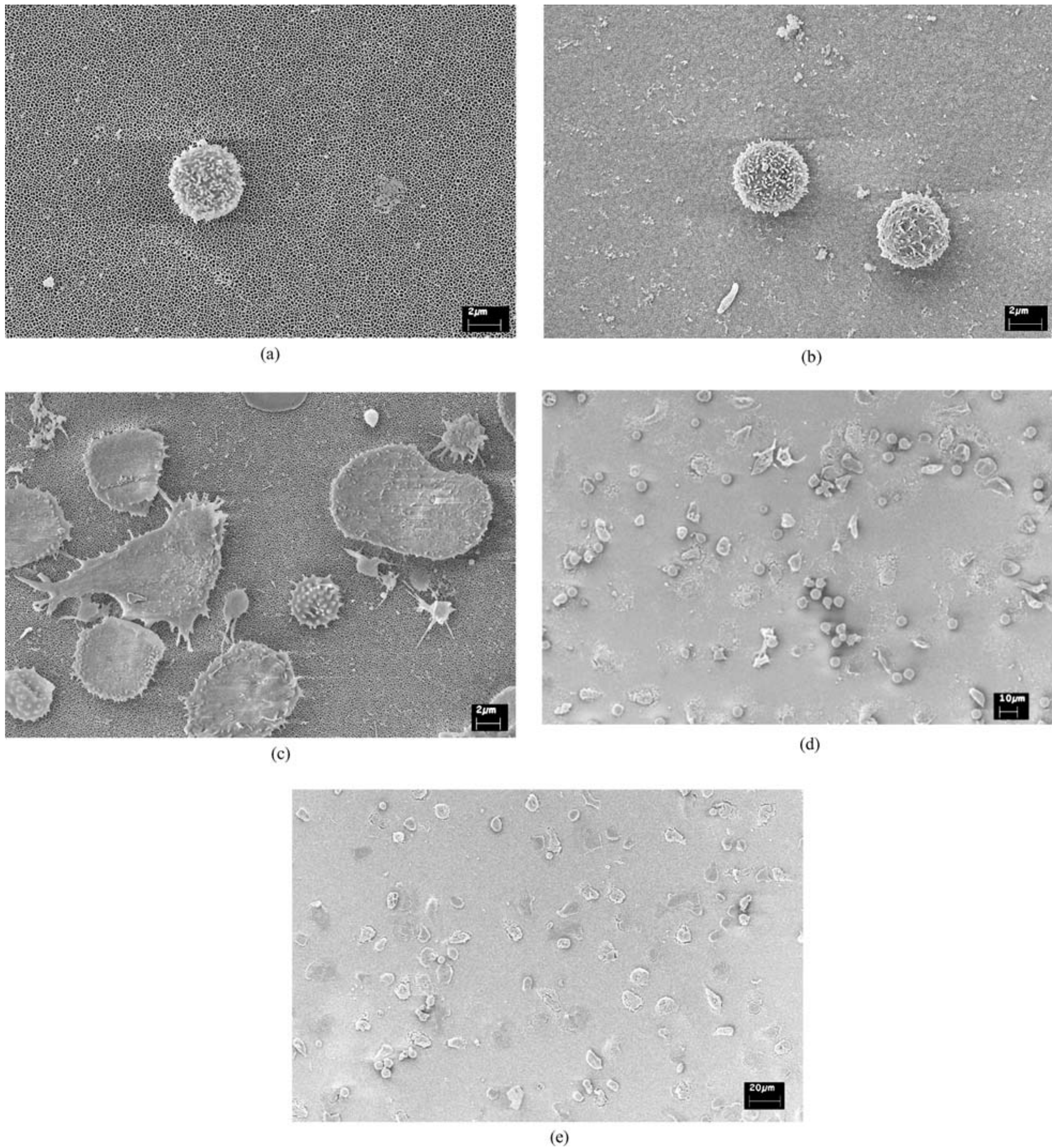

Fig. 1 (a) SEM micrograph of neutrophil on 200 nm pore-size alumina membrane pre-coated with collagen. (b) SEM micrograph of neutrophils on 20 nm pore-size alumina membrane pre- coated with serum. (c) SEM micrograph of neutrophils on 200 nm pore-size alumina membrane pre-coated with fibrinogen. Note the flattened morphology of the cells. (d) SEM micrograph of neutrophils on uncoated 200 nm pore-size alumina membrane. (e) SEM micrograph of neutrophils on uncoated 20 nm pore-size alumina membrane.

Osteoblast viability study (optical microscopy and alamar blue)

To study whether the survival of osteoblasts were affected by the activation of neutrophils and subsequently the release of granule constituents, a series of in vitro ostoblast viability studies were performed on both uncoated and protein-coated nano-porous alumina surfaces using optical microscopy and the alamar blue assay [25]. When looking at the light microscopy pictures one could see a clear difference in cell number and spreading between the differently coated samples, however no significant difference could be observed

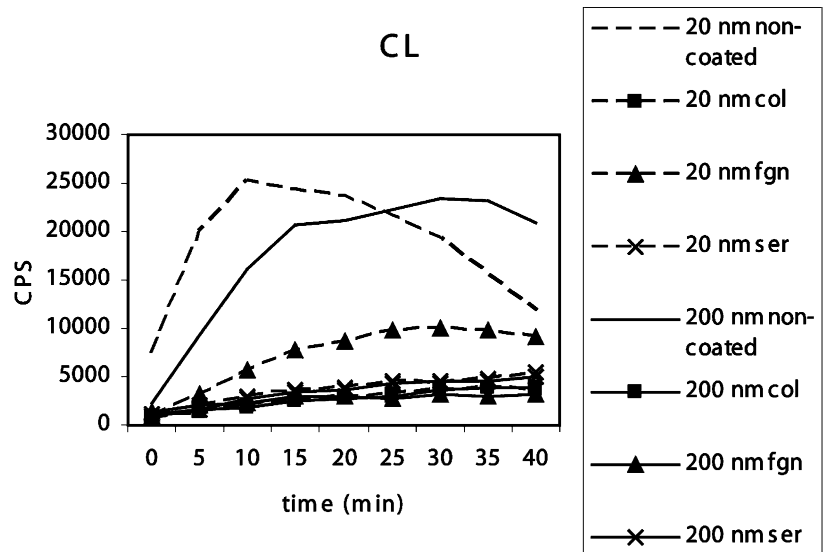

Fig. 2 Chemiluminescence graphs for neutrophils exposed to non-coated and protein-coated nanoporous alumina membranes with pore diameters of 20 nm and 200 nm in diameter. Each value represents the means of four samples.

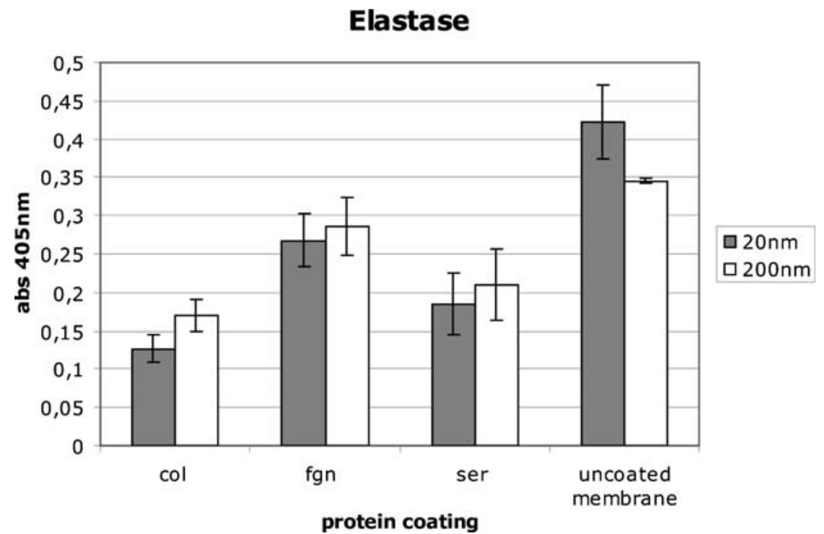

Fig. 3 Elastase activity on nano-porous alumina surfaces after a 30 min exposure to neutrophils. Each value represents the means of four samples.

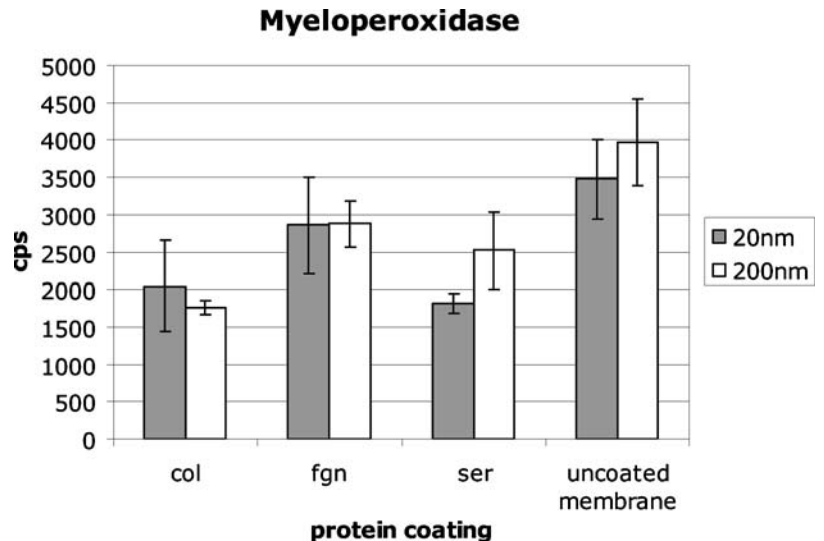

Fig. 4 Amount of MPO on nano-porous alumina surfaces after a 2 h exposure to neutrophils. Each value represents the means of four samples.

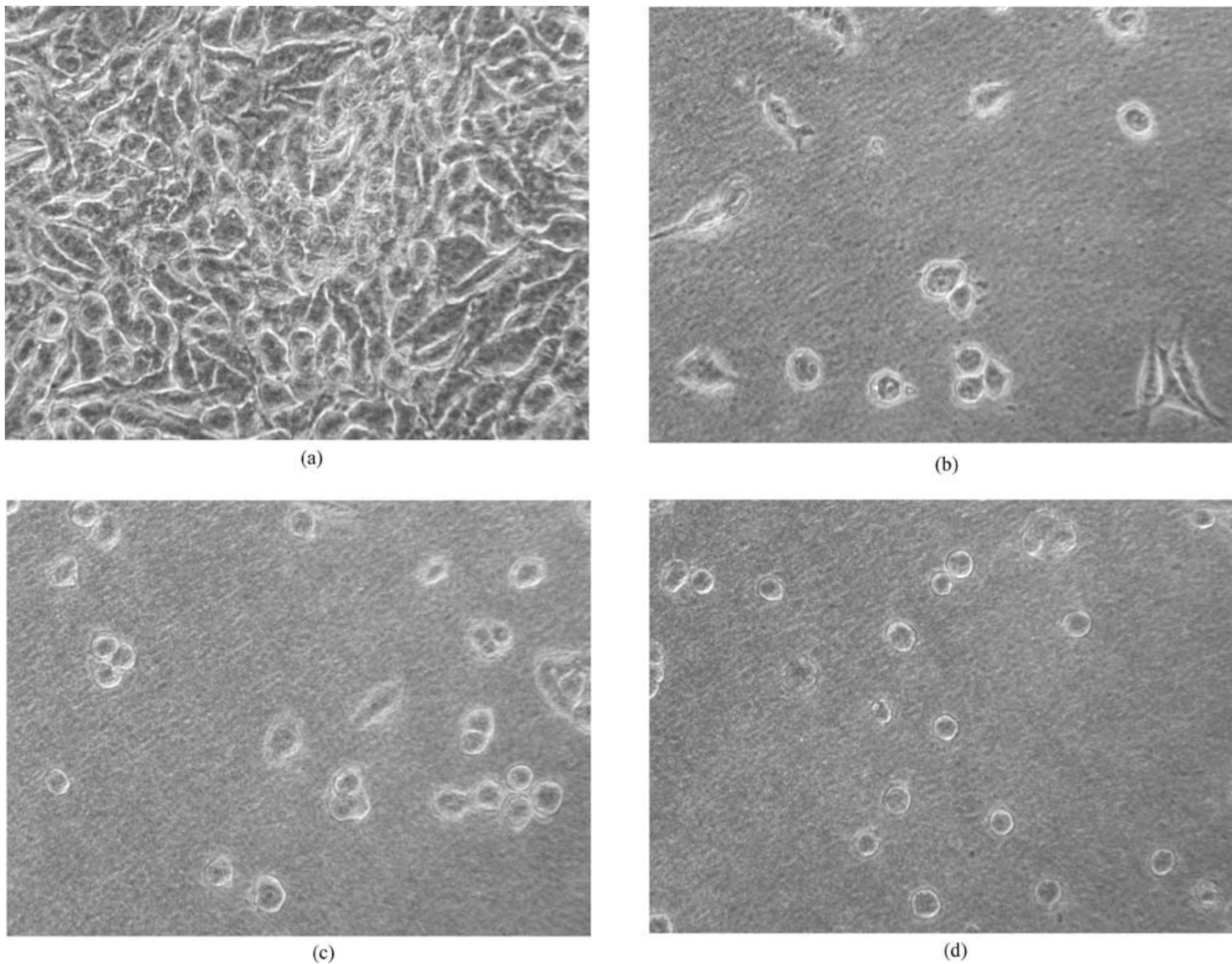

Fig. 5 (a) Light microscopy picture of a collagen-coated 20 nm pore-size alumina surface exposed to neutrophils and thereafter to osteoblastst for 24 h. (b) Light microscopy picture of a fibrinogen-coated 20 nm pore-size alumina surface exposed to neutrophils and thereafter to osteoblasts for 24 h. (c) Light microscopy picture of a serum-coated 200 nm pore-size alumina surface exposed to neutrophils and thereafter to osteoblasts for 24 h. (d) Light microscopy picture of a non-coated 200 nm pore-size alumina surface exposed to neutrophils and thereafter to osteoblasts for 24 h.

due to the membrane porosity. Alumina pre-coated with collagen showed significantly more adhered and spread out osteoblasts on the surface (Fig. 5a) than the fibrinogen-coated, serum-coated and uncoated alumina membranes were only very few rounded cells could be detected (Figs. 5b–d).

The alamar blue viability assay was performed on the non-coated alumina and collagen coated surfaces. As can be seen in the graph (Fig. 6) lower osteoblast viability was observed after 24 h on the non-coated surfaces compared to the collagen-coated ones. The latter surface showed the same degree of viability as the control surface i.e. osteoblasts cultured directly on an alumina (without protein coating or PMN incubation). These results clearly show that the degree of neutrophil activation and subsequently their granule enzyme release have a significant affect on the post-cultured osteoblasts.

Discussion

The interaction of inflammatory cells, including neutrophils, with an implanted medical device can bee considered an important determinant of biocompatibility, since these phagocytic cells represent one of the very first defence lines in the immune system [7]. During implantation biomaterials are exposed to blood and almost instantly the surface will be covered with a layer of plasma proteins [18]. Because this adsorption of proteins is much more rapid than the migration of cells, the species and composition of the proteinaceous film is likely to dictate the subsequent recruitment, adhesion and activation of phagocytes [7].

In this study we have shown that neutrophils react differently depending on the type of protein that has been pre-adsorbed to nano-porous alumina membranes. Collagen I

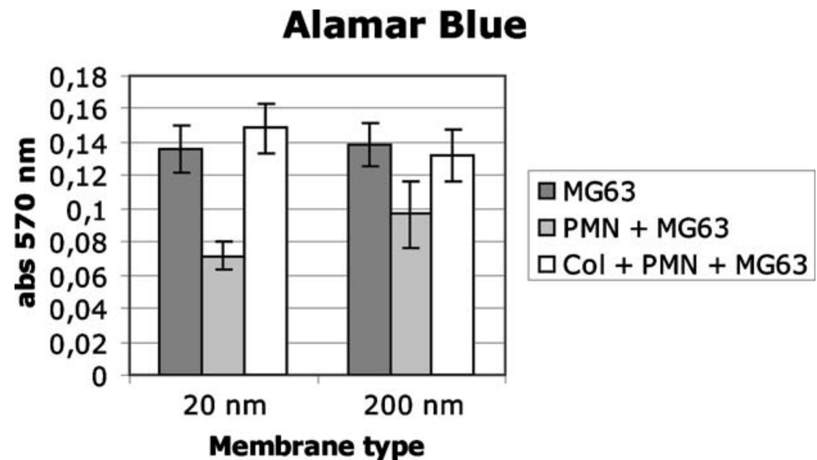

Fig. 6 Alamar blue assay results from osteoblastic cells cultured on uncoated and collagen coated alumina membranes for 24 h after a 2 h exposure to neutrophils. The graph also shows control surfaces where the osteoblasts were cultured directly on the membranes i.e. they were not pre exposed to protein or PMN. Each value represents the means of four samples.

was chosen because of its presence in bone and cartilage [28], since the substrate nano-porous alumina is evaluated for hard tissue replacement, [2–4]. Fibrinogen coating was used since it is well documented that when spontaneously adsorbed it is responsible for the pathogenesis of foreign body reactions [5, 21, 29]. Serum was also included in the study to more closely mimic the diversity of the body fluid *in vivo* situation. Adsorption of albumin and IgG was finally evaluated because of their high concentrations in serum and well known passivating (albumin) and activating (IgG) effects on neutrophils [19]. According to a study made by Takami *et al.* [30] albumin adsorbs to an alumina surface more readily than IgG when incubated in plasma. This is of course fortunate since an albumin-coated surface is less likely to trigger neutrophil and platelet adhesion [19, 31]. It has also been reported by Jenney *et al.* [32] that surfaces which strongly adsorb IgG promote long-term macrophage adhesion which is unfavorable since this might lead to chronic inflammation. Additionally IgG, a known stimulator of the complement system has been found to promote platelet adhesion and activation [31]. As can be seen in (Table 1), our study shows that on both the 20 and 200 nm mebranes approximately 40% more albumin than IgG was adsorbed. This experiment was done on a single protein basis, it is however very likely that the difference will become even more pronounced in serum since approx 45 mg/ml of serum consists of albumin compared to only 10 mg/ml of IgG [33]. This was in fact confirmed, when preliminary studies were done investigating the adsorption of proteins from diluted serum to nano-porous alumina using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (unpublished data). The desorption patterns showed virtually no sign of IgG while albumin could be detected in high amounts.

For all proteins used less adsorption was seen on the 20 nm membranes. The difference in amount of bound protein between the 20 and 200 nm pore-size membranes was however more pronounced for collagen and fibrinogen than for albumin and IgG. This is most likely due to the different sizes and shapes of the proteins. It is reasonable to assume that the smaller proteins albumin (65 kD) and IgG (150 kD) more easily will diffuse into and bind to the internal surface of the 20 nm pores while the larger proteins collagen (231 kD) and fibrinogen (350 kD) mainly bind to the macroscopic surface.

Incubation with serum gave the highest protein content on both membranes. This is most likely due to the fact that apart from the much higher concentration (3500 μg protein/ml compared to 250 μg/ml used for the single protein solutions) and the large amount of albumin, serum also contains many other proteins of various molecular weights, which are likely to bind to both the macroscopic and microscopic alumina surface. For the single proteins the largest adsorbed amount was detected for collagen and albumin.

When comparing these results with the neutrophil morphology and activation one can clearly see on both the SEM pictures (Fig. 1) and CL-intensity graph (Fig. 2) that collagen and serum (which contains high concentrations of albumin) seem to be the most beneficial surface coatings (regardless of membrane pore-size) for preventing activation of neutrophils. Fibrinogen on the other hand seems to be more activating on the 20 nm pore-size alumina compared to the 200 nm pore-size surface. The reason for this could be related to the size and shape of fibrinogen. Because of steric reasons this protein is less likely to penetrate the smaller 20 nm pores thus leading to accumulation of fibrinogen on the macroscopic surface. A pore-mediated orientation of the adsorbed protein might also be a likely explanation.

Decleva *et al.* has reported that neutrophils do not respond to tumor necrosis factor (TNF) with a respiratory burst when adhering to a surface coated with collagen I [34]. On the other hand, in contrary to our findings, Monboisse *et al.* [35] reports that collagen I triggers PMN activation when coated on 96-well tissue culture plates. The reason for these contradictory results is likely caused by the fact that very different surfaces were used. Because of the nano-porous structure

and the hydrophilic nature of the alumina, the surface is highly hydrated and presents an environment more similar to the *in vivo* situation. This characteristic should diminish adsorbed proteins from changing their native conformation due to hydrophobic interactions when adhering to the substrate [36]. Exposure of the reported activating sequences [37, 38] of collagen and subsequent neutrophil activation might thus not occur on the alumina.

As previously reported by Karlsson *et al.* [11], non-coated alumina membranes show a clear difference in neutrophil activation depending on the pore size. Higher Initial ROS levels were seen on the non-coated 20 nm pore-size membrane than on the 200 nm membrane, where the activation time was longer and the production of ROS started decreasing later. Thus the cells seem to be significantly affected by the nano-porous structure. However our present finding indicates that pore-size in the nano-range has very little influence on neutrophil activation when the membrane is coated with a single protein or with serum.

Neutrophils as mentioned above are one of the first cells to enter an inflammatory site, and when doing so, might become activated to release a wide variety of products that mediate the overall host response [39, 40]. Although the ability of adherent neutrophils to specifically release their granule components extracellulary in response to non-phagocytosable materials (frustrated phagocytosis) has been well established [27, 41], the effect of the granule enzymes on other cells present at the sight of implantation has been less well studied. As mentioned above neutrophil activation was seen to a higher extent on the uncoated, and fibrinogen-coated alumina surfaces than on the serum-coated and collagen-coated surfaces. These results were in agreement with the measured elastase activity and amount of MPO on the different surfaces, both showing lower values on alumina pre-coated with collagen and serum compared to uncoated and fibrinogen coated alumina (see Figs. 3 and 4). From these results one can conclude that neutrophil activation and thus release and binding of extracellular granular components to the latter surfaces were much higher.

Using a novel in vitro cell culture model, we further assessed the influence of alumina:phagocyte interaction on osteoblastic survival. As expected, a clear difference in viability could be seen.

Very few osteoblast had established and spread out on the non-coated, fibrinogen-coated and serum-coated surfaces after 24 h compared to the collagen-coated alumina (see Fig. 5). The surface-mediated phagocyte activation is most likely responsible for the decreased osteoblastic survival on uncoated surfaces, since many of the phagocyte products are cytotoxic. For example, elastase has a broad range of target substrates and can digest a large number of proteins among these are fibronectin [42–44] and collagen [45, 46], which are needed for osteoblast adhesion, differentiation and mineral formation. MPO catalyses the interaction of H_2O_2 with Cl^- to form hypochlorous acid HOCl, which also is very toxic for a broad range of microorganisms [47].

The results clearly show that the neutrophils have an affect on the surrounding environment. By releasing enzymes that in turn adhere to the substrate other cells such as osteoblasts will be affected. These are thus clearly important events when evaluating the biocompatibility of an implant. Association between neutrophil activation and tissue destruction has been seen in e.g. periodontites and peri-implantites (destruction of tissue around dental implants and teeth) due to accumulation of neutrophils and thus release of potent tissue destructive substances [48].

Overall these studies have shown that one can minimize the activation of neutrophils and subsequently increase osteoblastic cell viability to nano-porous aluminium oxide by coating the material with collagen I and that fibrinogen and IgG has less tendency to bind to this substrate than albumin. The above properties both contribute to make nano-porous alumina a good candidate for implant use.

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