Fibroblast growth and polymorphonuclear granulocyte activation in the presence of a new biologically active sol–gel glass

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The search for chemical devices to be used in clinical orthopaedics must find substances that are biocompatible and do not elicit inflammatory responses *in vivo*. To this end, a new form of glass has been prepared, composed of 8.1% CaO, 2.9% P_2O_5, 6.7% N_2O_5 and 82.3% SiO_2, using sol–gel procedures. In order to evaluate the *in vitro* biocompatibility of this glass, the proliferation of cultured murine fibroblasts and the activation of human polymorphonuclear leukocytes has been studied. The performance of the sol–gel glass has been compared with that of a biocompatible non-resorbable soda–lime glass. Unlike the soda–lime glass, the sol–gel glass neither caused the inhibition of fibroblast growth nor elicited a marked inflammatory response by polymorphonuclear leukocytes, as demonstrated by chemiluminescence assay for reactive oxygen metabolites.

1. Introduction

Implanted chemical devices are becoming increasingly important in medical care. These materials are generally inert and non-toxic, although they can trigger a variety of reactions, including inflammation, fibrosis, activation of blood coagulation and infection [1, 2]. In some cases, the release of products by implant-associated inflammatory cells has caused degradation of the implanted material [3]. In fact, these cells may generate low levels of reactive oxygen species which may be used to trigger the activation or inhibition of enzymes, cellular functions and the control of cell proliferation and death. Alternatively, the production of high levels of these species may cause tissue damage and indeed has been implicated in the pathogenesis of a number of human diseases such as adult respiratory distress syndrome, ischemia-reperfusion injury, xenobiotic toxicity, and iron overload states [4–7]. The factors that minimize inflammation will maximize biocompatibility. Biomaterials may induce an inflammatory reaction characterized by a rapid recruitment at the implantation site of polymorphonuclear cells and macrophages, which may produce large quantities of oxidant after their stimulation. Optimum soft-tissue compatibility may be achieved by restricting the inflammatory response, which is ideally obtained by limiting the cellular responses by modulating the cell functions of the host, or by selecting materials that elicit a minimal cellular response. Furthermore, implanted medical devices can elicit a fibrotic response. Fibrous capsule formation around implants has been considered a normal response [1].

Graft substitutes used for bone implants can be classified as bioinert or non-bonding and bioactive or bonding according to their surface reactivity and bone-bonding properties. Glasses based on a $CaO–SiO_2–P_2O_5$ composition form chemical bonds with natural bone and promote bone formation. This ability appears to be related to the modification of the glass surface due to dissolution, ionic substitution and precipitation reactions in body fluids, leading to formation of a silica gel with a hydroxycarbonate apatite layer similar to bone mineral [8]. Adsorption and incorporation of proteins into the material and adhesion and growth of cells lead to gradual bone tissue formation. The rate of this process depends on the chemical composition and physical characteristics of the glass [9]. These glasses, when obtained by melt, must contain 35–60 mol % SiO_2 to display bioactivity. However, it has been found that the upper limit can be increased to about 90% using glasses prepared by sol–gel techniques [10].

In this paper we report the preparation of a glass containing $\sim$80 mmol % SiO_2 obtained by sol–gel methods. This material induces normal fibroblast growth, with very little production of oxygen species by polymorphonuclear granulocytes.

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2. Materials and methods

2.1. Chemicals

Luminol, 2-phorbol-13-myristate acetate (PMA) and Ficoll-Hypaque were obtained from SIGMA.

Minimum essential medium (MEM) and Ham's F 12 culture media, foetal calf serum (FCS), phosphate buffered saline solution (PBS) were supplied by Hyclone. Plastics were supplied by Falcon. FCS was heat-inactivated at 58 °C for 30 min. Other chemicals were reagent grade and used without further purification.

2.2. Biomaterials

The glass identified as biocompatible non-resorbable (BCNR) was obtained from Seipi (Milan, Italy). It was a soda–lime glass prepared by melting a powder mixture (% by weight) of CaO (9%) – SiO_2 (71%) – Na_2O (14%) – K_2O (1%) – MgO (3%) – Al_2O_3 (2%) at 1450 °C. The melt was quenched and then pulverized into a fine powder (30–50 μm).

Sol–gel glass. This was prepared according to the following procedure.

A solution was prepared by dissolving $Ca(NO_3)_2$ $4H_2O$ (1.68 g) in 4.6 ml of a solution containing H_3PO_4 (0.164 g) and NaOH (0.0648 g). The resulting solution (pH 2.2) was added dropwise with vigorous stirring to a round bottomed flask containing $Si(OCH_3)_4$ (3.6 g), immersed in a cold water bath. The mixture was stirred vigorously at room temperature for three days until gel formation was evident. The wet gel was removed from the flask and partially dried in air for three days. Final drying was performed in vacuo (1.33 Pa) at room temperature for 32 h, yielding 2.68 g.

Surface area was 11 m^2/g, determined by BET (Micromeritics ASAP 2010).

The sol–gel glass was suspended in water (milliQ) for a 2-week-long test to determine the possible release of soluble ions. The water phase was periodically sampled and analysed for the presence of Ca^{2+}, Si^{4+}, PO_4^{3-} and NO_3^-. Ca^{2+} and Si^{4+} were determined by atomic absorption (Perkin-Elmer 5000), PO_4^{3-} was analysed spectrophotometrically (Perkin-Elmer Lambda 5) using the molybdenum blue test, while NO_3^- was determined by ion chromatography (Dionex DX 500).

2.3. Fibroblast culture

A murine fibroblast cell line (NIH 3T3) was used to test proliferation in the presence of glass. The cells were grown in a 1/1 mixture of MEM and HAM's F 12 supplemented with 10% (v/v) FCS at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2. Cells were plated at 8×10^4 density in a 35 mm Petri dish. The glass, suspended in the culture medium, was placed into the dish 3–4 h after cell seeding at the final concentration 1 mg/ml. At the end of treatment, glass was separated from culture medium by centrifugation at 1000 rpm for 10 min. The effects of the biomaterial were evaluated by determining cell growth and viability. Both the cells retrieved from the culture dish using trypsin-EDTA and the cells eventually adhering to the glass surface were evaluated. Viable cells were stained by treatment with 5 mM dichlorofluorescein diacetate in PBS for 30 min at 37 °C and the fluorescent cells were directly counted in a counting chamber using a fluorescence microscope (Nikon). Dead cells were blue stained by treatment with trypan blue in PBS and counted using direct light microscopy. Cells grown on a Petri dish without glass were used as a control.

2.4. Polymorphonuclear granulocytes (PMN) preparation

Blood (5 ml in K_3 EDTA) was drawn from 20 healthy donors, aged 20–30 years. PMNs were isolated by the one-step procedure using Ficoll-Hypaque density centrifugation [11]. The contaminating erythrocytes were lysed with distilled water. PMNs were resuspended in PBS and 5.0×10^5 cells/ml with 98% purity were obtained. Viability was tested by trypan blue staining. PMNs were stored at room temperature for a maximum of four hours.

2.5. Chemiluminescence (CL) assays

CL response of PMNs was measured by means of a highly sensitive LUMI-A (SEAS, Italy) luminometer [12]. The dead time of the instrument is about 30 s, due to the rotation of the sample holder required to position the sample in front of the photomultiplier. At first, a stable basal activity of 5×10^5 cells in the presence of 1 μM luminol was observed for 15 min, then the activation was started by the addition of glass suspension. Stimulation by PMA (0.33 μg/ml) was used as a control test.

3. Results

3.1. Synthesis of the sol–gel glass

This material was obtained by ordinary sol–gel methods [13] starting from tetramethoxysilane as SiO_2 precursor and then promoting the hydrolysis (sol formation) and condensation (gel formation) reactions using an acidic water solution containing Ca and P salts as precursors. Under the conditions reported in the experimental procedures section, the formation of a thick gel required about 3 days. Solvent evaporation (i-propanol and water formed during the condensation) to yield the final dry xerogel was performed partly in air and partly in vacuo. This led to a solid containing both oxides chemically bound in the three-dimensional gel network and encapsulated ions that were easily washed out with water.

The release of soluble species was checked during a period of two weeks and was found to occur only within the first hour. Species analysed were Si^{4+}, PO_4^{3-}, Ca^{2+} and NO_3^-. A summary of the glass composition before and after washing is shown in Table I. In the cases of Si and P, the amounts released were negligible with respect to the amount of both elements introduced into the glass. Conversely, in the case of Ca, it was found that 4.77 mmol were washed out (Table I), i.e. more than 60% of the total amount

TABLE I Molar composition of the sol–gel glass

Oxide	mmol (initial)	Content %	mmol (after washing)	Content %
P_2O_5	0.83	2.1	0.83	2.9
Na_2O	0.81	2.1		
CaO	7.11	18.0	2.33	8.1
N_2O_5	7.11	18.0	1.91	6.7
SiO_2	23.6	59.8	23.6	82.3

initially present. This was parallelled by an even higher loss of NO_3^- (5.2 mmol as N_2O_5), strongly suggesting that a large part of the initially added $Ca(NO_3)_2$ does not react during gelation but remains as such inside the gel bulk and can be easily washed out. Indeed, the total loss of nitrate can be accounted for by assuming that all the Na added in the starting synthesis mixture is washed out as soluble $NaNO_3$.

On the basis of these results, the mol % composition of the sol–gel glass can be calculated as indicated in Table I. This indicates that the SiO_2 content is well beyond the 35–60% range normally associated with bioactivity for glasses obtained by melt. Therefore, in view of the suggestions of Li *et al.* [10], it seemed interesting to check their bioactivity properties in a series of tests. Only thoroughly washed (3 h) sol–gel samples were used for all biological tests.

3.2. Effects on fibroblast growth

Growth tests were carried out on cells in culture in direct contact with the glass powders. The amount of powder was determined beforehand, given that an excessive concentration can interefere with the functional activities of cells and their assessment. Preliminary tests indicated that the optimal quantity was 1 mg for a 35 mm diameter Petri dish.

Cells cultured in the presence of sol–gel glass showed a proliferation pattern comparable to that of control cells, while cells incubated with BCNR glass had significantly lower growth (Fig. 1). The viability of the cells was consistently greater than 95%.

3.3. Effects on the chemiluminescence of PMNs

The maximum luminol-amplified CL response of PMNs and its time course significantly varied between the two glasses tested. Representative CL responses are shown in Fig. 2. As can be seen, the activation of PMNs was immediately induced by the addition of the BCNR glass to the cell suspension, with a plateau after 10 min. The maximum response was evoked by the addition of 0.75 mg/ml while no CL signal was elicited at concentrations below 0.2 mg/ml. The maximum value was four times lower than that of PMA-stimulated control cells, although the kinetics were similar. After the maximum of glass-induced CL was reached, a second stimulation with PMA produced a photon emission leading to a total CL response of about 50% of that obtained by PMA alone (Fig. 3).

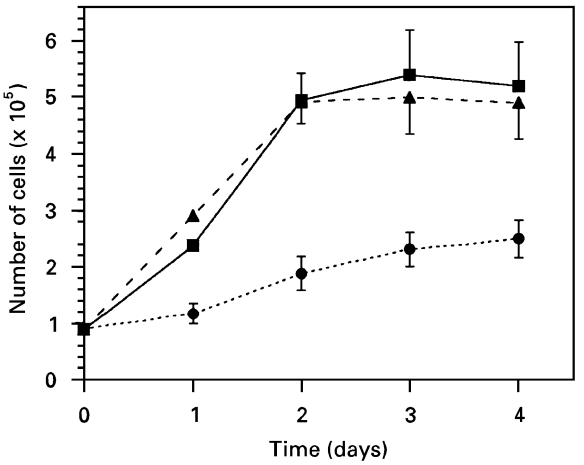

Figure 1 Growth rate of fibroblasts in the absence of glass (■) or in the presence of sol–gel (▲) or BCNR glass (●) at 1 mg/ml concentration. Data are means of triplicate determinations. The vertical bars are the standard deviations.

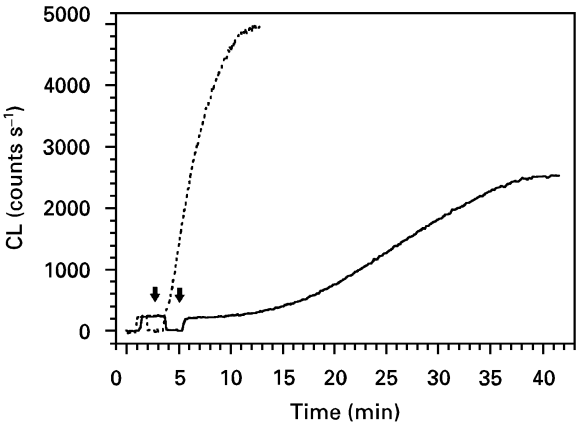

Figure 2 Time course of CL of PMN induced by glass. The reaction mixture (total volume = 1 ml) contained 5×10^5 cells, 1 μM luminol and 1 mg/ml glass, suspended in PBS pH 7.4. BCNR glass: dotted line; sol–gel glass: solid line. The arrows indicate the point of glass addition. The initial luminescence is due to PMN in the presence of luminol. The shutter of the luminometer was closed before additions to check for the instrumental baseline.

Different behaviour was exhibited by the sol–gel glass (Fig. 2). A large amount of material (1–2 mg/ml) and a pre-incubation of 10–15 min were necessary to elicit a CL signal. The maximum was reached after 40–50 min. While PMA and inert glass produced a nearly constant CL response (SD within 20%), sol–gel showed variability in the maximum response, but not in the shape (SD within 40%). Similar to that found for the BCNR glass, stimulation with PMA of sol–gel-treated PMN induced a secondary CL reaching about 50% of that obtained by PMA alone (Fig. 3).

4. Discussion

Stimulated phagocytes produce a large amount of superoxide anion (O_2^-) with the secondary generation of more oxidant species including hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\bullet$) and hypochlorous acid (HOCl). These phagocyte products play an important role in host defence and tissue injury. The key

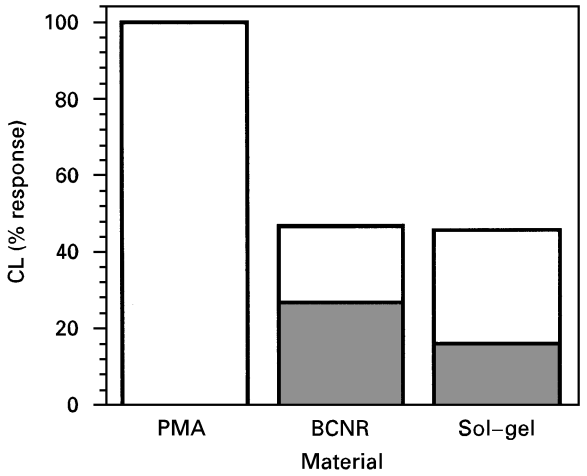

Figure 3 Maximum CL response of PMN induced by PMA or by glasses and secondary CL response of glass-treated PMN induced by subsequent stimulation with PMA (white column on the top of shaded column). PMA: 0.33 μg/ml. Glass: 1 mg/ml. The response induced by PMA alone was set to 100%. Data are means of eight determinations.

element in the generation of all of these radical species is the NADPH oxidase which catalyses the one-electron reduction of molecular oxygen to superoxide anion. The increase in oxygen consumption and reactive oxygen species (ROS) production is termed the respiratory burst [14–16]. It has been shown that all of the cellular activities associated with the burst can be attributed to the activation of the NADPH oxidase. Accumulating evidence suggests that an increase in the hexose monophosphate shunt associated with the respiratory burst provides NADPH as the electron source for oxygen reduction. Components of the oxidase system, such as cytochrome b-558 and flavoprotein, have been identified while there are at least three other components in the cytoplasma. Particulate stimuli such as opsonized bacteria or yeast cell wall fragments appear to induce the respiratory burst on the portion of the cell with which they come into contact. After phagocytosis, the products of the respiratory burst are contained within the phagosomes. Soluble stimuli appear to elicit the respiratory burst over the entire membrane surface of the cell either by direct perturbation of the cell surface or through receptor-mediated events. With any of these stimuli, there is an initial lag period (the length varies with the stimulus before any respiratory burst activity is detected) followed by an acceleration in radical oxygen species generation and oxygen uptake until a maximum velocity is attained within seconds or minutes. In many cases a subthreshold application of one stimulus appear to cause the respiratory burst oxidase to enter a primed state; subsequent stimuli cause a much greater level of radical oxygen species. Repeated application of the stimulus may induce an apparent deactivation of the respiratory burst enzyme.

The paradigm for the activation of the neuthrophil NADPH oxidase with the protein kinase C-activator phorbol myristate acetate (PMA) states that the oxidase assembles in the plasma membrane and ROS are released extracellularly. This paradigm has been challenged by recent results, which show that activation of oxidase, using both soluble and particulate stimuli, can occur without any release of oxygen metabolites into the extracellular medium [17–19]. Recently it has been suggested that a part of oxidase activity in normal neutrophils takes place in an intracellular compartment. Indeed, it has been demonstrated that PMA-induced CL is largely insensitive to scavengers of extracellularly released ROS. It has been indicated that PMA induces the production of a large amount of oxygen metabolites that are not released from the cells. In this case, the NADPH oxidase localized in the granules will be activated [19]. On the other hand, by cytochemical methods, it has been shown that oxidase activity may be enhanced at the plasma membrane level by activation with particulate stimuli [20].

Many procedures have been proposed to measure the ROS production. Among these techniques luminol-induced chemiluminescence has proved to be very effective in investigating the respiratory burst of activated phagocytes. Luminol is a small membrane-penetrating molecule that can be used to amplify respiratory burst activity both extracellularly and intracellularly. The interaction between neutrophils and PMA results in the production of a large amount of light. An inert glass elicited a CL response which was clearly less than that induced by PMA. The sol–gel material, instead, induced a very weak CL response. In fact, although the response was different depending on various individual donors, the lag phase was always long and the maximal amount of ROS production was significantly less than that induced by PMA or by inert glass, which served as a control. The demonstration that the glasses produced a small amount of ROS as compared to PMA may be due to the fact that these materials only elicit the extracellular production of ROS of the respiratory burst. The variability in the responses of the PMNs to sol–gel further documents that this material represents a weak stimulus. In all experiments, further addition of PMA to glass-stimulated PMNs led to a secondary response which was about 50% of that obtained by PMA alone. Altogether, these data show that these glasses are able to stimulate PMNs, but that they slowly activate the transductional events, which lead to NADPH oxidase activation or induce the production of messenger(s) that are unable to elicit a complete response. The long lag phase observed suggests that the sol–gel is unable to cause a normal assembly of the oxidase complex.

The sol–gel appears to be a non-toxic material, as demonstrated by its ability to induce normal growth of fibroblast cells. Fig. 1 shows a marked difference between the BCNR glass and the sol–gel glass that can be explained by Hench's consideration on the bioactivity of glasses [10]. Both glasses have relatively similar SiO_2 mol % content (BCNR 71.9% versus sol–gel 82.3%), however, only the latter, made by sol–gel, can be considered bioactive. Indeed, the materials also differ in chemical composition, as the sol–gel glass contains a significant amount of phosphorous which likely influences its biological properties.

The bioactivity properties make the sol–gel glass particularly suitable for clinical orthopaedic use.

Inflammatory responses have been observed around many types of biomaterial implants and have been associated with adverse effects for both the host and the implant itself. For instance, the major drawback of artificial hip implants is that the rate of implant failure and re-operation after 10 years is between 10 and 30% [1]. Aseptic loosening appears to be the predominant cause of implant failure and osteolytic changes, probably caused by inflammatory reactions, have been suggested to be responsible. During inflammatory responses products generated by inflammatory cells may damage the implant itself and or react with the biomaterials to generate toxic catabolites. Since the activation of neutrophils is influenced by the physical and chemical nature of the adherence substrate [21], the preparation of a biomaterial which minimizes the PMN response may be critical for the construction of such medical devices.

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