Monocytess-based in vitro assay for a preliminary biocompatibility assessment of blood-contacting devices

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Abstract
The biological evaluation of biomaterials is currently defined by the ISO-10993 norm in which parts four and five are dedicated to emo-compatibility and cell toxicity, respectively. Our study will provide a novel in vitro experimental approach for the biocompatibility assessment of biomaterials or medical devices using human primary monocytes as cellular model. In these new settings, human monocytes are exposed to a medium containing the extractable compounds derived from materials or devices; subsequently, cell toxicity and pro-inflammatory effects are analysed through MTT assay, flow cytometry and enzyme-linked immunosorbent assay (ELISA) methodologies. These experimental procedures offer the advantage to use a human and primary cell context belonging to the immune system, in order to accurately predict the nature of blood/device interaction occurring during a clinical application. To validate the reliability of this method, we also reported a comparative study between two different membranes showing a different level of biocompatibility. On the bases of these data, it is possible to state that this new experimental model represents a good approach to investigate the effects induced by a biomaterial on cell death and inflammation using human, primary monocytes.

Keywords
biocompatibility, biomaterials, blood-contacting devices, cell toxicity, extractable compound, inflammation, medical devices, monocytes

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Introduction
Several medical procedures employ biomedical devices that, according to their intended use, enter in contact with human tissues with a different grade of invasiveness. Medical devices (MDs) include a wide range of products, most of them come into direct communication with patient’s blood: from simple catheters and extracorporeal circuits to membrane oxygenators, haemofilters, stents and cartridge for therapeutic apheresis, to name but a few examples. Inside the human body, all these devices give rise to an immune system response. In particular, the direct contact with blood promotes plasma protein deposition on the biomaterial surface creating a layer of proteins able to activate several signalling cascades, mainly related to blood coagulation and complement activation. As a result, numerous plasma proteins are affected and immune cells and platelets are recruited, leading to

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adverse reactions including acute or chronic inflammation.\textsuperscript{1,2} In recent years, biomaterial engineers have strived to design materials capable of avoiding such detrimental effects by developing novel inert biomaterials or by improving the biocompatibility of well-known materials. Current strategies focus on the modification of surface properties, chemical composition, topographic characteristics and the use of specific coatings able to prevent inappropriate host immune response.\textsuperscript{3–7}

In order to perform a biocompatibility assessment of a MD or a biomaterial, in this article we propose a novel experimental approach where devices or materials will be in contact with immune primary cells, essentially primary monocytes.\textsuperscript{8,9} This in vitro model system gives the possibility to evaluate both cell toxicity and the inflammatory response caused by the system under test, using a single-type cellular model, reproducing in vitro the conditions of the clinical setting.

To demonstrate the reliability of this experimental planning, we carried out a comparative analysis between two different polysulphone membranes that will be indicated in this article as sample A and sample B. Polysulphone membranes are constituted by hollow fibres and are mainly used in blood purification therapies, such as haemodialysis/hemofiltration as well as donor and therapeutic apheresis. These membranes are commercially available in several configurations and are ethylene oxide (EtO) or beta rays sterilizable.

The aim of this study is to illustrate a new experimental approach for the biocompatibility assessment of materials or biomedical devices using human primary monocytes; furthermore, to demonstrate the different behaviour of A and B membranes in terms of cell toxicity and inflammatory response. These biological effects will be investigated, respectively, by MTT assay and flow cytometry analysis using propidium iodide and annexin cellular staining and through the measurement of released pro-inflammatory cytokines.

**Materials and methods**

*Monocyte purification*

Human normal monocytes were purified from adult peripheral blood specimens, recruited at the blood transfusion division of Policlinico of Modena, upon a written informed consent of voluntary donors. Human blood was diluted with phosphate-buffered saline (PBS) and peripheral blood mononuclear cells were isolated by density gradient centrifugation over a Ficoll-Hypaque gradient (Lympholyte, cat. # CL5010, Euroclone, Devon, UK). CD14\textsuperscript{+} cells selection was carried out using the immune-magnetic system ‘EasySep Human CD14 Selection Kit’ (cat. # 17858, Stem Cell Technologies, Vancouver, Canada), according to the manufacturer’s instructions. The percentage of purified CD14 positive cells was higher than 95\%, as assessed by flow cytometry analysis. Monocytes were cultured in Iscove's Modified Dulbecco’s Medium (IMDM) (cat. # ECM0192L, Euroclone) supplemented with 10\% heat-inactivated human serum AB (cat. # 14-490E, Biowhittaker, Walkersville, MD) and 1 mM L-glutamine (cat. # ECB3000D, Euroclone).\textsuperscript{10,11}

**Extract preparation**

The culture medium containing the extractable compounds was obtained from each sample by incubation in IMDM, without serum, at 37°C for 18 h and according to a material/medium ratio of 0.025 g/mL. At the end of the incubation time, the extracts were supplemented with 10\% human serum and used for cell culture. Medium incubated in the same conditions was considered as negative control.

**MTT assay**

To assure a good cell adhesion, 0.75 × 10\(^{5}\) human monocytes were seeded in a 24-well plate under normal culture conditions. Medium was then replaced with the undiluted extract obtained by the incubated materials or by medium from negative control sample, both supplemented with human serum. A positive control was also prepared by treating monocytes with a 0.45\% phenol solution in complete IMDM. Plate was maintained at 37°C in 5\% CO\(_2\) for 4 h and the extracts were then replaced with normal medium. Following 24 h, 100 \(\mu\)L of 5 mg/mL MTT solution (cat. # M5655, Sigma–Aldrich, St. Louis, USA) was added to each well and after 2 h of incubation, the precipitated formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The samples were finally analysed at 570 nm using a spectrophotometer (Enspire Multiplate Reader, Perkin Elmer Inc., Whaltman,
MA, USA). The percentage of metabolic activity and cell viability were calculated using the following formula: 

\[
\text{(% metabolic activity)} = \left( \frac{\text{test optical density}}{\text{control optical density}} \right) \times 100
\]

Each sample was analysed in triplicate.

**Apoptosis evaluation by flow cytometry**

To examine the distribution of cells in the different phases of cell cycle, \(1 \times 10^5\) cells were suspended in 500 \(\mu\)L hypotonic solution (50 \(\mu\)g/mL propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100) and then placed at 4°C in the dark for 10 min before flow cytometry analysis. Apoptotic cells were stained with the Annexin V–FITC Apoptosis Detection Kit I (cat. # 556547, BD Biosciences, Erembodegem, Belgium) following the manufacturer’s guidelines. Analysis of cells labelled for the assessment of cell cycle and apoptosis was then accomplished using a Coulter Epics XL-MCL flow cytometer (Coulter Electronics Inc., Hialeah, FL, USA). At least 10,000 events were counted for each sample to ensure statistical relevance. Analysis was performed in terms of positivity percentage.

**ELISA**

To perform enzyme-linked immunosorbent assay (ELISA), \(0.25 \times 10^5\) monocytes were seeded in a 24-well plate in 1 mL of normal medium, subsequently replaced with the same volume of fibre extracts. A positive control sample was also prepared by a 24 h treatment of normal monocytes with a combination of 100 ng/mL lipopolysaccharide (LPS; cat. # L2880, Sigma–Aldrich) and 20 ng/mL interferon gamma (IFN\(\gamma\); Roche Diagnostics, Mannheim, Germany) assuring a classical M1 monocyte activation. The levels of interleukin (IL)-6 and tumour necrosis factor alpha (TNF\(\alpha\)) cytokines were measured in the culture media following 2, 4 and 24 h of incubation with the considered extracts and using the corresponding ELISA kits (Cat. #D6050 and DTA00C, R&D System, Minneapolis, MN), according to manufacturer’s instruction. Each sample and standard were run in triplicate and the negative control was represented by complete medium.

In order to determine the cytokine concentration, a standard curve was created for each cytokine by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis. IL-6 ELISA exhibited a concentration range of 3.1–300 pg/mL, with a sensitivity of 0.7 pg/mL whereas the assay range of TNF\(\alpha\) ELISA test was 15.6–1000 pg/mL, with a sensitivity of 5.5 pg/mL. The obtained standard curves had an R\(^2\) coefficient >0.99.

**Statistical analysis**

All experiments were repeated at least three times and results were presented as mean ± SEM values. Pairwise comparison was carried out using Student’s t-test method. Results of statistical analysis were considered significant when exhibiting \(P\)-values <0.05 and are indicated by asterisks, *\(P<0.05\) or **\(P<0.01\).

**Results**

**Experimental model system**

In order to investigate the cytotoxicity effects induced by the considered membranes, we performed a set of experiments in which human normal monocytes were isolated from peripheral blood of healthy donors. These cells were carefully chosen as cellular model since monocytes belong to the immune system and, together with granulocytes and resident macrophages, are rapidly recruited and activated in response to a host material or device.\(^{1,4,9}\) In addition, the use of these primary cells provides the advantage to achieve a more reliable response than an established cell line, mimicking in vitro the conditions of a clinical treatment. In this experimental design, monocytes were conditioned with a culture medium containing the extractable compounds derived from the considered biomaterials. Following a period of 4 h, cell viability and inflammation response were analysed by MTT assay, flow cytometry and ELISA (Figure 1).

**Analysis of cell viability**

To assess the capacity of the membranes to modify cell viability, we first carried out an MTT assay. To ensure statistical relevance, three independent experiments were performed and each sample was run in triplicate. The results reported in Figure 2, panel a, demonstrated that both membranes do not
induce major toxicity, presenting a cell viability of about 85% as compared to the negative control sample. In order to further confirm these results, cell death was also investigated by two different flow-cytometric methods. In the first one, treated monocytes were incubated with a hypotonic solution containing propidium iodide to detect a sub-G1 peak in cell cycle analysis (data not shown), usually corresponding to cells undergoing apoptosis. In addition, cell viability was also investigated by annexin staining of monocytes upon exposure to material extracts (Figure 2(b)). In agreement with MTT assay, the results achieved by flow cytometry examination disclosed the lack of influence of both specimens on cell viability, as compared with the negative control sample.

**Evaluation of pro-inflammatory molecules release**

To better verify the capability of the considered membranes to induce an inflammatory response during monocytes–material interaction, we analysed the levels of IL-6 and TNFα cytokines in a time course experiment in which human monocytes were exposed to fibre’s extracts. ELISA was performed at 2, 4 and 24 h timing and the data obtained are reported in Figure 3, as mean ± SEM.
of three independent experiments; in each assay, samples and standards were run in triplicate. Interestingly, the results demonstrated that membrane B do not determine IL-6 and TNF\(\alpha\) release up to 2 h of incubation, whereas at 4 h we observed a moderate secretion of both cytokines. At the same time, our method highlights the ability of the extract achieved from fibre A to induce high levels of cytokine release, especially at 4 h and up to 24 h of monocyte exposure. Moreover, the data obtained show that the occurred effect of A sample is comparable to the one achieved in the LPS-positive control and that TNF\(\alpha\) disclosed a significant difference \(P=0.02\) between the two membranes, upon 4 h treatment. Therefore, these data clearly demonstrate that fibres A are able to trigger monocyte activation, causing a major pro-inflammatory response as compared to B specimen. In addition, these results support the sensitivity of our experimental approach since it allows obtaining more reliable indications about biocompatible membranes already used in clinical applications.

**Discussion**

In this article, we described a comparative study between two polysulphone membranes focusing our attention on two main biological processes, that is, the capability of these materials to induce cell mortality and to evoke a pro-inflammatory effect. To achieve this aim, we have developed a novel experimental setting in which human monocytes were exposed to an extract obtained from the materials under investigation. Subsequently, through MTT assay, flow cytometric analysis and ELISA tests, we demonstrated that both membranes do not modify cell viability whereas A specimen evokes a major inflammatory response. These data allowed us to state that B membrane is more biocompatible than A device.

In our opinion, this experimental model represents a good approach for a preliminary evaluation of biocompatibility, especially for materials or MDs that, in their intended use, are in direct contact with blood. At present, the International Standard Organization (ISO) regulates the experimental tests for the biological evaluation of biomaterial or biomedical devices. About cell toxicity, described in part 5, the ISO-10993 recommends the use of established cell lines including L-929, Balb/3T3 and WI-38. These cells offer homogeneous morphology and growth characteristics, facilitating reproducibility in ‘in vitro’ cytotoxicity testing. Nevertheless, with the exception of WI-38, these cells have a mouse origin and were derived from subcutaneous connective tissue (L-929), mouse embryos (Balb/3T3) and lung tissue (WI-38). This means that the considered cellular models show morphological and functional features very different from blood cells and cannot accurately predict the nature of the blood/device interaction occurring during a clinical application. Therefore, the results achieved from cytotoxicity tests might be controversial and different from what happens in vivo. The test ideated and
developed by us utilizes human primary cells, belonging to the immune system, and contributes to better simulate in vitro the conditions of a device during its use.

Furthermore, ISO-10993 part 4 provides general requirements to assess the biocompatibility in terms of coagulation, inflammation, complement activation and haemolysis following a direct contact between MDs and blood. Our experimental model system achieves preliminary but noteworthy results regarding the capacity of a MD or biomaterial to evoke a pro-inflammatory response. This aspect may be examined through the release of pro-inflammatory cytokines, such as IL-6 and TNFα, or by flow cytometric analysis of monocyte surface activation markers.

On the basis of these considerations, our experimental model system offers the advantage to achieve a preliminary biocompatibility assessment using a primary cell model as the monocyte, that is, a cell taking part in the human immune system. In addition, the results described in the article underline the high sensitivity of this approach, demonstrating the capacity of a biocompatible membrane, already used in clinical treatments, to induce a monocyte response. Second, this experimental setting can provide a useful and rapid test to achieve, in a preliminary manner, important information about the biological safety of a new material, of a material undergoing structural, chemical or surface modifications, or of a single component of a complex device/system.

Declaration of conflicting interests

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