Quantitative Response Measurement of Cell Substrate Interactions by RT-PCR

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Introduction

New synthetic methodologies have enabled a remarkable advance in the rational design of polymeric materials that actively control cellular and physiologic responses for use in tissue engineering applications. These methods, which afford precise control over molecular architecture, mass, and composition, produce well-defined materials that are being incorporated into scaffolds capable of supporting and regulating the adhesion, growth, and function of target cells while being minimally detrimental to normal cellular processes and surrounding tissues. Recently, NIST has developed numerous platforms of ECM components such as fibronectin, collagen, and actin in orthogonal gradients in thickness, temperature, morphology, and processing conditions using combinatorial methodologies.

High-throughput metrorlogies for the rapid and systematic evaluation of synthetic materials, which would elucidate a candidate’s potential biocompatibility, are needed. A model system to evaluate the biocompatibility of materials in vitro using real-time polymerase chain reaction (RT-PCR)4,5 has been developed within the Biomaterials group. Inflammatory responses play a prominent role in the biocompatibility of materials, as indicated by the induction of the cytokines interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α). These responses have been characterized for several materials used in biomedical applications.6,7 These RT-PCR studies have quantitatively documented the inflammatory response and yielded great insight regarding the initiation and propagation of the genetic cytokine profile of immune cells. The further development of improved methods for the quantification of cellular responses to biomaterials at the genetic level is of great importance. The extracellular matrix (ECM) has recently received considerable attention due to its importance in cell-cell signaling, wound repair, cell adhesion and tissue function.11 ECM is one of the environmental factors (along with hormones) that communicate with a cell nucleus, modifying nuclear structures and leading to selective gene expression. RT-PCR is being used to test the genetic expression profiles for inflammatory and ECM markers by RT-PCR will be described.

Experimental

Materials. Unless otherwise listed, all solvents and reagents were purchased from Sigma (St. Louis, MO) and used as received. Quant iTect SYBR Green RT-PCR Kit, and Rneasy Kit were obtained from Qiagen (Valencia, CA). Primer identification, isolation, and probe development was nearly identical to methods described previously.9,10

Cell lines. RAW 264.7 were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI (Life Technologies, Rockville, MD) supplemented with heat inactivated FBS (10% (mass fraction) Life Technologies, Rockville, MD) in 5% CO2: 95% air supplemente d with heat inactivated FBS (10% (mass fraction) Life Technologies, Rockville, MD).

mRNA extraction. Cells were plated in 150 mm x 25 mm non- pyrogenic polystyrene dishes (Daigger, Vernon Hills, IL). Specific cultures were incubated on 25 mm glass coverslips coated with the respective polymer film. Alternatively, nanoparticles were added to plated cells 24 h following seeding. The mRNA extraction protocol was followed according to the manufacturer’s specific conditions, except a 21-gauge needle was used to homogenize the sample. The RNA was treated with RNA Secure immediately following elution and stored at –20 °C. Standard spectrophotometric measurements were taken and a 2% (mass fraction) agarose gel stained with 10 μg/mL ethidium bromide (Sigma, St. Louis, MO) was used to image the RNA. Densitometry was performed using the Versa Doc imaging system (Bio-Rad, Hercules, CA).

Results and Discussion

RT-PCR and flow cytometry have afforded the characterization of several key cellular processes, including inflammatory cytokine production and apoptosis progression of macrophages in response to polymeric materials. Prof. Joachim Kohn and coworkers, of Rutgers University have developed a series of tyrosine-derived polycarbonates for use in orthopedic, tissue engineering and drug delivery applications.12-15 The materials have undergone extensive study, are FDA approved, and we hope to use them as a starting point for the evaluation of biocompatible material response tolerances. The characterization data for the respective polymers, which differ only by the length of the alkyl side chain group, are listed in Table 1. PCL is e-polycaprolactone, and the E, B, H, and O nomenclature on the tyrosine- derived polycarbonate correspond to the ethyl, butyl, hexyl, and octyl side chain alkyl groups, respectively.

Table 1. Characterization Data for Tyrosine Derived Polycarbonates

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mn (10^4)</th>
<th>Tg (°C)</th>
<th>Contact angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL</td>
<td>80.0</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>DTE</td>
<td>131</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>DTH</td>
<td>79.1</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>DTO</td>
<td>61.6</td>
<td>90</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1 depicts flow cytometry data for tyrosine-derived polycarbonates thin polymer films under identical solution conditions. Although the percentages detected by flow cytometry as a whole remain largely the same, the increases in the number of cell in early apoptosis cannot be ignored and suggest that simple “live-dead” analyses of materials do not accurately predict material performance. Further analyses of the way the genetic inflammatory and ECM profiles are affected by these materials using RT-PCR are currently in progress. It is anticipated that thresholds established...
using FDA approved materials can be used as a rapid and accurate biocompatibility screen to reduce the number of potential candidates, which are carried forward to further in vitro and animal testing.

Figure 2 contains data collected by RT-PCR, which measures gene copy numbers of the mRNA harvested from cells following exposure to a material. Below is the data acquired for the inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor- alpha (TNF-α) after 24 h of exposure to each of the tyrosine-derived polycarbonates, ε-polycaprolactone (PCL) and tissue culture polystyrene (TCPS).

The differences in TNF-α induction between the respective samples are negligible. Although small, statistically significant differences do exist between the induction properties for IL-1β between the respective samples and controls. While these increases would not prevent the use of these materials in vivo, we have demonstrated the ability to measure small response differences in biomaterials possessing very similar properties and chemical functionality. In addition, RT-PCR and its application are also relevant to other systems, including nanoparticles and peptide functionalized hydrogels, and research is currently underway in several areas.

Conclusions

The evaluation and identification of detrimental interactions between biological species and synthetic surfaces is a daunting challenge as the number of materials and control of physical variables increases. RT-PCR is a method for obtaining quantitative data that can provide valuable insight to the ways cells respond to the introduction of biomaterials and is an important tool that can be utilized when solving problems in tissue engineering.

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References