Biocompatibility of microplates for culturing epithelial renal cells evaluated by a microcalorimetric technique

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In the present study we have developed a microcalorimetric procedure which allows convenient investigation of biocompatibility in a microsystem. We examined the biocompatibility of a porcine renal epithelial tubule cell line LLC-PK_1 and a human primary renal epithelial tubule cell (RPTEC) with microplates composed of three different materials, i.e. Thermanox, transparent film and titanium. All three materials showed equal biocompatibility with LLC-PK_1 cells, judging from the attainment of steady-state power curves and the same rate of heat production per cell (2.5 μW / μg DNA). The human renal cells were poorly biocompatible with the Thermanox and transparent film. However, on titanium the RPTEC cell did adhere, as demonstrated by a steady-state power curve. The human cells also showed a higher metabolic activity (3.0 μW / μg DNA), than did LLC-PK_1 cells cultured on the same type of microplates. In research on biocompatibility there is a need for alternatives to experimental animal investigations. The present technique allows studies of cellular interactions with different biomaterials in a rapid and standardized manner and may therefore prove to be a useful screening procedure.

1. Introduction

Research in the area of biocompatibility has increased in importance during the last two decades. Areas of clinical interest are for instance, evaluation of vascular graft as substitute for venous autograph [1, 2] and development of biomaterials for osteosynthetic implants [3, 4]. So has also the need for development of micromodels as alternatives to *in vivo* studies within this research field.

At present, there are a number of different methods in use for evaluating biocompatibility, including scanning electron- and light microscopy [5–7]; measurement of cell proliferation [8], enzyme reactions [9] or RNA expression [10, 11], as well as cytotoxicity assays [7]. Furthermore, cell-biomaterial interactions also involve the generation and release of different kinds of mediators and the expression of several plasma membrane adhesion molecules belonging primarily to the selectin or immunoglobulin family.

There is a need for a convenient method for screening for biocompatibility, a method that measures both rapid events, including cell growth, and cell adherence to surfaces: Furthermore, it should be possible to sample the medium for analysis of compounds released by the cells. Preferably, the technique should allow study of specific biological processes and of recovery after exposure to effectors without disturbing the cell culture. An instrument, which may meet these demands is the microcalorimeter. This apparatus allows direct and continuous monitoring of the metabolic activity of living cells [12] and measurements can also be carried out under aseptic conditions, if necessary.

The aim of the present study has thus been to develop a sensitive and reliable procedure for evaluating biocompatibility of three different types of microplates with cultured epithelial renal cells using a microcalorimetric technique.

2. Materials and methods

2.1. Cells

LLC-PK_1 cells, a porcine renal tubular cell line (American Type Culture Collection, Rockville, Maryland), were obtained by culturing in 75 cm^2 plastic culture flasks with E199 medium (Gibco BRL, Life Technologies, Paisley, Scotland) supplemented with 10% fetal calf serum and gentamicin (10 μg/ml). Upon reaching confluence, the cells were harvested by treatment with 0.025% trypsin (Gibco BRL, Life Technologies, Paisley, Scotland) for about 5 min. When the cells had detached, fresh medium was added and the cells were washed to remove the trypsin. About 4×10^6

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cells/ml were seeded onto the microplates and allowed to settle overnight.

Primary human renal proximal tubule epithelial cells (RPTEC) (Clonetics Corporation, Walkersville, MD) were obtained by culturing in 75 cm^2 plastic culture flasks with E199 medium (Gibco BRL, Life Technologies, Paisley, Scotland) supplemented with 5% human serum, 40 ng epidermal growth factor/ml and 10 μg gentamicin/ml. Harvesting, washing and seeding were performed as described for LLC-PK_1 cells.

2.2. Microplates

The plastic foil (Thermanox) was purchased from Nunc, Inc. (Naperville, Ireland). Transparency film PP 2500 was the product of 3M Svenska AB (Sollentuna, Sweden). The surface modified titanium plates were a kind gift from Nobel Biocare (Gothenburg, Sweden). These plates were washed with detergent (basic pH), then rinsed with distilled water and finally washed with distilled water in a sonicator bath for 10 min. All three kinds of plates were sterilized with 75% ethanol before use.

2.3. DNA determination

DNA was extracted and quantitated employing the fluorimetric procedure (Model F–2000 fluorescence spectrophotometer, Hitachi Ltd, Tokyo, Japan) described by Shapira and Takashiba [13].

2.4. Microcalorimetry

The rate of cellular heat production was monitored using a 3-channel microcalorimeter of the heat conduction type (Thermal Activity Monitor 2277, Thermometric AB, Järfälla, Sweden) [14]. Briefly, this instrument is equipped with three separate channels, each consisting of a stainless steel reaction ampoule plus a reference ampoule, each ampoule having a total volume of 5 ml. The water bath was maintained at 37 °C. The sample to be measured is loaded into the reaction ampoule with Teflon seals and the corresponding volume of medium into the reference ampoule. The difference in heat production between the two ampoules (P_{value}) creates a heat flow through the thermopiles to the surrounding heat sink and a voltage output is generated. The rate of heat production per cell (P_{cell}) or per amount of DNA (P_{DNA}) is calculated from the P_{value} and the corresponding number of cells or amount of DNA. These instruments demonstrate a baseline variability of less than 1 μW during long-term runs (72 h) [14].

3. Experimental design

3.1. Microcalorimetric investigations

The cells cultured on microplates (placed in small Petri dishes) were carefully rinsed and transferred to the reaction vessel. The reference vessel was loaded with the corresponding microplate only. The vessels were allowed to equilibrate for 30 min prior to introduction into the measurement positions. The recordings lasted for as long as 2 h.

3.2. Recording of the rate of heat production by LLC-PK_1 cells cultured on microplates

Titanium, plastic foil (Thermanox) and transparent film were cut into rectangles of three different sizes (1 × 1 cm, 1 × 2.2 cm and 1 × 3 cm) and cells allowed to adhere to these plates for several days. In pilot experiments it was found that 2.2 cm^2 rectangles allowed adherence of the optimal number of cells, resulting in the highest relative heat production and long-term steady-state power curves. (Cell adherence was monitored in parallel using invert microscopy (Zeiss MC30, Oberkochen, Germany), except in the case of the titanium plates.

3.3. Measurement of heat production by RPTEC cells cultured on microplates

Titanium, plastic foil (Thermanox) and transparent film were put into small Petri dishes and the cells applied, allowed to settle, adhere and spread out for 4–10 days. These plates were cut into the same three sizes as described above and cell adherence monitored continuously using an inverted microscope (except in the case of the titanium plates).

3.4. Calculations

The signal from the microcalorimeter was transferred to a computer via a Pico ADC-16 connector (Pico Technology Ltd, UK). The rate of heat production, $P_{evaluated}$, in the population was expressed per unit of DNA (P W/g DNA), as well as the total heat output recorded.

4. Results

4.1. Recording of the rate of heat production by LLC-PK_1 cells cultured on microplates

LLC-PK_1 cells were cultured on Thermanox, transparent film and titanium plates. Judging from light microscopic examination, the same plating efficiency was achieved with the transparent film and Thermanox. Confluence was reached within 2–3 days. For comparison of the efficiencies of cell adherence to titanium with transparent film and Thermanox, it was necessary to determine cellular DNA and the total rate of heat production. As seen from Table I, the mean rate of heat production per unit DNA of LLC-PK_1 cells cultured on titanium plates, Thermanox and transparent film were identical and the total heat productions per unit area were in same range, indicating that the cells reached the same degree of confluence.

When these cells were cultured on microplates of three different sizes (1, 2.2 or 3 cm^2), heat output was approximately proportional to the area of the plates, i.e. 6–7 μW / cm^2 (Fig. 1). In populations cultured on 3 cm^2 plates, heat output started to decrease slowly after 1.5 h of incubation in the microcalorimeter. Populations cultured on plates with a smaller area demonstrated a stable heat production for at least 10 h (the longest time period tested in these experiments). Plates with an area of 2.2 cm^2 and cell confluence generated a total heat output

TABLE I Heat production by LLC-PK$_1$ cells cultured on microplates (area 2.2 cm^2) composed of different materials

	Transparent film	Thermanox	Titanium
Total heat production (μW)	16.9 ± 1.5 ($n = 15$)	16.0 ± 1.2 ($n = 19$)	17.3 ± 2.7 ($n = 5$)
Heat production/DNA (μW/μg DNA)	2.54 ± 0.19 ($n = 10$)	2.55 ± 0.28 ($n = 13$)	2.58 ± 0.23 ($n = 5$)

n: number of independent experiments.

TABLE II Comparison of total heat production by human renal proximal tubule epithelial cells (RPTEC) cultured on titanium, Thermanox or transparent film (area 3 cm^2)

	Titanium plates	Thermanox	Transparent film
Total heat production (μW)	7.2 ± 1.3 ($n = 20$)	3.4 ± 1.2* ($n = 8$)	0.3 ± 0.1* ($n = 4$)
Heat production/DNA (μW/μg DNA)	3.03 ± 0.15	ND	ND

n: number of independent experiments.
*$P < 0.001$ compared to the corresponding value with titanium plates.
ND: not determined (the number of cells was too low to allow reliable determination of the total DNA).

of around 15 μW and steady-state heat production lasted up to 3 h.

4.2. Measurement of the rate of heat production by primary human proximal tubule cells cultured on microplates

The human renal epithelial cells were found to attach poorly to transparent film, as demonstrated both by light microscopy and by microcalorimetry (Table II). However, RPTEC did attach to both Thermanox and titanium, although the cell survival was more pronounced on the titanium plates. As viewed under the light microscope, cells cultured on Thermanox plates did, however, become confluent rapidly, i.e. after 2–3 days (Fig. 2). Cells cultured on microplates (size 3 cm^2) generated steady-state power curves with a heat output of 3.0 μW/μg DNA versus 2.5 μW/μg DNA for LLC-PK$_1$ cells. However, the total heat output by the RPTEC was much lower than for LLC-PK$_1$ cells.

The total heat production rate by RPTEC cultured on 3 cm^2 titanium plates (Fig. 3) is higher than on Thermanox plates, i.e. 7.2 ± 1.3 μW versus 3.4 ± 1.2 μW, respectively (Table II). The presence of a larger number of cells on the titanium plates was

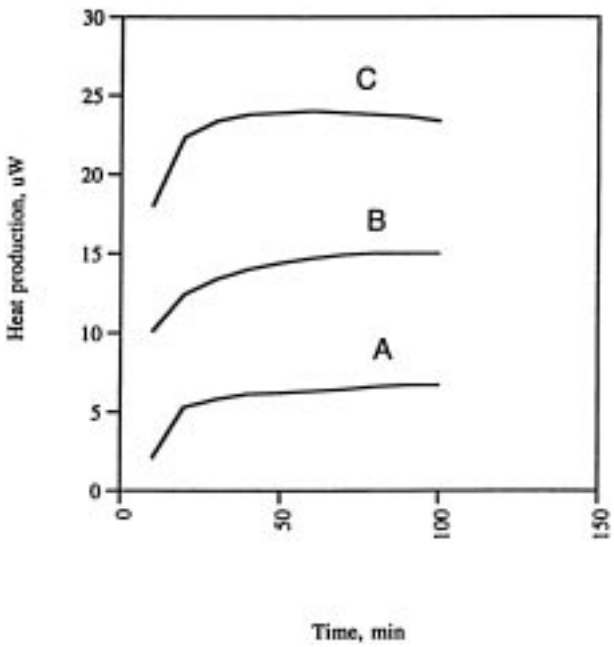

Figure 1 Power curves for LLC-PK$_1$ cells cultured on Thermanox plates of 3 different sizes. A. 1 cm^2, B. 2.2 cm^2, C. 3 cm^2. Culturing on transparent film and titanium resulted in similar power curves.

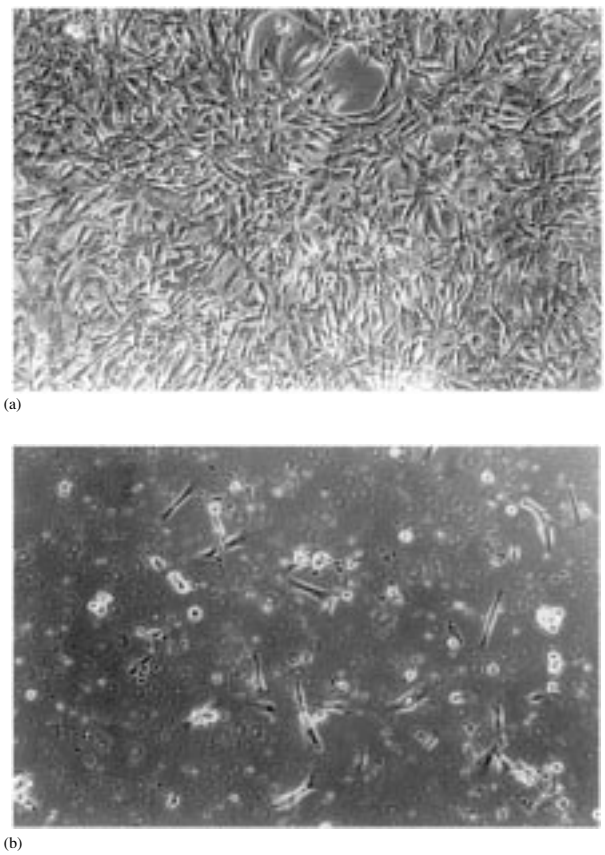

Figure 2 Light microscopy of human renal proximal tubule epithelial cells (RPTEC) cultured on two different plastic foils. (a): Thermanox, (b): transparent film. (× 320).

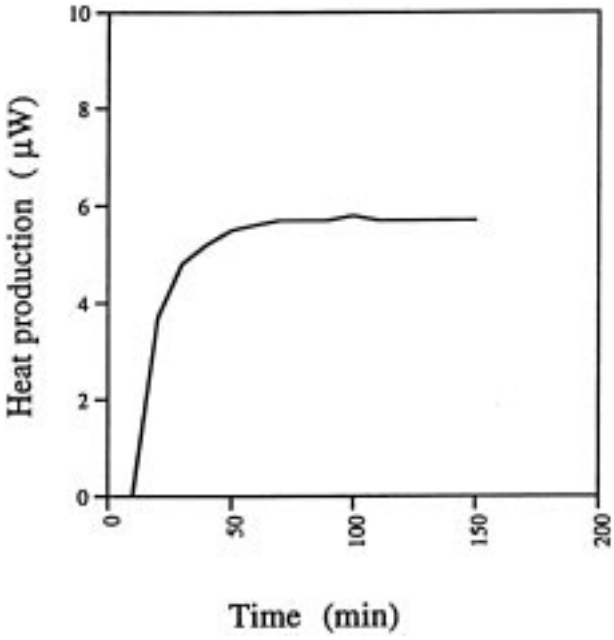

Figure 3 A typical power-curve for human renal proximal tubule epithelial cells (RPTEC) cultured on titanium plates (3 cm^2) for 6 days.

confirmed by DNA measurement. Corresponding measurements on microplates of Thermanox and transparent film 1 and 2.2 cm^2 in size were not performed, due to the low rates of total heat production (close to the baseline) found in pilot studies under these conditions. When viewed by light microscopy, only scattered cells were seen on these plastic plates. The rate of heat production by RPTEC cultured on titanium plates 1 and 2 cm^2 in size were lower than on 3 cm^2 plates, demonstrating the same dependence on plate size as seen with LLC-PK_1 cells.

5. Discussion

Investigation of the compatibility between cells and various materials is an interesting and important task in the development of biomaterials. Microcalorimetry appears to be one suitable technique for investigating biocompatibility. This technique has recently been employed for studying complex interactions between blood and vascular grafts [1, 2].

The biocompatibility of special materials can be divided into two phases, acute and chronic interactions. The acute phase involves, e.g. immediate cellular reactions at the blood-graft interface. These initial reactions are of the utmost importance, since they determine the magnitude of the cellular reaction. Several factors influence cellular interaction with the graft surface, including the morphological appearance of the surface, charge, porosity, chemical composition and hydrophobicity or hydrophilicity [15].

Usually, it is difficult and expensive to follow such acute cellular responses in animal models *in vivo*. Therefore, development of appropriate microcalorimetric procedures may turn out to be a powerful approach for examining the rapid and complex interactions, referred to as acute phase reactions, occurring between biological components and non-biological material.

In the present study we were interested in investigating biocompatibility with respect to human and non-human renal tubular epithelial cells. Most microcalorimetric investigations are carried out using cell suspensions, which may, however, not represent optimal experimental conditions. The use of monolayer cell cultures on microplates, as developed here, will allow long-term investigations, as well as recovery studies.

In our microcalorimeter model employed here, it is also possible to install a perfusion cell. This equipment provides sufficient oxygenation of the medium during long-term runs. However, it requires the use of cells cultured on a solid support.

In the present study we have been interested in identifying biomaterials suitable for culturing conventional cell lines and human primary cells. The three materials chosen were titanium (a biologically inert material used widely as an implant in dentistry), the plastic foil Thermanox and a transparent film. All three can easily be cut into microplates of standardized sizes which fit into the reaction and reference ampoules. LLC-PK_1 cells adhere equally efficiently to all three types of microplates tested, forming confluent monolayers, and the rate of heat production is proportional to the area of the plates, i.e. the number of cells present. For the largest plates (3 cm^2) used, a minor decrease in the power curve was observed after a 50 min steady phase. This is referred to as the ''crowding phenomenon'' and is a well-known and general phenomenon occurring with most cells, including unicellular organisms [16]. The decrease in heat production rate caused by this phenomenon may reflect limited diffusion of nutrients and oxygen, together with accumulation of carbon dioxide, lactic acid and/or other waste products when large numbers of cells are present. This crowding effect might well be avoided completely by using a perfusion cell (see above).

Primary human kidney cells, RPTEC, demonstrate more restricted growth compared to LLC-PK_1 cells. This is not unexpected, since primary cells are usually more dependent on the growth conditions than cell lines able to undergo an unlimited number of passages. RPTEC were found to adhere poorly to the Thermanox and transparent film, with the transparent film being the somewhat less biocompatible of these two plastics. We found that the primary human kidney cells adhere to and grow much better on the inert biological material titanium. RPTEC cultured on 3 cm^2 titanium plates demonstrated a steady-state power curve, indicating stable cellular metabolism. Indeed, these cells demonstrated a higher metabolism per cell compared to LLC-PK_1 cells cultured on the same biomaterial.

In conclusion, the microcalorimetric procedure employed here may be useful for evaluating biocompatibility. It may be useful both for determining the overall heat output generated from metabolic events, as well as for quantitation of cell adherence and basal metabolism. It is a rapid method, convenient to operate and with good reproducibility. The present method can in most cases replace more time-consuming light and electron microscopic investigations for quantitating of adhered cells. Furthermore, this procedure can be scaled down and performed with small amounts of cells and material.

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