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The covalent grafting of fibronectin at the surface of poly(ethylene terephthalate) track-etched membranes improves adhesion of rat hepatocytes

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[^{3}H]methylated fibronectin (FN) has been immobilized on the surface of poly(ethylene terephthalate) track-etched membranes (PET), carboxylated and then activated or not with water-soluble carbodiimide (WSC). Upon washing in 10% SDS or in the nutritive medium used for hepatocytes cultivation (supplemented with 15% newborn calf serum), respectively, 76% and 43% of [^{3}H]FN are released from the unactivated PET membranes and those WSC activated. This difference is almost totally abolished when the -NH_2 functions of FN have been fully acetylated, to impair their reaction with activated -COOH groups. These results strongly suggest that part of FN is covalently grafted to the activated -COOH of PET but that, in addition, FN is also adsorbed on this surface. Rat hepatocytes were inoculated on PET membranes on which FN had been adsorbed and/or grafted. Image analysis clearly indicates that, during the first hours of culture, the FN immobilization on WSC activated, carboxylated PET membranes, significantly favours the adhesion of hepatocytes. After 24 h, the difference between these substrates decreases, probably due to the reconditioning of the PET surface by extracellular matrix constituents secreted by the hepatocytes. Our results confirm that the nature of the protein–polymer interaction strongly affects cell behaviour.

1. Introduction

Anchorage-dependent cells require a substrate and an extracellular matrix in a correct conformation to favour and maintain adhesion. The hepatic parenchyma is a highly differentiated epithelium characterized, in particular, by a polarized architecture responsible for many of the liver-specific functions. Cultivated *in vitro*, under unappropriate conditions, hepatocytes rapidly loose their differentiation. One of the methods reported to allow the hepatocytes to keep their specific functions over long durations is the coating of the culture substrate with an extracellular matrix (ECM) [1, 2]. These constituents must, however, remain adsorbed on the cultivation substrate in sufficient amounts and in correct conformations to be able to activate the cell receptors responsible for cell adhesion, spreading and differentiation. The amounts as well as the conformation of the proteins adsorbed on the culture substrate are affected by the surface properties of the polymer [3, 4] and also by the composition of the nutritive medium. In order to circumvent this problem, several authors have proposed to immobilize, by covalent grafting, cell adhesion and/or growth factors onto different polymers [5–7].

Our aim was to study the adhesion of rat hepatocytes on the surface of poly (ethylene terephthalate) (PET) track-etched membranes on which fibronectin (FN) has been immobilized under conditions which should promote the adsorption or the covalent grafting of the protein. The PET membranes allow one to cultivate many different cell types [8] and are used, in our laboratory, as substrate for CaCo two cells in a cell culture system as an *in vitro* model of the human intestinal epithelial barrier [9, 10]. Fibronectin (FN) was selected as cell adhesive protein because it is a major component of the ECM of hepatocytes and plays an important role in diverse and complex biological functions such as the attachment and spreading of cells [11–13].

Results indicate that FN molecules may be immobilized on the surface of WSC-activated, carboxylated PET membranes in a way that strongly suggests a covalent grafting. The adhesion of rat hepatocytes is faster on these substrate than on those on which FN has been simply adsorbed.

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2. Experimental procedures

2.1. Materials

The cultivation substrates, manufactured and kindly provided by Whatman SA (Louvain-la-Neuve, Belgium) are made of poly (ethylene terephthalate) in the form of transparent track-etched membranes with 1.6×10^6 pores of 0.45 μm/cm^2 whose surface has been specially treated for tissue culture.

Inorganic reagents were obtained from UCB (Braine l'Alleud, Belgium) or from Merck (Darmstadt, Germany). Water (HPLC grade) was obtained with a MilliQ system (Millipore, Bedford, MA). 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride 98 + % (WCS) and 2-[N-morpholino] ethanesulfonic acid (MES) were obtained from Janssen Chimica (Beerse, Belgium). Bovine plasma FN was obtained from Gibco BRL (Paisley, UK) and calf skin type I collagen from Boehringer (Mannheim, Germany). Polyoxyethylenesorbitan monolaurate (Tween 20) was purchased from Sigma (Saint Louis, MI, USA) and sodium dodecyl sulfate (SDS) from Merck. All the other biochemical products were obtained from Sigma, Boehringer or Merck.

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL. The synthetic nutritive medium consisted in basal defined medium (BDM), prepared on a customary basis by Gibco BRL; it was originally described for the culture of hybridoma cells [14] and adapted for rat hepatocytes [15].

FN was radiolabelled with NaB[^{3}H]$_4$, purchased from Amersham (Little Chalfont, UK). Aqualuma cocktail (Lumac, Basel, Switzerland), Soluene 350, Pico-Fluor 350, Midi vial polyethylene flasks and Tricarb 1600 TR liquid scintillation analyser (Packard, San Diego, CA) were used for radiochemical determinations.

2.2. Protein modification and labelling

FN was labelled with NaB[^{3}H]$_4$ by reductive methylation of part of the accessible ε-NH_2 functions of lysine residues [16]. For some experiments, the partially methylated FN was further acetylated with acetic anhydride by the method of Fraenkel–Conrat [17].

2.3. Chemical modification of the PET membranes

Native PET membranes, previously sterilized by autoclaving for 20 min at 120 °C and 10^5 Pa pressure, were modified according to Marchand *et al.* [18] to modulate the chemical composition and the hydrophilicity of the polymer surface. Briefly, the samples were oxidized with 5% (w/v) $KMnO_4$ in 1.2 N H_2SO_4 for 1 h at 60 °C, washed with 6 N HCl and rinsed with water to restore a pH of 7.4; these samples were named PET-COOH.

2.4. Biochemical modification of the PET membranes

Sterile native PET membranes were coated for 2 h with bovine skin type I collagen at 33 μg/ml in phosphate buffer saline (PBS; pH 7.4) and used as reference to standard culture conditions. For the immobilization of FN on the PET surface, sterile -COOH membranes were activated or not with a 0.1% (w/v) WSC in 0.1 M MES buffer, pH 4.5, for 1 h at room temperature followed by washing with MES buffer and PBS and further incubated with FN at 1 μg/ml in PBS for 2 h at room temperature.

In all cases, the substrates were then washed once with PBS containing 0.1% Tween 20 (v/v) and twice with PBS. After protein adsorption and/or grafting, samples were washed for 18 h with either PBS or 10% (w/v) SDS. Following washings, samples were rinsed once with PBS-Tween 20, twice with PBS and then analysed by radiochemical assays.

2.5. Assay of protein on surfaces

The amount of [^{3}H] label remaining on the surfaces was assayed by liquid scintillation counting of the sample-associated radioactivity. Each sample disc was dissolved by immersion overnight in 1 ml of soluene and placed in Midi 20 polyethylene vials. Afterwards, 2 ml of pico-fluor cocktail were added and the radioactivity was determined, with automatic correction for quenching and transformation into DPM. This method avoids underevaluating the amount of tritium as a result of quenching by the polymer (unpublished observations).

The amount of [^{3}H] label present in liquid samples was assayed by counting the medium in polyethylene flasks with 2 ml of aqualuma cocktail. After correction for the specific activity of the protein and the open surface of the polymer disc, results were transformed to ng of labelled protein/cm^2. A blank value was determined with a sample of polymer without any treatment with protein.

2.6. Isolation and culture of rat hepatocytes

Hepatocytes were isolated from male Wistar rats (150–200 g) by the modified collagenase perfusion technique of Seglen [19] and Wanson *et al.* [20]. Viability was determined by trypan blue exclusion and was found to be $\geqslant$ 85%. Cells were cultured in inserts [8] consisting of PET membranes embedded between two rings of polypropylene. These inserts were adapted for 6-well microplates and formed a bicameral system with, respectively, 2 ml of nutritive medium in the lower and 1.5 ml in the upper compartment. Cells were inoculated at 2×10^5 cells/cm^2. Cells were first cultured at 37 °C in DMEM medium containing 4 mM glutamine, 25 mM glucose and 15% (v/v) NCS. After 4 h incubation, the medium was replaced by a nutritive synthetic serum-free medium (BDMH) adapted from Jin *et al.* [15].

2.7. Morphological analysis

The number of rat hepatocytes on the different substrates was determined with a Macintosh AV840 using the NIH1.54 Image Analyser program. Briefly, the culture medium was removed after 0.5, 1, 2, 4 and 24 h;

cells were washed three times with PBS, fixed with formaldehyde 5% (v/v) for 10 min and stained by Mayer's hematoxylin and 1% (w/v) eosine Y. The membrane was then cut out of the insert, mounted on glass and observed with the phase contrast microscope (Labovert, Leitz) coupled with a colour video camera (JVC TK 1280E) and a monitor (JVC TM-10E) at a magnification of 400×.

2.8. Statistical analysis

ANOVA 2 test was used to evaluate the statistical significance of measured differences between samples.

3. Results and discussion

3.1. Adsorption or grafting of the FN

In a first set of experiments, carboxylated PET membranes activated or not with WSC were incubated with FN, radioactively methylated (Met[^{3}H]FN) in conditions adapted to block only part of the accessible -NH_2 functions. Carboxylated PET membranes were also incubated with Met[^{3}H]FN further acetylated (Met + Ac[^{3}H]FN) by an excess of acetic anhydride to block all the accessible -NH_2 groups and make them unreactive with the surface activated -COOH groups of the PET membranes.

In order to determine the nature of the immobilization of FN molecules on the surface of the PET substrates, washings were then carried out for 18 h with either 10% SDS, an anionic detergent or PBS. The amounts of [^{3}H]label remaining associated with the surfaces after these washings were assayed by radiochemical analysis.

Fig. 1, clearly indicates that the WSC activation of the accessible -COOH functions of PET membranes does not significantly ($p = 0.973$) change the amounts of Met[^{3}H]FN remaining associated with the membranes after 18 h washing in PBS. In contrast, 18 h washing in 10% SDS, detaches 43% of Met[^{3}H]FN from the WSC-activated -COOH membranes, versus 76% in the absence of WSC activation. This suggests that at least part of FN could be covalently grafted to the carboxylated polymer by a chemical reaction between the -NH_2 functions of the protein and the activated -COOH functions accessible at the PET surface.

Whatever the conditions, the amount of Met + Ac[^{3}H]FN immobilized on the membranes is lower than that found with the corresponding Met[^{3}H]FN, indicating that slight modifications of FN considerably affect its interaction with the surface of the polymer. In addition, the washing in SDS releases almost entirely the [^{3}H]label from the PET surface activated or not by WSC, demonstrating that reactive -NH_2 functions are required for a stable immobilization.

In a second set of experiments to evaluate the stability of the FN immobilization on the membrane in conditions as close as possible to those used during the cultivation of hepatocytes, carboxylated PET membranes, activated or not with WSC and preincubated with Met[^{3}H]FN, were reincubated in DMEM containing 15% NCS, in the presence or absence of rat hepatocytes. (It should however be noted that membranes washed for 18 h in 10% SDS, as described above, were cytotoxic for the hepatocytes (results not shown)). The amounts of Met[^{3}H]FN remaining associated with the membranes were determined after 0.5, 1, 2, 4 or 24 h incubation and found to be not significantly different ($p = 0.50$). Therefore, the results illustrated in Fig. 2 represent the mean of the values obtained after the different durations of reincubation. They show that, for all the experimental conditions, a mean of 75.6 ± 2.6% of the protein

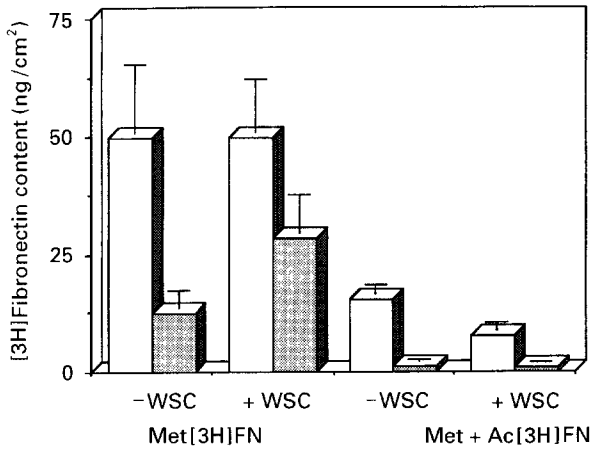

Figure 1 Fibronectin (FN), partially methylated (Met[^{3}H]FN) or partially methylated and then further peracetylated with acetic anydride (Met + Ac[^{3}H]FN) was incubated, at 1 μg/ml, with PET membranes carboxylated as described in Materials and methods and activated (+) or not (−) with WSC for 2 h at room temperature. The substrates were then washed for 18 h with PBS (□) or 10% SDS (w/v) (■). The amount of Met[^{3}H] radiolabelled FN remaining on the surfaces was assayed by liquid scintillation counting and expressed in ng protein/cm^2 of open surface. Means of five independent experiments ± SEM are given.

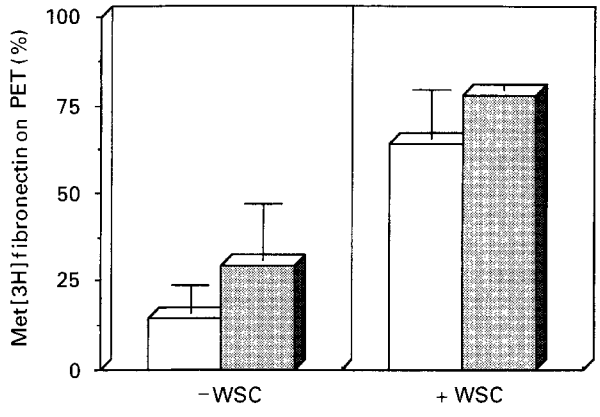

Figure 2 Met[^{3}H]FN at 1 μg/ml was incubated with carboxylated PET membranes, activated (+) or not (−) with WSC for 2 h at room temperature. The substrates were washed for 18 h with PBS and then inoculated at 37 °C without (□) or with (■) 2×10^5 rat hepatocytes/cm^2. After different durations, the culture medium was collected whereas the substrate was washed three times with PBS. Radioactivity associated with the substrates was assayed by liquid scintillation counting. Results are illustrated as percentage of total [^{3}H] protein associated with the membrane before cell inoculation. Means of four independent experiments ± SEM are given.

remains associated with the activated -COOH membrane, versus 20.4 ± 2.6% for the unactivated samples.

All these results clearly demonstrate that washing with either SDS or nutritive medium detaches the bulk of FN adsorbed on the membrane in the absence of WSC activation of the -COOH functions and which resisted a PBS washing. The activation of these functions with WSC significantly ($p < 0.05$) decreases the amounts of FN which are released by these washings. This effect is suppressed after complete acetylation of the $-NH_2$ groups of FN suggesting that this chemical function is involved in the immobilization process and strongly supports a covalent linkage of FN to the activated -COOH groups of the membrane. Nevertheless, our data indicate that, in addition to a covalent grafting, FN is also adsorbed on the activated PET membranes and that part of the adsorbed FN resists 18 h washing in 10% SDS.

3.2. Adhesion of hepatocytes

In order to further evaluate the biocompatibility of the membranes on which FN has been immobilized, adult rat hepatocytes were inoculated on carboxylated membranes, activated or not and incubated with 1 μg/ml FN, as above, before cell inoculation. As control, cells were also cultured on native PET membranes as such or after coating with a high concentration (33 μg/ml) of collagen (our standard culture condition). After 1, 2, 4 or 24 h at 37 °C, the substrates were washed and the adhering cells were fixed and stained. The number of cells/cm^2 and their size (μm^2/cell) were determined by image analysis.

Fig. 3 illustrates that the hepatocytes cultured at 37 °C on WSC-activated carboxylated PET membranes treated with FN, adhere within 1 or 2 h and that their number/cm^2 reached values close to those observed on collagen-coated membranes. In contrast, the adhesion of the cells on native membranes and on carboxylated membranes on which FN has been adsorbed require a lag phase of 2 to 4 h after which the number of cells/cm^2 increases almost proportionally to the duration of culture. Up to 4 h, in all conditions, each hepatocyte occupies an average area of 385.6 ± 38.4 μm^2.

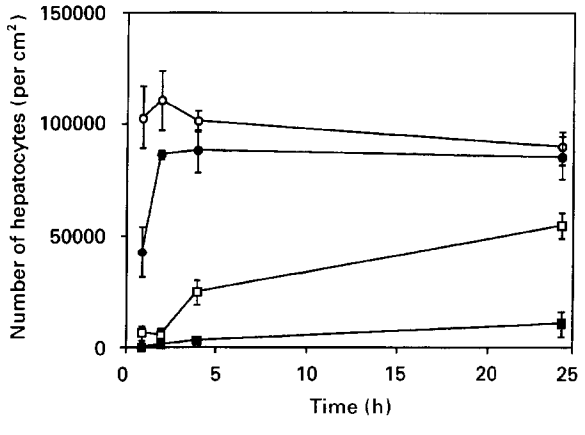

Figure 3 Rat hepatocytes (2×10^5 cells/cm^2) were plated on carboxylated PET membranes, activated (–●–) or not (–□–) with WSC and incubated with FN at 1 μg/ml or on native PET membranes (–■–) or coated with 33 μg/ml of collagen (–○–). After different durations of culture at 37 °C, the culture medium was removed. Cells were washed three times with PBS, fixed and stained. The number of cells was determined by image analysis. Means of five independent experiments ± SEM are given.

After 24 h, the differences between the substrates tend to diminish in terms of cell number. This is probably due to the synthesis and the secretion by hepatocytes of ECM constituents [21–23] which would progressively adsorb on the substrates making easier cell adhesion and spreading. Indeed the mean area increases significantly ($p = 0.002$) to 535.8 ± 71.2 after 24 h, indicating a spreading of the cells on the PET membranes.

4. Conclusions

Our experimental conditions, namely the carboxylation of PET track-etched membranes and their activation with WSC, allow stable immobilization of FN, resulting, most probably, from its covalent grafting. This immobilization resists washing for 18 h in 10% SDS or reincubation in a nutritive medium containing 15% NCS, in contrast to what is observed after the simple adsorption of the protein on the surface of the membrane. On a substrate on which FN has been grafted, the adhesion of rat hepatocytes is accelerated. Nevertheless, in conditions in which protein synthesis and secretion occur and with a normal energetic supply, the cells seem to progressively recondition the surface of PET substrate promoting adhesion and spreading.

We are now developing improved culture substrates which do not require precoating of the surfaces with extracellular matrix constituents. It is particularly important to understand the interactions between cultured cells and synthetic polymer surfaces. With that in mind, this study represents a first step in the development of surfaces optimized to support the attachment and functions of specific cell types. Further, the microporosity of the polymer can lead to the development of cell culture systems allowing a cell polarity close to the *in vivo* situation, by grafting adequate biomolecules onto the surface.

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