Cell–biomaterial interactions: role of transglutaminase enzyme

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Cell interaction(s) with biomaterial undergo a multistep-step paradigm of adhesion, contact, attachment, and spreading. However, the molecular mechanism(s) underlying the cell anchorage on a material surface is not yet well understood. Transglutaminases, a family of calcium-dependent enzymes, has been implicated in the interactions between cells and extracellular matrix. To investigate the functions of this enzyme in cell–material interactions, Balb-C 3T3 fibroblasts were cultured, stably expressing high levels of tissue transglutaminase (TGase), on dishes precoated with thin layers of the hydrophilic hydrogel poly (2-hydroxyethyl methacrylate) (polyHEMA). Results suggest that in the presence of a relatively high poly (HEMA) dilution, the transfected cells showed a more complex morphologic pattern, characterized by many spread and flattened cells, in comparison with a control culture. These studies support the hypothesis that the overexpression of tissue transglutaminase may contribute both to changes in cellular morphology and adhesiveness.

1. Introduction

The biomaterial/tissue interface is a dynamic region which contains a great deal of important information concerning the interaction(s) of tissues with implanted biomaterials [1].

The events at this interface play a critical role in determining the fate of implants; in fact, cell interaction(s) with biomaterial undergo a multistep-step paradigm of adhesion, contact, attachment, and spreading [2]. Recently, it has been demonstrated that attachment and spreading of human-derived bone cells on stainless steel and titanium, during the first 90 min of a cell culture attachment assay, is a function of adsorption of serum vitronectin onto the surface [3]. Indeed the formation of a protein layer at the tissue–biomaterial interface has generally been recognized as the first event which occurs after implantation and seems to mediate the interactions which occur subsequently between the surface and the cells. Albumin, the most abundant protein in blood, has been shown to passivate a biomaterial surface if it is adsorbed onto the material prior to body contact [4]. In contrast, pre-adsorbed fibrinogen or fibronectin enhances cell attachment [5, 6]. However, the molecular mechanism underlying cell anchorage on a material surface is not yet well understood. Recently a family of calcium-dependent enzymes, transglutaminases (TGases), has been implicated in the interactions between cells and extracellular matrix. Moreover, the enzyme is thought to play a role in events related to the assembly of the matrix, wound healing and excessive tissue repair.

TGases catalyse the postranslational modification of proteins referred to as the R-glutaminyl-peptide, amine-γ-glutamyl transferase reaction, which leads to the formation of an isopeptide bond either within or between polypeptide chains [7, 8]. Since it appears that nature has not provided an enzyme for the cleavage of the transglutaminases crosslink in proteins, the action of these enzymes results in the formation of "irreversibly" crosslinked, often insoluble supramolecular structures. TGase crosslinked matrix proteins have been shown to promote cellular adhesion and the induction of tissue transglutaminase has been

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shown to be correlated with morphological changes and increased adhesivity in several cultured cell lines [9, 10].

To investigate the functions of this enzyme in cell–material interactions, we have cultured Balb-C 3T3 fibroblasts, stably transfected with a constitutive tissue transglutaminase expression plasmid.

In the present study we examine the variation in cell shape of transfected mouse fibroblasts as a function of the hydrophilic hydrogel poly(2-hydroxyethyl methacrylate) (polyHEMA) film thickness, to evaluate the capability of the polymer to promote cell adhesion.

2. Materials and methods

2.1. Poly(HEMA)-coated plates preparation

Poly(2-hydroxyethyl methacrylate) [poly(HEMA)] (Sigma, Milan, Italy) coatings were applied to 24-well culture plates (Falcon, Becton Dickinson, Milan, Italy) by adapting a previously described technique [11].

An initial 12% poly(HEMA) in 95% ethanol (EtOH) stock solution was prepared; plates were precoated with prewarmed various dilutions of poly-HEMA in EtOH. Dilutions of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} represent solutions of 1.2%, 0.12%, 0.012%, and 0.0012% poly(HEMA) in EtOH, respectively. Then the plates were allowed to dry at 37 °C overnight and the cells were seeded. Each poly(HEMA) concentration was represented in triplicate.

2.2. Cell culture

Balb-C 3T3 cells were grown in αDME medium (Gibco Laboratories) supplemented with 10% heat-inactivated FBS (Hyclone Laboratories), 10% Serum Plus (Hazleton Research Products Inc.), 50 u/ml penicillin, and 50 μg/ml streptomycin (Gibco). Balb-C 3T3 cells, transfected with 10 μg pSG5-TGase and 1 μg pSV2-Neo plasmid by calcium phosphate procedure [12], were grown in αDME supplemented with 10% FBS in normal growth media containing 400 μg/ml G418 (Gibco).

2.3. Protease detachment assay

Adhesivity analyses were carried out by the following procedure: Control and transglutaminase-transfected Balb-C 3T3 cells were grown in Petri dishes, 5 μl of ^{3}H-labelled amino acids mixture (1 mCi/ml, specific activity 204 mCi/mg; ICN) were added to each dish 12 h before the assay. Monolayers of cells 24 h after reaching confluency were rinsed twice with αDME medium and 1 ml of 0.125% bovine trypsin preparation (Gibco) in Dulbecco's PBS was added. The dishes were then placed on a rotary shaker at 80 rpm at room temperature and 50 μl aliquots were taken each 10 min from the dishes. At the end of the incubation, the aliquots containing detached cells as well as the residual adherent cells (solubilized in 1% SDS) were quantitated by scintillation spectrometry.

2.4. Morphometric analysis

The adherent cells grown on poly(HEMA) layers with different concentration, were fixed with methanol and stained with Giemsa. Photomicrographs of cells were taken on an Olympus inverted microscope.

3. Results and discussion

Cell adhesion on a biomaterial can be divided into two steps: attachment, which is regulated by physicochemical factors, and spreading, in which specific biological molecules are involved. The dependence of cell attachment to the surface free energy or the water content of material may modify the first step, while the presence of specific molecules derived from serum and/or from plasmamembrane, such as fibronectin and collagen, can affect the second one.

Indeed, the adhesiveness of plastic culture dishes can be permanently reduced in a graded manner by applying increasing concentrations of poly(HEMA). The mechanism(s) by which poly(HEMA) reduces the adhesivity of plastic is not entirely understood. As poly(HEMA) is a hydrophilic hydrogel of neutral charge, it may act by reducing the net negative electrostatic charge of the plastic. It is also possible that the low amount of protein adsorbed on poly(HEMA), as well as the nature of the protein layer on the polymer, plays an important role in the poor *in vitro* cell attachment and proliferation on this material. In fact, it was reported that the attachment of fibroblasts is closely related to the type of adsorbed protein rather than to the conformational state of adsorbed protein [13]. Major plasma proteins such as serum albumin suppress the attachment of fibroblast cells on a biomaterial, while specific molecules, as exemplified by fibronectin, accelerate this attachment. Recently it has been shown [14] that cell spreading may be progressively inhibited in serum-free medium by overlying fibronectin-coated bacteriological dishes with poly(HEMA) coats of various thicknesses. Cell-substratum contacts that do form when this technique is used occur within defects in the polymer layer that are large enough to permit binding of multiple cell-surface receptors.

The important conclusion is that whatever the mechanism by which poly(HEMA) can reduce adhesivity of plastic, cells respond by a change in shape. Furthermore, it is emphasized that time-lapse films show that for substrates of low adhesivity, equilibrium shape is actually the average shape maintained by each cell as it continually spreads out and retracts. However, the extent of this transient spreading is less when the adhesivity of the substratum is diminished.

By review of the scientific literature it seems that the attachment of the cells to a substratum is relative only to extracellular factors. In fact, all the data reported ascribe the modifications of cell shape to extracellular factors, while no mention is made of any other possible mechanism(s) related to the same cells that in some way could modulate the attachment on a polymer. Indeed, until now the important variables

associated with the cells that may improve adhesion are unclear.

In several recent reports the induction of tissue transglutaminase activity in cells has been related with alterations in cellular morphology [15] and adhesive properties [16]. There is increasing evidence for tissue transglutaminase activity in cell spreading. A number of adhesion proteins such as fibrin(ogen) [17], fibronectin [18], vitronectin [19], collagen type II, osteonectin/BM-4/SPARC and osteopontin/SSP-1 have been shown to be glutaminyl substrates for tissue transglutaminase. Fibronectin and fibrinogen have been shown to bind to the surface of hepatocytes and endothelial in-suspension culture and become cross-linked into the pericellular matrix. This process is mediated by tissue transglutaminase, as shown by inhibition with TGase-specific antibodies and the formation of fibrinogen Aα-chain polymers, charateristic for TGase action [20]. In agreement, a large fibronectin-containing insoluble protein polymer, associated with plasma membranes in liver, was shown to be formed by the action of a TGase. Tissue transglutaminase covalently stabilizes lamino-nidogen complexes in the homoaggregate structure thought to occur in basement membranes and often co-localizes in the extracellular space with the laminonidogen complex and with fibronectin [21]. Tissue transglutaminase may modulate cell–matrix interactions [22], thereby facilitating the assembly of the matrix, and play a role in events related to wound healing.

In our work we observed that the overexpression of the human tissue transglutaminase in the Balb-C 3T3 fibroblasts increased cellular adhesion on the plastic surface materials.

In order to examine the molecular basis of this effect, several biological studies were carried out. To determine whether expression of tissue transglutaminase produced a generalized effect on cellular proliferation, we measured the proliferate activity of control and transglutaminase-transfected 3T3 cell clone by ^{3}H-thymidine incorporation during log-phase growth. There was no significant difference between the growth rate of the control and transglutaminase-transfected cells on the plastic surface (Fig. 1). In contrast, in the presence of increasing thicknesses of poly(HEMA), associated to a decrease in the number of available contacts of the cells with the plastic, there was an impairment of DNA synthesis. At the higher concentrations of poly(HEMA) the behaviour of the two cell lines (control and TGase-transfected fibroblasts) was not significantly different in terms of thymidine incorporation. Decreasing poly(HEMA) thickness resulted in enhanced proliferation of both fibroblast cell lines. However, TGase-transfected cells increased in number when cultured on relatively high poly(HEMA) dilution (10^{-3}), while at the same poly(HEMA) concentrations control cells did not grow and lost viability over time. On low poly(HEMA) coating concentrations, control cells exhibited a moderate adhesivity associated to an intermediate growth rate. Indeed, for cells subject to anchorage dependence, shape is modulated by

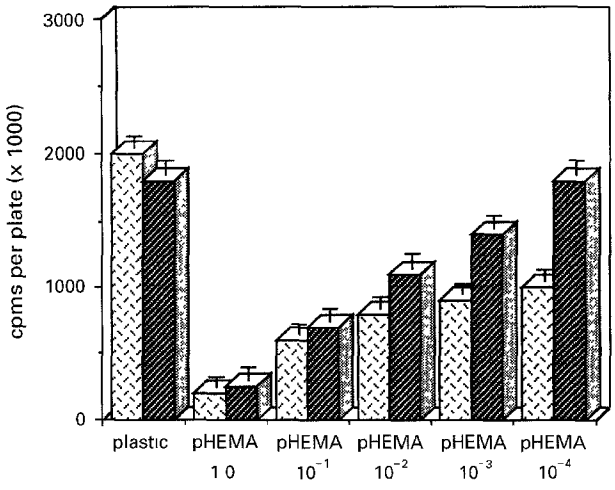

Figure 1 Growth rate of control (▨) and transglutaminase-transfected (▩) Balb-C 3T3 cells plated on increasing thickness poly(HEMA) substrates and plastic surface. The cells in log-phase growth were pulse labelled with ^{3}H-thymidine and the incorporation of thymidine into DNA was measured by TCA precipitation and scintillation spectrometry. Values presented represent the mean ± SD of three separate experiments.

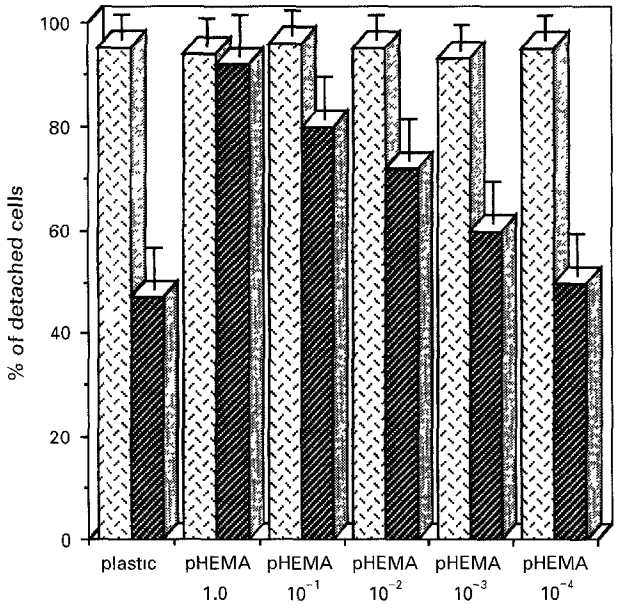

Figure 2 Trypsin-induced detachment of control (▨) and transglutaminase-transfected (▩) Balb-C 3T3 cells plated on poly(HEMA) substrates and plastic surface. Monolayers of control and transfected cells were subjected to trypsinization as described in Section 2. Values, expressed as the percentage of total cells that have detached at the end of the 30 min incubation, represent the mean ± SD of triplicate determinations.

substrate adhesiveness, and only when cells are spread to the appropriate degree can DNA synthesis proceed.

Protease detachment assays were performed with both the Balb-C 3T3 control and the stably transfected cell lines after plating on a nontoxic transparent film of poly(HEMA) of varying thickness. At the 10^{-3} poly(HEMA) concentration the TGase-transfected Balb-C 3T3 were more resistant to protease detachment than the control cells (Fig. 2). In fact, the transfected clone included many cells that required prolonged exposure to the trypsin preparation before

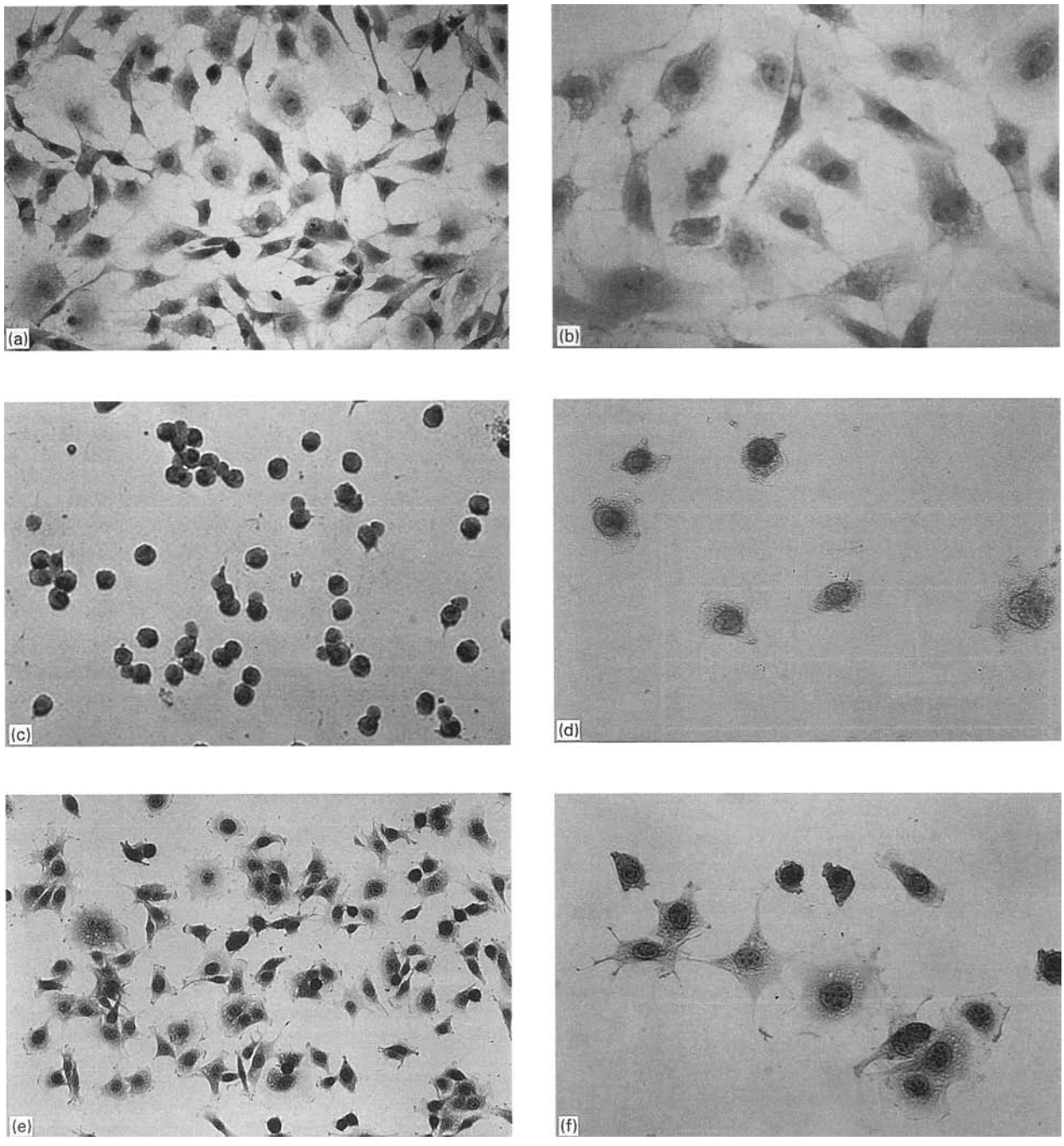

Figure 3 Morphological appearance of control and transglutaminase-transfected cells. Control (a) and TGase-transfected cells (b) grown on plastic surface. Transglutaminase-transfected cells plated on high concentration of poly(HEMA) (c, d), and on dilution (10^{-3}) of poly(HEMA) (e, f). (Original magnification a, b, c, e, ×200; d, f, ×400).

they were detached from the culture dish. To evaluate the relationships between TGase expression and cellular morphology, we used phase-contrast microscopy of methanol-fixed control cells and TGase-transfected cells. Unlike the control Balb-C 3T3 cells (Fig. 3a), the transfected cells (Fig. 3b), showed a marked flattening of their morphology indicating increased cell-substratum interactions on the plastic surface. In the presence of high poly(HEMA) concentration, the control cells were indistinguishable from transfected Balb-C 3T3 cells in terms of cell attachment and spreading. The majority of cells showed a typical round shape morphology, with scarce well-developed lamellipodia. Only a few flattened cells were occasionally present on the material surface (Fig. 3 c,d.). In the presence of 10^{-3} diluted poly(HEMA), the transfected cells showed a more complex morphologic pattern. These cultures were characterized by many spread and flattened cells in comparison with control culture (Fig. 3 e,f.).

The observations made with the transglutaminase-transfected cells strongly support the idea that increased expression of tissue transglutaminase can be associated with dramatic alterations in the pattern of interactions between cells and their substratum.

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