Differentiation of THP-1 and U937 cells in presence of synthetic hydrogels

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Abstract — We investigated the in vitro differentiation activity of two hydrogels: polyalkylimide (PAI) and polyvinyl alcohol (PVA) based hydrogels on THP-1 and U937 cells. PAI and PVA are synthetic injectable fillers, currently used for repairing serious or congenital or caused by traumas aesthetic defects. Recent studies show that hydrogels are involved in several differentiation process, i.e. osteogenic and chondrogenic differentiation. We analysed the differentiation degree, by using Nitro blue tetrazolium (NBT) assay, exerted by the incubation of cells with hydrogels up to 3 days. In addition, to mimic natural condition, we also carried out the experiments in presence of fibronectin, a component of tissue extracellular matrix. Induction of macrophagic differentiation by 50 ng/ml TPA (12-O-tetradecanoylphorbol-13-acetate) for up to 3 days has been used as positive control. We found that PAI and PVA induced differentiation in a cell type- and hydrogel- dependent manner. A differentiation degree comparable with those achieved with TPA was measured with PAI for both cell lines and with PVA only for THP-1 cells.

Key words: differentiation, macrophages, Polyalkylimide hydrogel, Polyvinyl alcohol hydrogel, THP-1 cells, U937 cells.

INTRODUCTION

Hydrogels are one of the most promising and versatile materials with many potential applications. They are highly hydrated polymer networks composed of water-soluble macromolecules held together by crosslinks formed via chemical bonds, ionic interactions, hydrogen bonds, hydrophobic interactions, or physical bonds (Peppas et al. 1986). The properties of the hydrogels, such as permeability, mechanical strength, and biocompatibility, can be easily engineered for specific applications (Jen et al. 1996). Their high water content allows easy exchange of nutrients and wastes with surrounding environment. In addition, the tissue-like elasticity of hydrogels plays a pivotal role when they are integrated into the human body. Many studies are focusing on hydrogel scaffolds for the partial or full regeneration of organs. These materials are based upon natural polymers, including alginates (Rowley et al. 1999), collagen (Yaylaoglu et al. 1999), gelatin (Tabata et al. 1999), and agarose (Griffith 2000), but also synthetic polymers such as poly(hydroxyethyl methacrylate) (Oxley et al. 1993), poly(ethylene oxide), and poly(acrylonitrile)sodium methallyl sulfonate). Recently polyalkylimide (PAI) (Pacini et al. 2002) and polyvinyl alcohol (PVA) (Nakamura et al. 2001) based hydrogels are used in reconstructive surgery, in order to repair serious or aesthetic defects, congenital or caused by traumas.

As a general rule, the ideal biomaterial should be biodegradable and bioresorbable to support the reconstruction of a completely normal tissue without elicits inflammation. In practice, the insertion of an artificial implant into the human body evokes a sequence of healing processes and host responses including a range of vascular, humoral, and cellular responses, known collectively as inflammation (Anderson 1993; Thomsen and Gretzter 2001). The use of biocompatible materials in implants could result in a progression of the inflammatory response from a high intensity, acute phase to a low-activity, quasi-equilibrium state, called the foreign body reaction (Jenne et al. 1998). The macrophage is central to direct host
inflammatory and immune processes; thus, its response to biomaterials is extremely important in understanding material-mediated host response (Kao 1999). The macrophages are one of the first cells to arrive at the tissue-implant interface from recruitment of blood monocytes (Futami et al. 2000; Sennery et al. 1993) and have a pivotal role in modulating the repairing process, mediating phagocytosis, and producing a myriad of cytokines for control of wound healing and cell recruitment as well proliferation (Dipietro et al. 1998; Dipietro 1995; Kovacs and Dipietro 1994). Adhesion and/or interaction of these elements to the material represents a crucial condition in determining the biocompatibility of implanted devices (Zhao Q. et al. 1991; Zhao Q. H. et al. 1993).

The human monoblastic leukaemia cell lines (U937 and THP-1) have been extensively used as model systems for many purposes including the study of the macrophagic differentiation. In fact, various physical and chemical agents can induce these cell lines to differentiate into mature monocytes. Differentiation is characterized by permanent alterations in a variety of cellular parameters, compared to an appropriate undifferentiated phenotype. Such alterations affect morphology, metabolic pathways, and cell growth. U937 and THP-1 cells represent different stages of monocytic development, promonocytes and monocytes respectively, (Tsudhia et al. 1980) and a variety of agents, including dimethyl sulfoxide (DMSO) (Nakamura et al. 1990), retinoic acid (Olsson and Breitman 1982), vitamin D₃ (Olsson et al. 1983), cytokines (Harris et al. 1985; Testa et al. 1988) and phorbol esters, in particular 12-O-tetradecanoylphorbol-13-acetate (TPA) that is the most potent agent (Hass et al. 1989), can promote their differentiation in the monocyte/macrophage pathway.

Aim of the present study is the investigation of the in vitro interaction of two synthetic injectable hydrogels, polyalkylimide (PAI) and polyvinyl alcohol (PVA), with U937 and THP-1 cells. Measurement of differentiation into macrophages will be analyzed by means of biochemical and morphological assays.

**MATERIALS AND METHODS**

**Cell culture** - THP-1 cells, leukemic cell line derived from a patient with acute monocytic leukaemia and human U937 myeloid leukaemia cells, were cultured in RPMI-1640 medium (Cambrex, Verviers, Belgium) supplemented with 10% foetal bovine serum, 2 mM L-glutamine (Cambrex), 100 IU/ml penicillin and streptomycin solution (Sigma, St. Louis, MO) and 10000 U/ml nystatin (antimycotic solution) (Cambrex) in a 5% CO₂ humidified atmosphere at 37°C. The cells were maintained in 75 cm² flasks at the concentration ranging from 2 x 10⁵ to 1 x 10⁶ cells/ml by passage every 3 to 4 days.

**Hydrogels** - Poly-alkyl-imide (PAI) hydrogel is a polyacrylic polymer containing alkyl-amide-imide groups, crosslinked by a redox method. PVA is a synthetic hydrogel consisting of poly-vinyl-alcohol (8%), highly purified (99%) and nonpyrogenic water, crosslinked by freeze-thawing cycles, according to a physical process. Both hydrogels containing 94.96% of pyrogen free water.

**Cytotoxicity evaluation: MTT assay** - Cell viability was evaluated by MTT assay. The culture medium has been replaced with 1 ml/well of MTT (Sigma, St. Louis, MO) solution (1 mg/ml in culture medium without phenol red). After 2h of incubation at 37°C the solution was removed and 1 ml/well of DMSO was added. After 10 minutes of incubation in condition of slow shaking the absorbance was read at 540 nm.

**Induction of differentiation** - To induce differentiation, cells were seeded into 6-wells flat-bottom plates (5 x 10⁶ cells/ml), in the presence of a fibronectin substrate, and incubated for 3 days with 50 ng/ml TPA (12-O-tetradecanoyl-13-phorbol acetate) (Sigma, St. Louis, MO) or 50 mg/ml PAI and PVA. The solution of fibronectin (100 µg/10ml of PBS 0.2 M pH 7.4) was left in the culture wells for 18 hours at 4°C followed by 1% BSA in PBS for 90 minutes at 12°C before each experiment.

**NBT assay: reduction of NBT** - Nitro blue tetrazolium (NBT) (Sigma-Aldrich) assay, performed according to the method described by Rook et al. (1985), was used to evaluate differentiation degree. Briefly, after each treatment, cells were incubated with 0.1 mg/ml NBT in culture medium, filtered before use, for 2 hours at 37°C; they were then washed three times with methanol. The amount of NBT-formazan produced is directly associated with differentiation degree and it can be determined spectrophotometrically (DU 640B Spectrophotometer (Beckman Coulter)) at 630 nm after solubilization of crystal in 1 ml of solution
KOH 2M/DMSO (460µl KOH and 540 µl DMSO).

Morphological modifications - In order to evaluate the morphological modifications after the differentiation in presence and in absence of TPA, PAI and PVA, the cells were stained with haematoxylin/eosin and observed with light microscopy, Eclipse 80i microscope (Nikon, Japan).

Statistical analysis - Statistical analysis were performed using one way analysis of variance (ANOVA) with 95% confidence limits. Data are presented in text and figures as mean ±SD.

RESULTS

Cytotoxicity - The presence inside the cells of blue crystals derived from the transformation of diformazan in salt crystals (MTT test) indicated that both U937 and THP-1 cells are viable and metabolic active also in the presence of hydrogels. The percentage of viable cells decreased in the presence of PAI more than in the presence of PVA only for THP-1 cells (Fig. 1). Indeed, THP-1 cell line is more susceptible to the presence of hydrogels: 20% less viable THP-1 cells in the presence of PAI versus control cells. Viability of THP-1 cells grown in the presence of PVA remained unchanged and was significantly higher for U937 cells (Fig. 1).

PAI and PVA hydrogels induce differentiation in U937 and THP-1 cell line - The differentiation achieved with the incubation up to 3 days of cells with the two hydrogels was compared with the percentage of spontaneous differentiation in in vitro culture, i.e. untreated cells as the negative control, and with the percentage of differentiation achieved with TPA incubation, i.e. cells treated with phorbol ester as positive control. The insoluble, blue coloured compound (diformazan) formed by the reducing substances (i.e. esterase acid) synthesized during differentiation, indicated that both PAI and PVA induce cells to differentiate in macrophages. The dark blue insoluble particles produced can be easily observed under a light microscope (data not shown) or measured at the spectrophotometry (Fig. 2). The two cell lines behaved differently after 3 days of culture in presence of the hydrogels. PAI only was able to induce differentiation of U937 cells. PVA-induced differentiation was not significant different from control. However, the percentage of differentiation in the presence of PAI never reached the level obtained with TPA, remaining always lower of about 30% (Fig. 2). Conversely, both PAI and PVA induced differentiation of THP-1 cells. In this case, PVA was a better inducer of about 30% than PAI. Indeed, at 3 days of incubation with hydrogels, the degree of macrophagic differentiation obtained with PVA was not significant different from that obtained with TPA (Fig. 2).

The differentiate condition as mature macrophages implies an adhesion growth to the culture

Fig. 1 — Viability of THP-1 and U937 cells in presence of PAI or PVA hydrogels evaluated by MTT assay. Values have been normalized for the control, that have been considered as 100%, and are the mean of three independent experiments done in triplicate. Differences from control: * p< 0.05. CTRL: untreated cells; PAI: Poly-alkyl-imide; PVA: poly-vinyl-alcohol
plates; as a consequence cell shape is dramatically modified, since undifferentiated U937 and THP-1 cells have a round shape and growth in suspension. Both cells have scarce cytoplasm and large nucleus. After differentiation with TPA, almost all cells attached to the culture plates and in particular for THP-1 cells developed aggregates by extending pseudopodia to each others (Fig. 3). Similar morphology was observed when THP-1 cells are cultured in the presence of hydrogels. The amount of attached cells is related to the differentiation degree: thus, in the presence of TPA or PVA much more cells are adhering to the substrate than when they are cultured with PAI (Fig. 3).

Conversely, when U937 cells are growth in presence of PAI, even if the number of substrate attached cells is very small compared to PVA, their shape is very similar to mature macrophages; indeed, a very irregular flattened shape with cytoplasmic protrusion is observed (Fig. 4b); furthermore in presence of PAI, U937 cells attach to hydrogel in large number (Fig. 4a).

**DISCUSSION**

In this work we have analysed the ability of two synthetic hydrogels, PAI and PVA, largely used for aesthetic surgery reconstruction, to induce macrophagic differentiation of U937 and THP-1 cells. The main goal of implanting any material is to obtain an appropriate host tissue response for the particular application (HUNT and SHOICHET 2001; SOSKOLNE et al. 2002). In the last few years, a growing request for biocompatible materials for implants in human tissues has been determined by the increased use and continuous progress in surgical techniques. PAI is very eligible for serious surgical reconstructions than for common aesthetic defects, since it has no mutagenic, allergenic or immunogenic effect and high biocompatibility (RAMIRES et al. 2005). PVA is currently regarded as non-toxic and tissue-compatible material and, due of its high biocompatibility, softness and flexible mechanical properties, high oxygen permeability, and better comfort, is commonly used for soft contact lenses. In addition, PVA is also used for long-term implants, including bioartificial pancreas, cartilage, nonadhesive film, and esophagus or scleral buckling material (NAKAMURA et al. 2001).

In the design of an implant material, any knowledge of interaction and mutual modifications between cells and material is fundamental for the positive fate of an implant. Previous studies carried out in our laboratory have indicated that both hydrogels are highly biocompatible, since they scarcely affected cell viability and cell growth of U937 cells, mouse fibroblasts 3T3 cells and human isolated lymphocytes and never provoked necrosis (DINI et al. 2005; RAMIRES et al. 2005). In this study we have found that both hydrogels are not cytotoxic also for THP-1 cells and promote the *in vitro* macrophagic differentiation. It could be hypothesised that when implanted *in vivo* PAI and PVA could also promote recruitment of macrophages (personal communications) since the presence and the activity of macro-
Fig. 3 — Inverse microscope micrographs of U937 (left) and THP-1 (right) cells after treatment with TPA (50ng/ml), PAI and PVA hydrogels (50 mg/ml). CTRL: untreated cells; TPA: 12-O-tetradecanoylphorbol-13-acetate; PAI: Poly-alkyl-imide; PVA: poly-vinyl-alcohol.
phages in the implant zone belongs to the natural foreign host reaction. Both hydrogels are good inducers of differentiation for both cell types with same differences: the degree of differentiation is cell-type and hydrogel dependent. PAI induces differentiation much more than PVA in U937 cells, the opposite is found for THP-1 cells. This could be due to the fact that U937 and THP-1 cells represent different stages of differentiation: U937 cells are promonocytes, while THP-1 cells are monocytes. Furthermore, differentiation degree in PVA- treated THP-1 cells is the same to TPA treated cells.

Many substances are known to induce monocyte/macrophage differentiation and TPA is the most effective one. In a previous work we achieved the higher degree of macrophagic differentiation of U937 cells after 3 days of culture with 50 ng/ml TPA better than with glutamine deprivation, 10% dimethyl sulfoxide (DMSO) and 100 mM/L Zn++ (Pagliara et al. 2005).

The surface characteristics (Jenney and Anderson 2000) dictates adhesion and survival of monocytes and macrophages. Surface topography is known as one of the major determinants of implant performance in vivo and influences cell behaviour in terms of cell adhesion, cell selection, mechanical interlocking, cell orientation and topographic (contact) guidance, tissue organization, cell shape, production of local microenvironments (Brunette 1996), and production of growth factors and cytokines (Kieswetter et al. 1996). The response of surface topography (roughness) to cells including fibroblasts, epithelium, and osteoblasts has been extensively studied (Kieswetter et al. 1996; Brunette 1999; Wieland et al. 2002). For example, many studies have investigated the effect of surface topography on the ability of cells to attach, proliferate, differentiate, and secrete extracellular matrix and proteins (Takebe et al. 2003; Martin et al. 1995). The surface of materials used in our experiment is very different: AFM analysis has revealed that PAI was much more structured than PVA, that showed an amorphous structure (Dini et al. 2005). This difference can explain the adhesion of U937 cells to PAI but not to PVA.

In conclusion, the results indicated that synthetic hydrogels PAI and PVA are not cytotoxic and can induce in U937 and THP-1 cells macrophagic differentiation, in a manner cell (promonocytes, U937 or monocytes, THP-1) and material types dependent.

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Fig. 4 — Light micrographs of ematoxylin/eosin stained U937 cells attached to fibronectin substrate after treatment with PAI (in (b) arrows indicate the cytoplasmic protrusions); (a) U937 cells attached to PAI hydrogel.
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