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Hyaluronic acid based materials for intestine tissue engineering: A morphological and biochemical study of cell-material interaction

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Abstract A wide number of gastro-intestinal disorders are associated with structural alterations of this district leading to an impaired gastrointestinal function. The study of cell material interactions represents one of the major issues for the development of tissue engineering purposes. Benzyl esters of hyaluronic acid are promising materials because they exhibit good tissue compatibility and are available in various configurations. In this work they have been studied for the possible application of intestinal cell growth and functioning.

The preliminary investigation on the morphologic and biochemistry data obtained by monitoring the growth and differentiation of intestinal epithelial cells on two hyaluronic acid benzyl esters is reported. Two types of materials structures were studied: a three dimensional matrix and a macroporous flat sheet membrane. Caco-2 cell line was used: these cells undergo spontaneous enterocytic differentiation after several days in culture. The differentiation status of these cells grown on different materials was used as a parameter of biocompatibility and cell functioning. The status of cell growth and differentiation was monitored by studying cell morphology using scanning electron microscopy. The results obtained were confirmed by biochemical determinations. Although both the configurations of the two polymers exhibited good compatibility with respect to intestinal cells, only the flat sheet membrane proved to induce cell differentiation, leading us to the conclusion that it is a promising substrate for the proposed application.

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1 Introduction

A wide number of primitive gastro-intestinal disorders are associated with structural alterations of this district leading to an impaired gastrointestinal function, either in terms of absorptive properties or motility.

Short bowel syndrome [SBS] is a clinical condition often occurring when less than one third of the normal jejunal-ileal length is left [1, 2]. The main clinical manifestations of this syndrome are: refractory diarrhea, steatorrhea, weight loss, vitamin deficiency, anemia malabsorption and malnutrition [3]. Several pathologic conditions may lead to such state; they normally tend to be different as the patient's age changes: in the newborn, both congenital conditions like short bowel, multiple intestinal atresia, megacolon [Hirschsprung's disease], celoschisis, meconium-induced peritonitis, as well as acquired conditions like volvulus and necrotizing enterocolitis can lead to SBS. In the childhood and the adulthood SBS is most commonly caused by abdominal trauma, Crohn's disease, radiation-induced enteritis and mesenteric ischaemia [4].

Parenteral nutrition can only be considered as a temporary remedy, for that after a variable time liver dysfunction

may occur [5, 6], accompanied by cholestasis, cholelithiasis, cirrhosis and bone demineralization [7]. At present none of the known surgical procedures can give satisfactory results in terms of expectancy of life and quality of life. Intestinal transplantation, for example, remains the only-yet clearly uneasy and expensive-procedure for those cases where the intestinal tract is too short, but beside the complications of immunosuppression, graft-versus-host disease [8, 9] and infections, it is accompanied by the problem of donor shortage.

Tissue Engineering is one of the major focuses of biotechnological research today, with the expectation that this type of biohybrid technology will ultimately transform the practice of restorative clinics. The most ambitious tissue engineering schemes assume that specific tissues and organs will be restored, repaired or replaced by tissues that are engineered *in vitro* [10].

Based on all the facts just mentioned researchers are trying to obtain portions of intestine *in-vitro* utilizing tissue engineering techniques [11, 12]. These layers should offer a biocompatible substrate for autologous cell adhesion and spreading, so that potentially "functional" bio-engineered intestinal "matrix" segments would be easily available for transplantation in SBS patients.

Aim of this study was to evaluate the potential application of hyaluronic acid based biomaterials for intestinal tissue growth. Hyaluronic acid is a naturally occurring, biocompatible and biodegradable linear polysaccharide composed of unbranched repeating disaccharide units of glucuronic acid and *N*-acetyl glucosamine, linked by β 1–3 and β 1–4 glycosidic bonds. It is present in all soft tissues of higher organisms and in particularly high concentrations in the synovial fluid and vitreous humor of the eye [13]. It has been extensively studied over the past twenty years especially with regards to the wound healing process. Foetal wound healing and scarless wound healing studies have shown that hyaluronan plays a major role in the process [14]. Consequently, modified hyaluronan polymers have been developed to be used in a number of finished medical devices that take advantage of the material's properties, such as promoting cell motility and differentiation in wound healing [15, 16]. Moreover, thanks to its unique rheological properties it is currently being used in viscosupplementation and viscosurgery [17, 18], in ophthalmic surgery [19, 20] and in cosmetic applications [21]. Many efforts have also been focused on the production of HA based films or sponges for implantation, charged with therapeutic agents [22–24]. For delayed release and /or prolonged effect. Because hyaluronan is a highly hydratable viscous gel, its utility as a medical device is limited. The residence time of the natural gel is too short for many applications. Considerable effort has been made to modify the material such that it can be processed into a variety of usable forms for the medical marketplace. For example, the HYAFF® polymers are esters of hyaluronic acid obtained from the esterification of hyaluronic acid with benzyl alcohol. These derivatives have significantly different physico-chemical properties compared with native HA but maintain the inherent biocompatibility of the native polymer. The product's properties such as degradation rate, hydrophobicity, finished product form and others can be tailored by varying the amount of esterification and other processing factors.

In this study we analyzed the validity of hyaluronic acid benzyl esters as supports for human intestinal cells attachment, growth and differentiation. The effects of material composition and macroporosity variations on intestinal cell growth and functioning were also evaluated. The materials involved in the study were the following: Hyalograft® and Laserskin®; both materials are HA benzyl esters [HYAFF polymers] but differently moulded: Hyalograft® appears as a three-dimensional non-woven scaffold, whereas Laserskin® is a flat sheet macroporous membrane. Both the materials have been previously used to assess hepatocyte cell culture and metabolism for artificial liver purposes [25]. Little is known about the interactions between HA esters polymers and intestinal cells. To this end we used the CaCo-2 cells which were involved in all *in-vitro* studies; these cells are well known to undergo spontaneous enterocytic differentiation after several days in culture, with microvilli spreading out the apical side of cells. They also form tight junctions and express apical enzymatic activities in a way similar to what can be normally observed in normal mature small intestinal epithelium [26, 27]. Our hypothesis was that the follow up of the differentiation of CaCo-2 cells grown on different materials could be a useful tool to study intestinal cells biocompatibility with respect to the materials under investigation. The investigation of CaCo-2 cells differentiation on HA benzyl esters has been performed using scanning electron microscopy to assess whether cells would attach, grow and normally differentiate by given time points of cell culturing. Alkaline Phosphatase enzyme activity assessment also served to confirm the differentiation degree of the cells. Both the assays proved to be good parameters to assess intestinal cells biocompatibility.

2 Materials and methods

2.1 Materials

The benzyl esters of hyaluronic acid were kindly provided by Fidia Advanced Biopolymers (FAB, Abano Terme Pd, Italy): a flat sheet macroporous membrane already used for *in vitro* growth of keratinocytes to treat extensive burns (Laserskin®) [28] and Hyalograft® (referred to as Hyaff

3D), a three dimensional non woven scaffold already used for fibroblast cell growth. Transwell microporous polycarbonate culture chambers (0.4μ pore size Corning-Costar, Milan, Italy) were used as positive control. As negative control a polyurethane flat sheet membrane, medical grade was used: this was done because it had been previously demonstrated in our laboratory that this material although can allow attachment and spreading of CaCo-2 cells, it does not allow their differentiation into enterocytic-like cells. Sterile Hyalograft®, Laserskin® and polyurethane were cut under sterile conditions in 2×2 cm^2 wafers prior to cell seeding.

2.2 Cell culture

The CaCo-2 human colon cancer cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% foetal bovine serum (FBS) (Invitrogen Laboratories, Milan, Italy) 1% nonessential aminoacids, penicillin (100 units/mL), streptomycin (100 μg/mL) (Sigma–Aldrich, Milan, Italy) and fungizone (50 μg/mL) (Invitrogen Laboratories). The cells were maintained at 37 °C in a 5% CO_2 95% air humidified atmosphere. The differentiation status of the cells was followed on days 3, 10, 20 and 35 after seeding.

2.3 Assays of cell growth on biomaterials

Cells were seeded at a density of 9×10^4/cm^2 in 24 mm diameter Transwell polycarbonate culture chambers (0.4 μm pore size), on HYAFF 3D, Laserskin® and Polyurethane. The media were changed every 48 hrs. Cell growth on the materials was determined by the total protein determination according to the Bradford colorimetric assay method.

2.4 SEM analysis

At the designated time points (days 3, 10, 20 and 35 of culture) cells grown on the different materials were washed three times with cold PBS, fixed with 2.5% glutaraldehyde/PBS solution and then post-fixed with 1% Osmium tetroxide/PBS solution (Sigma Chemicals); then they were dehydrated with ethanol/water at increasing concentrations starting from 30% and up to 100% ethanol. The samples were then dehydrated with a Critical Point Drier (Emitech, K850), sputter coated with gold (Emscope SC 500) and observed under a SEM (Leica, Cambridge S440).

2.5 Alkaline phosphatase assay

Alkaline Phosphatase (AlP) activity was measured in whole cell lysates and served as a marker for enterocytic differentiation. At the designated time points (days 3, 10, 20 and 35 of culture) triplicate cultures were washed three times with cold Phosphate Buffered Saline (PBS) solution (pH 7.4), scraped (except for cells grown on HYAFF 3D) and collected into 2 mM Tris-HCl/50 mM mannitol (pH 7.2) (final volume 1 mL) and sonicated on ice and assayed (5 and 10 μL). Due to the fact that scraping was not possible for the cells grown on HYAFF 3D, these samples were directly sonicated. AlP activity was measured by kinetic determination of p-nitrophenylphosphate hydrolysis using a commercial kit (Boehringer). The results are expressed in milliunits per mg of protein.

3 Results and discussion

Tissue material interactions are affected by the specific hyaluronic acid chemical structure, which is suspected of playing a vital role in cell motility and cell-cell interactions [29]. Hyaluronan binds to cells through three main classes of cell surface receptors, the main cell surface receptor being CD44 which is the most widely distributed in the body [30]. Whether bound to cells or the extracellular matrix components, its hydrophilic nature creates an environment permissive for migration of cells to new tissue sites, while its free radical scavenging and protein exclusion properties offer protection to cells and extracellular matrix molecules against free radical and proteolytic damage. We previously reported [31] the possible use of hyaluronic acid based derivatives in the treatment of gastric ulcers. In this work hyaluronic acid based materials have been studied to assess their biocompatibility with respect to intestinal cells. The differentiation status of CaCo-2 cells was used as a parameter of biocompatibility and was followed up by SEM observations and alkaline phosphatase activity determination. Figure 1 shows cells at three days of culture on Transwell, Laserskin®, Hyaff 3D and Polyurethane. Cells seeded on Transwell and Laserskin® (Fig. 1 A, B) appear as a homogeneous layer. Cells grown on Hyaff 3D (Fig. 1C) show the same flat morphology although the number of cells adhering to the surface of the material seems to be much lower when compared to the other two materials (Fig. 1E). On the other hand cells seeded on polyurethane (Fig. 1D), as expected, did not adhere well enough to form a uniform homogenous layer.

After 10 days in culture, cells grown on Transwell and Laserskin® showed microvilli on nearly all the surface of their apical membrane (Fig. 2 A, B) leading us to the consideration that they had started to differentiate into enterocytic-like cells. Cells grown on Hyaff 3D although showing an increase of spreading over the material's fibres, did not seem to have started differentiation yet as displayed by the absence of microvilli on their apical surfaces (Fig. 2C). Cells grown on the negative control [polyurethane] showed many extroflexions on their apical side. Such structures appeared similar to microvilli to some extent, though their greater thickness and

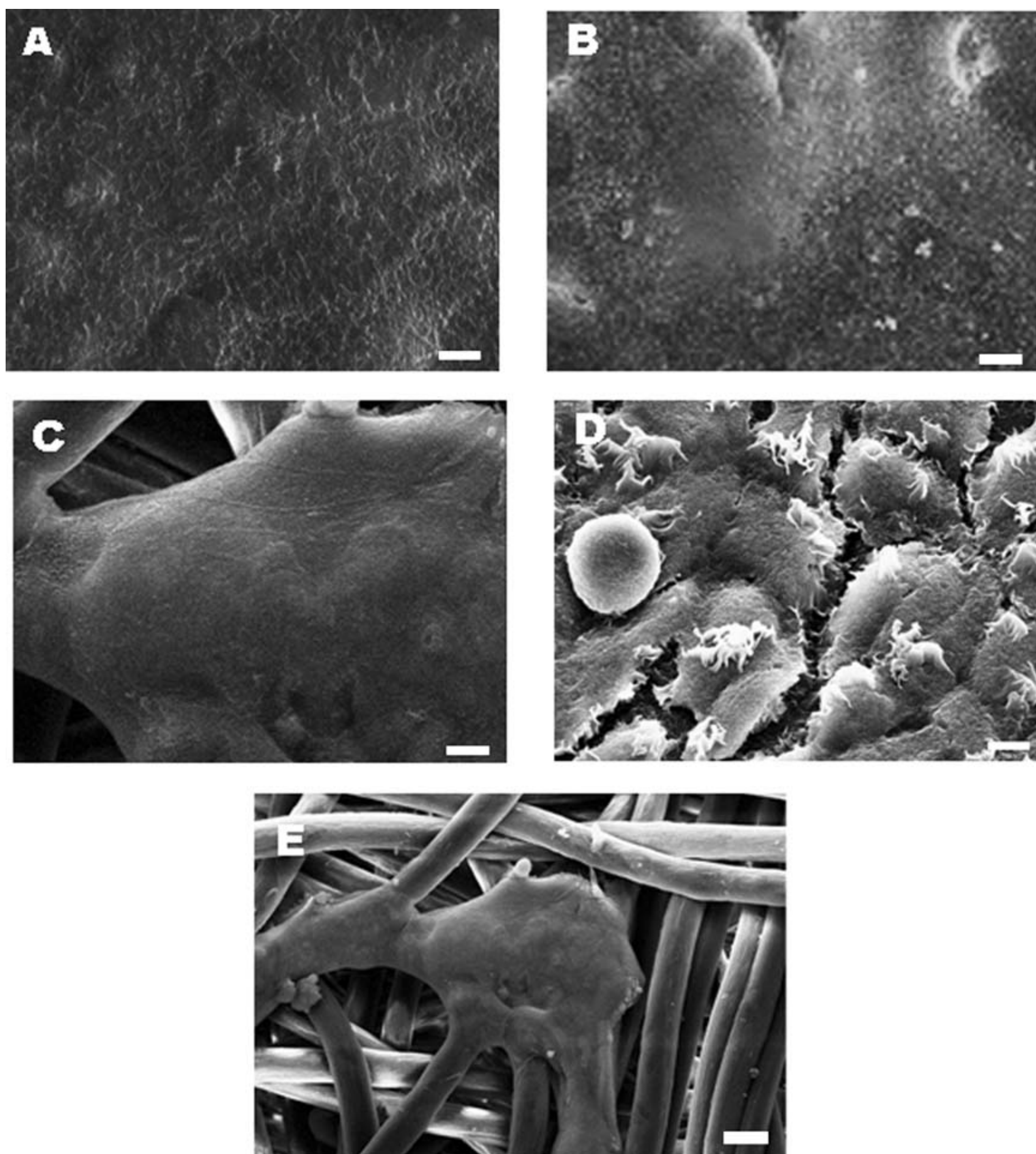

Fig. 1 CaCo-2 cells at 3 days of culture on different materials; A: cells on Transwell polycarbonate chambers; B: cells on Laserskin®; C: cells on HYAFF 3D; D: cells on polyurethane; E: cells on HYAFF 3D at lower magnification. Scale bar A-D: 5 μ. E: 10 μ

random orientation (Fig. 2D) did not allow us to consider them as normal microvilli.

Figure 3 shows SEM micrographs of the cells after 20 days of culture. The presence of microvilli on the apical sides of all the cells grown on Transwell (as expected) and Laserskin® (Fig. 3A, B) indicates that the differentiation process is completed. This is consistent with data reported in literature [32], that after 21 days of culture on Transwell, Caco-2 cells show microvilli on their apical side. Cells cultured on Laserskin® exhibited morphologic appearance comparable to that seen for cells grown on Transwell. Cells cultured on HYAFF 3D seemed to have differentiated in some areas (data not shown) while in other areas they show signs of suffering (Fig. 3C). This could be due to the fact that the fibre's meshes were of different sizes and showed large grooves between one another. The large fibre mesh led the cells to grow and differentiate along the fiber axis only in some areas of the material. However, although the scaffold provided a large area for cell attachment and growth, it did not fully permit to all the cells to differentiate. Cells grown on polyurethane (Fig. 3D) continued to show extroflexions on their apical side: this was expected as we previously observed that CaCo-2 cells show this behavior when grown on this material [33].

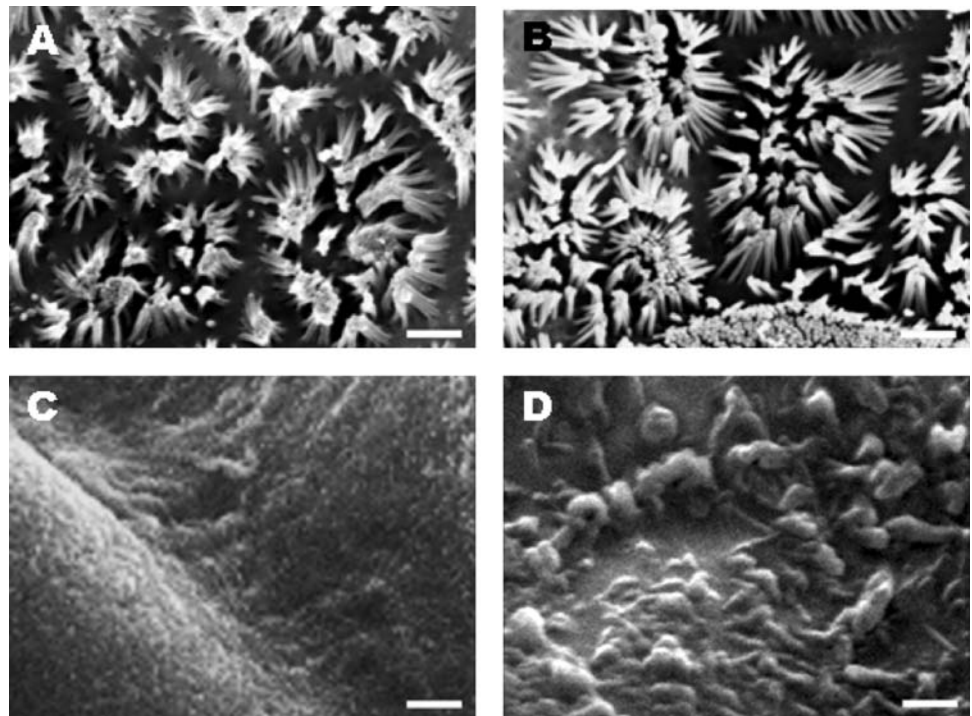

Fig. 2 CaCo-2 cells at 10 days of culture on different materials; A: cells on Transwell polycarbonate chambers; B: cells on Laserskin®; C: cells on HYAFF 3D; D: cells on polyurethane; Scale bar A–D: 1 μ

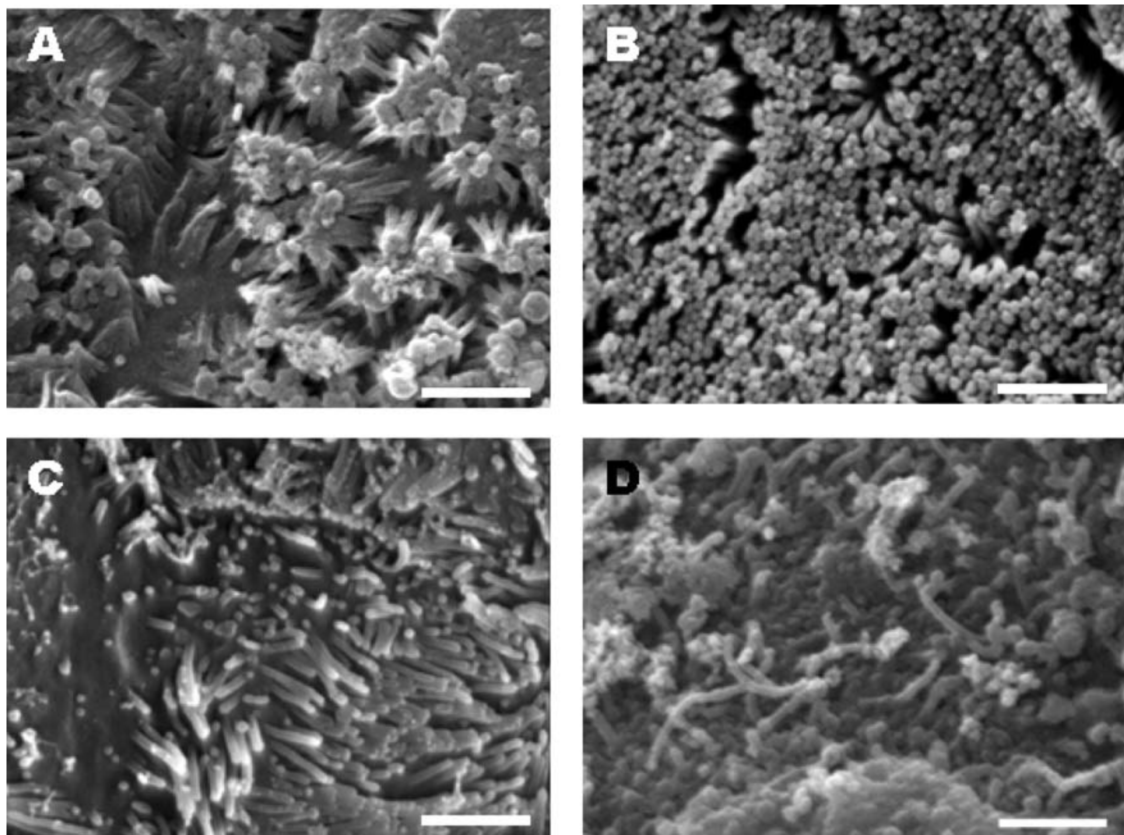

Fig. 3 CaCo-2 cells at 20 days of culture on different materials; A: cells on Transwell polycarbonate chambers; B: cells on Laserskin®; C: cells on HYAFF 3D; D: cells on polyurethane; Scale bar A–D: 1 μ

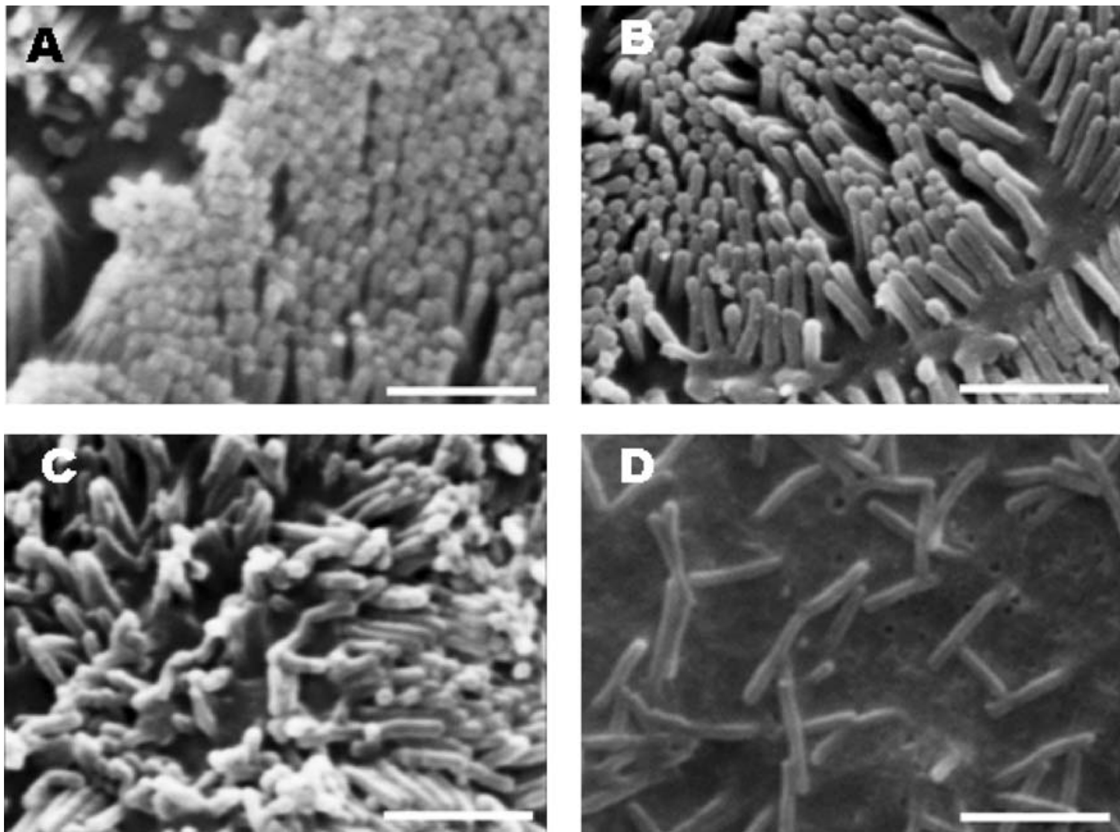

Fig. 4 CaCo-2 cells at 35 days of culture on different materials; A: cells on Transwell polycarbonate chambers; B: cells on Laserskin®; C: cells on HYAFF 3D; D: cells on polyurethane; Scale bar A–D: 1 μ

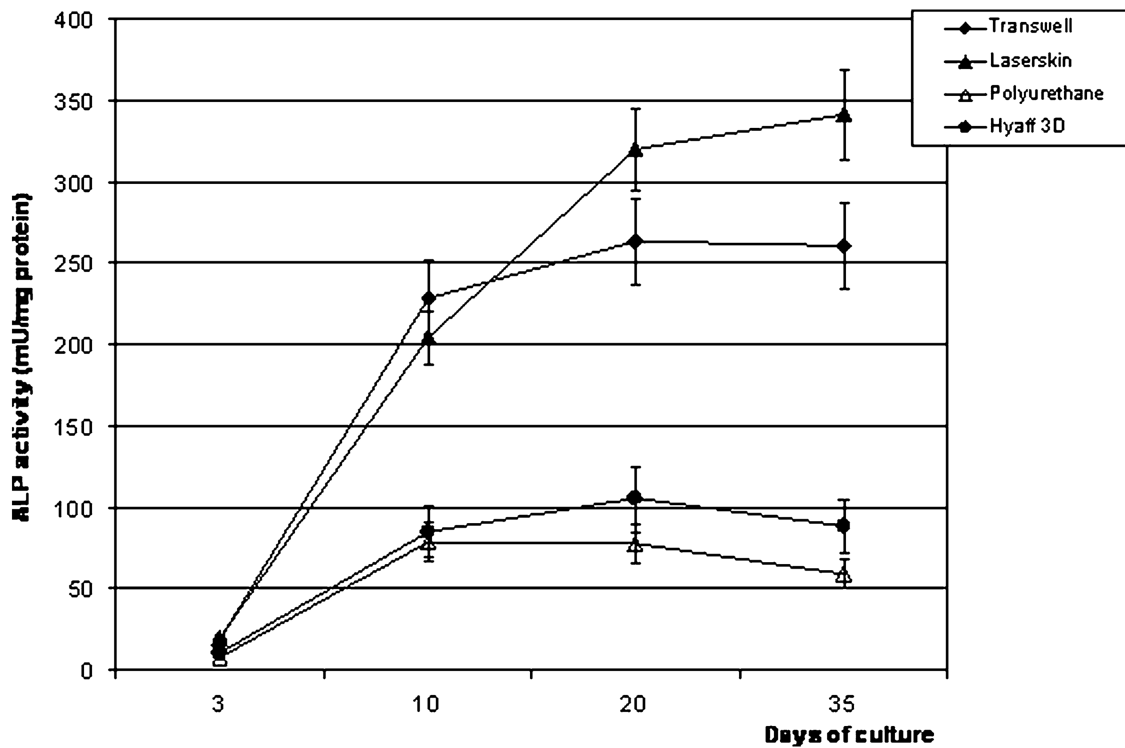

Fig. 5 AlP specific activity of CaCo-2 cells grown on the materials. The activity is expressed as mUnits per mg of protein. The values reported in the figure represent the means ± the standard error (error bars) of two independent determinations in which each point was performed in triplicate

Long term culture of the cells on these materials (35 days Fig. 4A–D), confirmed the results obtained at 20 days of culture on all the materials. Cells grown on Laserskin® displayed a differentiation status that could be considered even better that that seen for cells grown on Transwell. Cells grown on HYAFF 3D and polyurethane showed a morphology which resembled what seen at 20 days of culture leading us to the consideration that the material's chemical composition positively influences intestinal cells adhesion, growth and differentiation and makes it a possible promising substrate for intestinal tissue repair. Beside this consideration, from the biological point of view, the three-dimensional structure of this material should be modulated in a different way in order to obtain a scaffold able to promote not only the formation of an epithelial layer, but also to provide an environment able to allow new vessel ingrowth and matrix deposition.

CaCo2 cells show during the differentiation process increasing activity, above others, of the enzyme alkaline phosphatase (a hydrolase typical of the brush border). Therefore the determination of AlP activity has been used to confirm the results obtained with SEM observations. As far as the determinations carried out on cells grown on Transwell and Laserskin® were concerned, the results obtained confirmed what had been observed with scanning microscopy (Fig. 5). AlP activity of cells grown on Transwell reached a plateau while cells grown on Laserskin® showed higher activity at the same points in this way resembling the data obtained with SEM observations. This could lead to the consideration that hyaluronic acid esters mould in flat sheet membranes are good substrates for intestinal tissue growth and differentiation better than the microporous polycarbonate itself and therefore appear to be good candidate for the application under investigation. Although cell determination on Hyaff 3D showed high number of cells (data not shown) the AlP activity showed that the differentiation was not completed in all the areas of cell growth like what had been previously observed at the SEM while the low AlP activity exhibited by the cells grown on polyurethane could be ascribed to the low number of cells grown on the material's surface.

4 Conclusions

The preliminary investigation reported in this paper suggests that polymers made of benzyl esters of hyaluronic acid in sheet membranes are promising substrata for intestinal cells adhesion and growth. Cells cultured on the HA benzyl ester macroporous membrane differentiated even better than those grown Transwell polycarbonate membranes, in particular in long terms, while the material's shape (Hyaff 3D) seemed to dramatically affect cell differentiation, even if the chemical compositions were equal. Therefore Laserskin® seems to be a suitable material to be used in those pathologic conditions in which intestinal tissue growth is required. Moreover the use of morphologic parameters for the study of the differentiation status of the cells proved to be a useful tool to assess these materials biocompatibility.

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