Gut acellular matrix for the in vitro study of Enteric Nervous System cells

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Abstract

Enteric nervous system (ENS) cells respond to the intestinal extracellular matrix (ECM) signals changing their proliferation rate, migration and differentiation. In this study, we explored in vitro the adaptive response of primary ENS cell cultures to the stimulation of gut acellular matrix (AM) defining the gene expression profile of neuronal functionality markers. Scanning electron microscopy was used to detect the acquisition of specific morphological features.

Intestinal AM was prepared using an enzyme-detergent treatment. Primary rat enteric cells were isolated from the myenteric plexus of postnatal rats using an enzymatic method and seeded on intestinal AM in the presence of exogenous neurotrophic factors. The morphological properties and the expression of specific differentiation markers were evaluated by Scanning Electron Microscopy (SEM) and wholemount fluorescent staining. In order to verify the synergic activity of soluble factors and AM, the gene expression of neurotransmitter receptors was evaluated by qPCR in ENS cells cultured in SM conditions in the presence or not of AM.

The development of interconnected ganglion-like structures and the expression of neurotransmitter receptors suggested that gut matrix engineered with ENS cells could be useful for medical applications of regenerative medicine or for the in vitro assessment of tridimensional culture system of ENS.

Introduction

In the last decade, the acellular matrices obtained by a detergent-enzymatic method [1], have been demonstrated to optimize in vitro and in vivo cell viability providing a near-physiologic environment. Showing high structural heterogeneity for responding to mechanical, chemical, nutritional, immunological and bacterial stimuli, ECM serves to organize cells in microenvironment, to direct site-specific cellular regulation and to elicit cellular responses, such as proliferation and differentiation. Rather than merely providing structural information, it plays an instructive role that is critical for the maintenance of tissue

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homeostasis or the initiation and progression of pathologies. To date, the influence exerted by ECM in the regulation of Enteric Nervous System is far from being clear. Under inflammatory bowel diseases (IBD) [1-5], dysfunctional motility, hypersensitivity and interruptions in the neuronal network are commonly observed and correlated to ECM disruption [6-8]. In this context, the development of a three-dimensional cellular model could be beneficial to investigate ENS-gut matrix interactions under in vivo-mimicking in vitro conditions. In the past decade, ENS progenitor cells isolated from rodents or humans [9-13] have been successively used to assess two-dimensional neuronsphere cultures. Lacking an adequate scaffold, ENS cells are considered as limited to study in vitro the mechanisms underlining gut homeostasis or diseases.

Biological matrices obtained by decellularization of native tissues [14] have been successfully used in pre-clinical animal studies or clinical applications of regenerative medicine [14-17]. Under in vitro settings, AMs support cell adhesion, growth, differentiation and functionality [18,19] due to a network architecture resembling native ECM [20]. After implantation, compelling evidence has shown their ability to target tissue specific regeneration [17,21]. In this work, we adapted the decellularization method defined by Conconi et al. (2005) to obtain an optimized rat 3D culture system based on gut acellular matrix and primary ENS cells [22]. Providing cells with intestinal adequate architecture, matrix composition and exogenous soluble factors, this model could be useful for researchers to gain more translatable and clinically relevant data in the research of gut dysfunctions.

Materials and methods

All animal procedures were performed according to D.Lgs n. 26/2014 which warrants care of experimental animals in Italy. The research project was approved by the Italian Health Department according to the art. 26 and 31 of above-mentioned D.Lgs.

Gut acellular matrix

Intestines were obtained from adult Sprague Dawley rats (3-months-old) and rinsed four times in phosphate-buffered saline (PBS), 1% antifungal and antibiotic agent (Amucina, sodium hypochlorite, Angelini ACRAF S.p.A., Ancona, Italy). Specimens from small intestine were treated according to Conconi et al. [19]. Briefly, the luminal surface of each sample was treated with 4 U/mL Dispase II (Roche Diagnostics, Monza, Italy) to remove the epithelial monolayer. Then, six decellularization cycles were performed as follows: sterile Milli-Q water supplemented with 1% of antibiotic solution (Sigma-Aldrich, Milan, Italy) for 72 h at 4°C, 4% sodium deoxycholate (Sigma-Aldrich) for 4 h, and 2000 U deoxyribonuclease-1 (DNase-I) (Sigma-Aldrich) in 1 M NaCl (Sigma-Aldrich) for 3 h. Matrices were stored in PBS at 4°C until use. The presence of cellular elements was verified histologically [haematoxylin/eosin (H&E) (Sigma-Aldrich) and DAPI staining (Invitrogen, Thermo Fisher Scientific, Monza, Italy)] on cryosections. In parallel, the presence of DNA and RNA was investigated digesting tissue specimens with TRIZol Reagent (Invitrogen), according to the manufacturer's instructions. The analysis was performed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and DNA/RNA samples extracted from native intestines were used as reference. Acellular matrices (AMs) were stored at 4°C, in PBS supplemented with 1% antibiotic solution.

Haematoxylin/Eosin

AMs were longitudinally cut to obtain samples of approximately 5 mm². After fixation in cryostat embedding medium (Kililik, Bio-Optica, Milano, Italy), sections with 7 μm thickness were prepared using a Cryostat DM2000 (Leica Microsystems) and transferred on glass microscopic slides. Staining with haematoxylin/eosin (H&E, Sigma-Aldrich) was carried out according to standard protocols.

Scanning electron microscopy (SEM)

Seven-micron thick sections were permeabilized with Triton X-100 (Sigma-Aldrich) for 30 min. To prevent non-specific binding of antibodies, blocking was performed by incubation in PBS supplemented with 1% (w/v) bovine serum albumin (BSA, Sigma-Aldrich). Then, the samples were incubated with rabbit anti-rat laminin antibody (CosmoBio, Tokyo, Japan) overnight, at room temperature (RT). After washing in PBS, binding specific sites were revealed by incubation with secondary antibody goat anti-rabbit-FITC (Santa Cruz Biotechnologies, Inc., Heidelberg, Germany) at 4°C, for 1 h. Negative controls were prepared treating AM sections with only secondary antibody. After mounting with Anti-Fade Mounting Medium (StereoScan-205 S, Cambridge Instruments, Cambridge, UK), the analysis was performed using a DM2000 (Leica Microsystems, Wetlzar, Germany).

Isolation and culture of ENS cells (ENSc)

ENSc were isolated from intestine of 3-day-old Sprague Dawley rats according to Schäfer et al. [23]. Overlying tissue was stripped out and the smooth muscle layer was extracted for digestion at 37°C in Hank’s balanced salt solution (PAN, Aidenbach, Germany), 50 ng/ml trypsin-chymotrypsin inhibitor (Sigma-Aldrich), 1 mg/ml collagenase type 2 (Worthington, Lakewood, USA) and 200 μg/ml DNase (Roche, Basel, Switzerland). Myenteric nets were collected and then treated with 1X Accutase (PAA, Pasching, Austria) at 37°C for 10 min. The cells were seeded (2x10⁴ cells/cm²) and cultured for seven days under standard conditions in complete growth medium (CCGM) composed of Neural Base P (PAA) 2% (v/v) neuronal stem cell supplement (PAA), 1% (v/v) bovine serum albumin (Sigma-Aldrich), 0.1% (v/v) β-mercaptoethanol (Invitrogen), 1% (v/v) penicillin/streptomycin (Invitrogen) and 1% (v/v) L-glutamine (Sigma-Aldrich), 10 ng/mL EGF (ImmunoTools, Friesoythe, Germany), 20 ng/mL bFGF (ImmunoTools) and 10 ng/mL GDNF (ImmunoTools).

Assessment of 3D model cell culture

AMs were cut into pieces of approximately 5 mm², stretched on a glass coverslip and then transferred into 24-well plates (Falcon, BD Biosciences, Milan, Italy). Stainless steel rings were used to anchor the samples to the bottom of plates. Primary neuronsphere were obtained by culturing ENS cells for 7 days and, after enzymatic disruption with 1X Accumax solution (Sigma-Aldrich) for 15 min at 37°C, were mechanically dissociated using a 27 Gauge needle. Single cell suspensions were seeded at a density of 2x10⁴ cells/cm² on the outer side of AMs. At three different time points (3, 7 and 14 days), the three-dimensional cultures were fixed and prepared for SEM analysis, wholomount immunofluorescent staining and gene expression analysis.
**Wholemount immunofluorescence staining**

The samples were fixed using BD Cytofix (BD Biosciences, Milan, Italy) for 15 min and 1% (v/v) BSA for 2 h at 4°C for blocking unspecific binding sites. For intracellular staining, the incubation with 0.5% (v/v) Triton X-100 (SigmaAldrich) was performed for 30 min, at RT. The incubation with mouse anti-rat tubulin β III (Merck Millipore, Vimodrone (MI), Italy) and rabbit anti-rat α-SMA-FITC (Abcam, Cambridge, UK) antibodies was executed overnight, at RT. The detection of tubulin β III required the incubation for 3 h at RT with a secondary antibody goat anti-mouse PE (Santa Cruz Biotechnology). After mounting with Anti-Fade Mounting Medium (Immunological Sciences), the samples were examined using a fluorescence microscope DM2000 (Leica Microsystems, Wetzlar, Germany).

**Gene expression study**

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Milan, Italy) and quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Gene expression analysis was performed using RT² Profiler™ PCR Array Rat Neurotransmitter Receptors (Qiagen, Hilden, Germany), according to manufacturer’s instructions. The list of target genes is reported in Supplementary data (Table S1). RNA isolated from ENSc cultured on polystyrene plates was used as control. The Rat neurotransmitter receptor RT² Profiler PCR array profiles the expression of 84 important cytokine genes. Data were acquired using DNA Engine Opticon® Real Time Thermal Cycler (MJ Research, St. Bruno, QC, Canada) and analyzed using the RT² Profiler data analysis Web portal (SA Biosciences, Washington DC, US), applying the 2^ΔΔCt method. Hypoxanthine phosphoribosyl transferase 1 (Hprt1) was used as housekeeping gene.

**Statistical analysis**

The p-values were calculated based on a Student’s t-test of the replicate applied on 2^ΔΔCt values for each gene in the control group and treatment group. Fold-change values less than one have been considered a down-regulation; fold-change values greater than two have been interpreted as an up-regulation. Data were analyzed using the RT² Profiler data analysis Web portal (SA Biosciences, Washington DC, US), applying the 2^ΔΔCt method. In the analysis, p < 0.05 was considered statistically significant.

**Results**

**Gut matrices**

In comparison to the native gut (Figure 1A), samples submitted to six cycles of decellularization showed to be lacking mucosal/submucosal layers (Figure 1B). Compared to controls (Figure 1C), no signs of nuclei and cytoplasmic components were detected in AMs by DAPI staining (Figure 1D). Furthermore, more than 99% of nucleic acid content (DNA: 2.82 ± 0.4 ng/µL; RNA: 13.17 ± 4.99 ng/µL) was removed by decellularization with respect to the native gut (DNA: 523.20 ± 11.31 ng/µL; RNA: 1552.04±15.23 ng/µL) (see graph in Supplementary data). By scanning electron microscopy, the outermost layer showed a flat surface, with fibers longitudinally arranged (Figure 1E) and gut architecture almost preserved (Figure 1F), as suggested by the detection of laminin through Immunofluorescence (IF) (see Supplementary data).

**SEM**

After 3 days of culture, ENSc were attached and spread on AMs (Figure 2A). From 7 (Figure 2B) to 14 days (Figure 2C), numerous colonies started to acquire morphological specialization and ganglion-like interconnected structures were detectable.

**Immunofluorescence (IF)**

In parallel, wholemount immunofluorescent staining confirmed the ganglion-like appearance and the positivity for the expression of tubulin β III, a typical structural component of neuronal connection and differentiation during 3 (Figure 3A), 7 (Figure 3B) and 14 (Figure 3C) days. The presence of cells positive for the expression of α-SMA Muscle Actin (α-SMA) and negative for tubulin β III underlined the ability of ENSc to differentiate also into glial-smooth muscle like cells.

**Gene expression profile**

PCR data confirmed that AMs compared to the traditional bi-dimensional culture system enhance the in vitro functionality of ENSc. The analysis performed with RT² Profiler PCR Array (Figure 4) showed a significant expression change (fold change > 2 with p-value < 0.05) in 29 of 84 total analyzed genes in ENSc cultured on AMs compared to control samples on polystyrene dishes. The expression resulted increased for 11 genes (Avpr1a, Gabra1, Gabre, Grin2a, Htr2a, Sstr1, Sstr2, Tspo, Actb, Ldha and Rplp1), while a decreased expression was detected for 18 targets (Adra2a, Avpr1b, Cckbr, Chrm1, Chrm3, Drd2, drd5, Gabra5, Gabrab, Grn2c, Grn4, Hrh4, Htr1a, Npy5r, Ntsr2, Sstr4 and Tacr1). Twelve genes resulted to be unaffected by the culture conditions.
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system (B2m, Brs3, Chrne, Car1, Gabbr1, Gabbr2, Gabra2, Gria3, Gpr, Hcrt2, Htr7, Tacr3) while remaining 47 targets were not expressed or expressed at non-detectable level (Ct-value > 35) in both culture conditions. Data from this analysis were reported in a Volcano plot showing fold-change and \( p \)-value for ENSc seeded on AMs compared to cultures on polystyrene dishes. Uregulated and downregulated genes were indicated with red and blue dots, respectively. Genes with unchanged expression level were reported with black dots.

**Figure 2.** ENS cells formed ganglion-like structures on acellular matrices. SEM analysis at 3 (A), 7 (B) and 14 (C) days of culture on AM. Magnification: X100

**Figure 3.** ENS cultured on acellular matrix expressed tubulin \( \beta \) III. Immunofluorescence analysis of tubulin \( \beta \) III (red), u-SMA (green), DAPI (blue) on ENS cells cultured for 3 (A), 7 (B) and 14 (C) days on acellular matrices. Magnification: x100

**Discussion**

Biologic scaffolds obtained from tissue or organ decellularization have been successfully used in both pre-clinical studies and human clinical applications [24]. In the last decade, several decellularization protocols have been investigated to define the optimal method for obtaining acellular matrices with minimal residual cellular material and low-grade ultrastructure alterations [14]. In this paper, gut ECM
different receptors, control cells showed to be active in the development of neuron-like cells with high responsivity to Dopamine, Serotonin and GABA but not to glutamate, confirming the potentialities of AM to enhance neuronal in vitro differentiation. A remarkable finding of the present study was that some genes, such as L-lactate dehydrogenase A chain (LdhA), that is notably related to energetic metabolism and, converting pyruvate to lactate during aerobic glycolysis. Yang et al. reported that L-lactate stimulates in neurons the expression of genes related to synaptic plasticity through a mechanism involving the activation of N-methyl-D-aspartate (NMDA) receptors, such as Grin2a [45]. Moreover, it also protects neurons against Glutamate-mediated excitotoxicity [46]. Acellular matrix was demonstrated to positively modulate the gene expression of beta-Actin (Actb), a protein recently reported to contribute to neural crest ontology [47], directional growth cone motility [46-50] and development of dendritic spine processes [51]. The active process of neuronal maturation was strongly confirmed by the formation of tubulin β III-positive ganglion-like structures on acellular matrix.

**Conclusions**

The synergic activity exerted by intestinal AM and neurotrophic factors promoted the maturation of ENS cells into interconnected ganglion-like structures expressing genes related to Acetylcholine, GABA, Glutamate, Serotonin and Somatostatin receptors. Our findings validate an effective approach of cell/matrix-based system to potentiate in vitro the differentiation abilities of ENS cells.

**Author contributions**

Study conception and design, C.M.T.; Execution of experiments, S.S., P.M., and T.A.; Collection and assembly of data: S.S. and P.M.; Manuscript writing, S.S., P.M., and D.L.R.; Manuscript editing: P.M., T.A., D.L.R., and C.M.T.; Data analysis: C.M.T.; D.L.R.; Data interpretation and final approval of manuscript: D.L.R. and C.T.M.

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**Conflicts of interests**

Authors declare no conflicts of interest.

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