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Orginal article

# Reciprocal stimulation of pancreatic acinar and stellate cells in a novel long-term *in vitro* co-culture model

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ABSTRACT

*Background/objectives:* Pancreatic stellate cells (PSCs) are the key fibrogenic cells in the pancreas. Acinar cell injury is known to trigger PSC activation. To facilitate the experimental analysis of the crosstalk between acinar cells and PSCs, an *in vitro* system for their long-term co-cultivation was developed.
*Materials and methods:* PSCs and acinar cells capable of retaining their secretory phenotype in long-term *in vitro* culture were obtained from mouse pancreata. A dual-chamber co-culture model was built in 24-well format with acinar cells seeded in the wells and PSCs in tissue culture inserts. Acinar cell-3T3 fibroblast co-cultures served as controls. After 4-day maintenance, the acinar compartment was analyzed for cell morphology, secretory capability, necrosis (HMGB1), apoptosis (TUNEL) and inflammation (NFκB). PSCs were analyzed for migratory activity and extracellular matrix (ECM) protein expression. The results were compared to parallel monocultures.
*Results:* Acinar cells in monoculture and in co-culture with fibroblasts exhibited a healthy monolayer arrangement and an ability to respond to 0.1 nM caerulein stimulus by increased amylase release. Co-culture with PSCs caused marked changes in acinar cell morphology and rendered them insensitive to secretagogue stimulus. Activation of NFκB and necrotic changes, but not apoptosis, were identified in co-cultured acinar cells. Co-culture increased the migratory activity and ECM protein expression of PSCs.
*Conclusions:* Humoral interactions between acinar and PSCs in co-culture were shown to reciprocally affect their cellular functions. With its two separable cell compartments the co-culture system provides a versatile culture setting that allows independent manipulation and analysis of both cell types.


## Introduction

The structural and functional integrity of the exocrine pancreas is locally maintained by its component cells via a network of regulatory interactions. An orderly glandular arrangement and secretory function of acinar cells is dependent not only on the immediate surroundings in the acinar cell compartment [1] but also on the physical and chemical cues provided by the ductal [2,3] and endocrine compartments [4] as well as cells residing in the underlying mesenchyme [5].

In the healthy pancreas the periacinar and periductal mesenchyme is occupied by pancreatic stellate cells (PSCs) in a number constituting 4–7% of that of acinar cells [6]. PSCs exhibit in healthy tissue a quiescent phenotype characterized by rare proliferation and an abundance of cytoplasmic lipid droplets [5]. Owing to their ability to produce extracellular matrix (ECM) proteins as well as matrix-digesting enzymes, the main task of PSCs in tissue homeostasis is considered to be the maintenance of a balanced turnover of the ECM and the basement membrane [5,7]. The observed expression of cholecystokinin receptors [8] as well as acetylcholine [9] in PSCs suggest that they also may play a part in intrapancreatic regulation of exocrine secretion.

PSCs help to re-establish tissue homeostasis after injury in any one of the cellular compartments by rapidly transforming into an activated myofibroblast-like phenotype with an increased potential to proliferate, to migrate and to produce and deposit ECM proteins [5]. Paracrine regulation of PSC activation is mediated by pro-

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fibrogenic stimuli derived from neighboring cells and reciprocally modulated by autocrine loops and paracrine feedback effects exerted by activated PSCs [10]. In acute pancreatitis, acinar cells injured by noxious stimuli are considered to be responsible for releasing the first inflammatory signals which mediate the recruitment of circulating inflammatory cells into the site of injury and trigger PSC activation [11,12]. Persistent PSC activation is the major cause of the development of fibrosis in chronic pancreatitis and pancreatic cancer [5].

The etiological factors and pathophysiology of pancreatitis have been experimentally elucidated using *in vivo* animal models [13] as well as isolated pancreatic acini in short-term *ex vivo* settings [14–18]. Isolated and *in vitro* propagated PSCs in turn have been used to identify PSC-activating pro-fibrogenic growth factors and cytokines [5]. In addition to purified mediators, PSC activation has been demonstrated in response to short-term *in vitro* exposure to cell lysates and cell secretions, including conditioned supernatants from injured acinar cells [19–21]. Besides short-term single cell type models, more elaborate long-term co-culture systems are needed in order to be able to experimentally address questions related to acinar cell and PSC interactions in the pathophysiology of fibrosis development and its potential management in pancreatitis.

We have recently developed cell culture techniques for long-term *in vitro* maintenance of mouse [22] and human [23] pancreatic acinar cells and demonstrated with the former the possibility of acinar cell cryopreservation for on-demand use without dedifferentiation [24]. These methods have been the basis of the present study whose aim was to examine the possibility of creating a co-culture system in which the interactions between acinar cells and PSCs could be studied on a long-term basis *in vitro*. In this methodological paper we describe the development of a culture system that enables the maintenance of mouse acinar cells in co-culture with PSCs for a minimum of 4 days. With its two separable cell compartments the present co-culture model provides a versatile experimental setting that allows independent manipulation and analysis of both cell types.

## Materials and methods

### *Cells*

Primary mouse pancreatic acinar cells were prepared from pancreata of 6–7-week-old male mice (C57BL/6JOlaHSD, Harlan, The Netherlands) and cryopreserved and thawed for experiments according to previously published protocols [22,24]. The culture medium for acinar cells consisted of DMEM/F12 with a 3-fold supplementation of DMEM amino acids, 2 mM sodium pyruvate, penicillin (50 U/ml)/streptomycin (50 μg/ml) and 0.01% soybean trypsin inhibitor, all purchased from Life Technologies, Grand Island, NY; 25 ng/ml EGF and 5 μg/ml insulin from Sigma St. Louis, MO and 0.2 mg/ml growth factor-reduced Matrigel (BD, Franklin Lakes, NJ).

Mouse PSCs were obtained using the explant outgrowth method [25,26]. Briefly, whole mouse pancreata were minced and the pieces were pipetted into T75 culture bottles in 7 ml culture medium consisting of DMEM/F12 supplemented with 10% fetal calf serum (FCS), L-glutamine and penicillin (100 U/ml)/streptomycin (100 μg/ml), all provided by Life Technologies. After 7–9 days of culture the outgrown PSCs were collected, reseeded in T75 bottles and allowed to grow to near confluence. After two passages 1:3 the cultures were detached and cryopreserved in liquid nitrogen.

Mouse 3T3 fibroblasts obtained from ATCC (Manassas, VA) were used as control cells. Their propagation was performed in the same medium as that of PSCs.

### *Choice of medium*

Preliminary experiments were performed to assess the viability and growth of PSCs and 3T3 fibroblasts in the FCS-free acinar cell-specific medium. Both cell types were seeded into 96-well plates at a density of 2500 cells per well in their normal FCS-containing medium and allowed to attach for 3 days. The cells were thereafter incubated for 7 days in acinar cell-specific or in FCS-containing medium. The media were renewed every 48 h. Relative cell numbers were assessed on days 4 and 7 using the colorimetric crystal violet assay as previously described [27]. Eight determinations were used to calculate the mean optical density in three independent experiments.

### *Co-culture*

Co-culture was performed in a 24-well format in acinar cell-specific medium. In order to permit cell–cell contacts of acinar cells in culture, 50 μl aliquots of suspension containing approximately 10,000 cells were applied on the center of each well. The cells were allowed to attach overnight after which 250 μl fresh culture medium was added in each well. A similar amount of PSCs was seeded onto cell culture inserts that were placed in a separate 24-well plate (ThinCert, 8 μm pore size, Greiner Bio-One, Frickenhausen, Germany) and allowed to attach overnight. The inserts were then transferred into the wells containing acinar cells and co-cultures were maintained up to 4 days. Half of the medium in each well was replaced by fresh medium every day. Parallel controls consisted of monocultures of either cell type and of co-cultures set up with mouse 3T3 fibroblasts in place of PSCs.

### *Analysis of acinar cells*

The morphology of acinar cells in mono- and co-cultures was examined under a phase-contrast microscope (Nikon Eclipse Ti-S, Tokyo, Japan).

To determine the effect of co-culture on the secretory capability of acinar cells, their amylase release was determined both in basal and caerulein-stimulated conditions. For this, acinar cells from parallel monocultures and acinar-stellate or acinar-3T3 fibroblast co-cultures were rinsed with fresh culture medium, after which they were incubated in 100 μl of medium without (basal secretion) or with 0.1 nM caerulein (Sigma). Duplicate wells were prepared for each culture type and treatment. After 1 h of incubation at 37 °C the media were collected for amylase assay on a Cobas c111 autoanalyzer (Roche, Mannheim, Germany). A similar volume of medium containing 0.1% Triton X-100 was then added in each well and the plates were incubated for another 1 h. Cellular amylase content was determined in the obtained cell lysates. Amylase release was calculated as the percentage of total amylase content.

Antibodies against high mobility group box 1 (HMGB1) and nuclear factor-κB (NFκB) p65 (both from Abcam, Cambridge, UK) were used to analyze by immunocytochemical means the effect of co-culture on necrosis and inflammation, respectively. Prior to successive incubations with primary and secondary antibodies (Novex Histostain Plus kit, Life Technologies), the cells were fixed in 4% paraformaldehyde and permeabilized in 94% ethanol or in 0.3% Triton X-100 as previously described [22,27]. Immunoreactive proteins were visualized with diaminobenzidine (DAB; Novex Liquid DAB Substrate Kit, Life Technologies). Hematoxylin was used as counterstain.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Promega, Madison, WI, USA) was performed according to the protocol of the manufacturer to examine the level of

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