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Cell viability of fibroblasts to pifenidone and sirolimus A future concept for drug eluting stents

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ABSTRACT

Background: Currently one of the major problems that interventional pulmonologists have to face is the increased proliferation of fibrinous tissue on the site of the stent placement, and usually at the two ends.
Materials and methods: The drugs rapamycin and pirfenidone were chosen for our experiment. Fibroblasts were also cultured in order to administer pirfenidone and rapamycin in different concentrations. The following cell viability methods were used: (a) Senescence – Cell Titer Assay, (b) Necrosis – Cyto Tox Assay and (c) Apoptosis – Caspase-Glo 3/7 Assay.
Results: Rapamycin has minimal to no effect on fibroblasts regarding apoptosis, senescence and necrosis. 0.1 to 1 μM. Pirfenidone concentrations lead to an elevated cell metabolism because cells try to evade the cytotoxic effect of the drug. Increasing Pirfenidone concentrations lead to higher apoptosis rates. 10 μM pirfenidone induces the highest apoptosis rates in this experiment and reduce cell viability to a minimum.
Conclusion: Necrosis is unaffected by the investigated drugs.

1. Introduction

There are several cases where either due to benign or malignant reasons tracheal stenosis occurs (Bolliger et al., 2006; Giannoni et al., 2004; Hellmich et al., 2003; Tsakiridis et al., 2012). Malignant reasons include the growing of cancer tissue within the bronchus or there is also the case where stenosis occurs from pressure outside the lumen from lung cancer (Porpodis et al., 2012; Wilson and Moran, 1997). There is also the case of a benign disease such as; tracheomalacia, where stenosis occurs with breathing (Collard et al., 1996; Giannoni et al., 2004). Therefore stent placement is necessary, either to keep an airway open or to prevent early disease recurrence. However, stent placement has been observed to induce fibrinous tissue proliferation. The reasons proposed include; local tissue stress and local tissue hypoxia which activates an inflammatory cascade releasing chemokines and cytokines (Ito et al., 2013). The vascular endothelial factor (VEGF) is also activated either due to the stent placement, but also debulking in the case of malignancy (Gudehithlu et al., 2005). Debulking of cancer tissue is necessary in cases of lung cancer as previously described (Bolliger et al., 2002). Several classification systems have been proposed to characterize the length and type of tracheal stenosis (Freitag et al., 2007). Stent placement should only be performed by experienced interventional pulmonologists, since complications could be very severe (Bolliger et al., 2002; Zarogoulidis et al., 2012a). Moreover; the initiation of the fibrinous tissue formation has no specific time. The process could start immediately after stent placement if the previously factors are present or after several hours/days. Currently there are several stents on the market and the choice of the stent is based on various local factors and usage (Breen and Dutau, 2009; de Groot et al., 1995; Freitag et al., 1994a). There are different techniques of stent placement based on the location, length and usage (Freitag et al., 1994b; Freitag et al., 1997). Currently several adjuvant treatments have been proposed with stent placement in order to inhibit the granuloma tissue formation like the mitomycin C, local steroid administration, however; with minimum results (Parker et al., 2013). Currently there are drug eluting stents with sirolimus, zotarolimus, everolimus and paclitaxel that the cardiologists use to inhibit lumen stenosis by regulating the mammalian target of rapamycin (mTOR) pathway (Zarogoulidis et al., 2013a).

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Pirfenidone is a drug available in several countries as oral administration and is effective against idiopathic pulmonary fibrosis (Cottin, 2013). Pirfenidone inhibits the tumor necrosis factor-β (TNF-β) fibrogenic mediator and subsequently the production of collagen (Hewitson et al., 2001; Lin et al., 2009). Moreover, pirfenidone reduces the production of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) making it a additional arrow in the quiver of modern pharmacology as an anti-inflammatory and anti-fibrotic agent (Card et al., 2003; Kakugawa et al., 2004; Oku et al., 2008). Rapamycin on the other hand is a well known immunosuppresent that is used to prevent organ transplantation rejection. It prevents activation of T cells and B cells by inhibiting their response to interleukin-2 (IL-2). Furthermore, it has been found to be especially effective against kidney transplantation. Rapamycin is considered a macrolide (Pritchard, 2005; Zarogoulidis et al., 2012b). Rapamycin blocks the mTOR by by directly binding the mTOR Complex1 (mTORC1) (Zarogoulidis et al., 2013a). The chief advantage sirolimus has over calcineurin inhibitors is its low toxicity toward kidneys. In our current study we investigated different drug concentrations of rapamycin and pirfenidone in fibroblast cell cultures, in an effort to identify as a future concept if these drugs could be one day used for drug eluting stents against the formation of granuloma tissue.

1.1. Cell line experiments

Human lung fibroblast cell line MCR-5 was obtained from the American Type Culture Collection (Manassas, VA, USA). MCR-5 was cultured in Eagle's Minimum Essential (MEM) medium (life technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (life technologies) at 37 °C in a 5% CO_2-humidified atmosphere. Cells were grown until 85% to 95% confluency, then washed with phosphate-buffered saline (Invitrogen) and trypsinized with 1 ml of 0.05% trypsin – 0.53 mM ethylendiaminetetraacetic acid, phenol red (life technologies). Trypsination was stopped by adding fresh medium to the reaction. Approximately 10 μl were transferred to a hemocytometer (Brand GmbH und Co KG, Germany) for cell counting purposes. Approximately 1000 cells per well (100 μl) were seeded into Microplates 96/U (Eppendorf, Hamburg, Germany) that are suitable for luminescence and fluorescence detection. The cells were allowed to attach overnight at 37 °C and 5% CO_2. At the next day the medium was removed and fresh medium containing either one of the cytostatics, 10% DMSO or without additive was applied to each well. Pirfenidone (Sigma–Aldrich, MO, USA) was applied in final concentrations of 0.1 μM, 1 μM, and 10 μM. Rapamycin (Sigma–Aldrich) was applied in final concentrations of 0.1 μM, 1 μM, 10 μM, and 100 μM. All concentrations contained ≤1% DMSO despite from 10 μM pirfenidone that contained 10% DMSO. Cell cultures containing cytostatics, 10% DMSO and blank medium were incubated for three days at 37 °C and 5% CO_2. After three days necrosis, apoptosis and cell viability were assessed by using the following luminescence assay systems: CytoTox-Glo™ Cytotoxicity Assay (Promega), Caspase-Glo® 3/7 Assay (Promega) and CellTiter-Glo® Luminescent Cell Viability Assay (Promega). The assays were performed as recommended by the supplier. Per cytostatic drug and luminescence assay at least triplicates were measured. Luminescence was assessed using a SpectraMax L Luminescence Microplate Reader (Molecular Devices, CA, USA). Luminescence (relative luminescent units; RLU) was measured using photon counting and the integration time was adjusted to 1 s. Temperature of the SpectraMax L was kept between 21.0 °C and 23.0 °C during measurements.

1.2. General considerations

The experiments were performed twice. Each experiment was performed on one 96 well plate. Plates cannot be compared directly without additional normalization. Concentrations of Pirfenidone were 0.1 μM, 1 μM and 10 μM. 10 μM Pirfenidone contained the highest DMSO concentration (10% DMSO). Therefore 10% DMSO in medium was also tested. All other drug concentrations contained ≤ 1% DMSO. Rapamycin was tested in final concentrations of 0.1 μM, 1 μM and 10 μM on plate 1. On plate 2 Rapamycin was tested in final concentrations of 1 μM, 10 μM and 100 μM to assess the effect of even higher concentrations. All investigated concentrations were based on literature examples. The negative control contained medium without any admixture.

2. Results

2.1. Senescence – Cell Titer Assay

The assay reagents lead to a total lysis of all cells releasing ATP into the medium. ATP-content is directly proportional to viable cells present in the medium. High RLU means high ATP-content in the cells (highly viable cells). Low RLU means low ATP-content in the

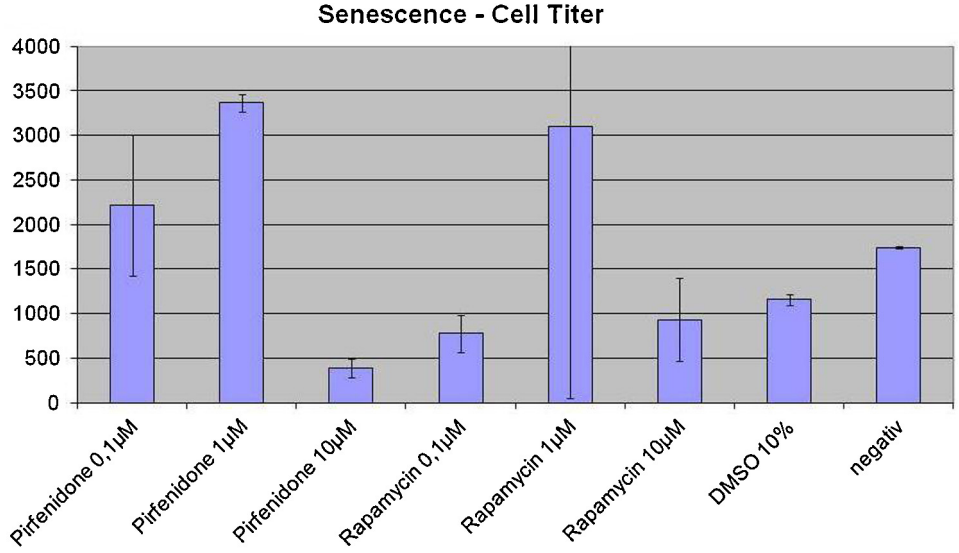

Fig. 1. Senescence – Cell Titer Assay.