COMPARATIVE STUDY OF THE CYTOTOXIC EFFECTS OF TWO *P. AERUGINOSA* CYSTIC FIBROSIS ISOLATES

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ABSTRACT

The study addresses the cytotoxic effects of co-cultivation of lung epithelial cells A549 with two clinical strains of *P. aeruginosa* isolated from the same patient with cystic fibrosis pre- (Pa48) and post-tobramycin (Pa64) inhalatory treatment. We examined the effects of 2 h co-cultivation of the lung cells with 10^5 bacteria/ml by applying the trypan blue exclusion test. The results show a high *in vitro* cytotoxic effect of the two strains if compared with the widely studied strain PAO1. They differ in the mode of action. While trypan blue penetration is over the whole area of the A549 monolayer co-cultivated with strain Pa48, both strains Pa64 and PAO1 affect individual loci of neighboring cells leaving other areas of the monolayer apparently intact.

Key words: *Pseudomonas aeruginosa; Cystic fibrosis; alveolar cells; actin*

Cystic fibrosis is an autosomal recessive genetic disorder, associated with impaired chloride and sodium ions transport. Impairment of ion balance leads to complications in lung, pancreas, intestine and liver functions. In addition CF patients are suffering of chronic lung infections caused by *Staphylococcus aureus*, *Haemophilus influenzae*, or *Pseudomonas aeruginosa* [Mitchell et al., 2006]. The interaction of *P. aeruginosa* strains with animal cells involves an arsenal of virulence factors including the Type III secretion of one or more effector molecules [Strateva, Mitov, 2011]. The injection of these into animal cell cytoplasm may switch on different processes among which cytotoxic effects are of great concern. Among the variety of tests for cytotoxicity, the most often appliied is the trypan blue exclusion test [Ramirez et al., 2012]. The aim of this study is to analyse the cytotoxic effects of two strains of *P. aeruginosa* isolated from the same patient with cystic fibrosis pre- (Pa48) and post-tobramycin (Pa64) inhalatory treatment.

Materials and Methods

P. aeruginosa strains

The study included two CF strains of *P. aeruginosa* isolated at one-year interval from the same patient, pre- (Pa48) and post-tobramycin (Pa64) inhalatory treatment. Strain PAO1 was used as a reference strain. The strains were cultivated overnight at 37°C in TSB. Bacteria were peletted, resuspended in 0.9% NaCl and calibrated to 10^9 CFU by MacFarland standard. They were then added to antibiotic-free DMEM medium to final concentrations of 10^5.

Alveolar cells cultivation and innoculation

A549 cells were grown to 80-90% confluence on 24-well plate in DMEM medium supplemented with 10% FBS and antibiotics. The medium was withdrawn, the cells were washed in three changes of PBS to remove antibiotics, and then the DMEM-suspended bacteria were added. The co-cultivation was performed at 37°C, 5% CO_2 for s up to 2 hours.

Assessment of membrane permeability with a modified trypan blue exclusion assay

After incubation, the samples were washed three times with PBS to remove unadherent bacteria, and stained in vivo with 200 μl 0.8% trypan blue in PBS per well. Negative control was alveolar cells cultivated in the same plate without bacteria. As a positive control for membrane permeabilisation, A549 cells permeabilised 5 min with 0.5% Tween 20 were used. Each sample was

observed under Olympus inverted microscope and several microphotographs of each well were taken immediately after staining.

Results and discussion

A significant amount of publications on *P. aeruginosa* cytotoxicity have been done on the model of clinical strains from patients with keratitis. In these studies, most frequently amounts of 10^7cfu/ml bacteria are included [e.g., Fink-Barbancon et al., 1997; Wang et al., 2013]. Our preliminary experiments with such a bacterial burden from the tested cystic fibrosis strains resulted in a wide-spread detachment of the A549 monolayer. For this reason we lowered the dose and here reported data are from experiments with 10^5 bacteria/ml. PAO1, which we included in the study as a reference strain, is a widely investigated laboratory model. Its Type III secretory system is characterized by the presence of ExoS, ExoT and ExoY, and the absence of ExoU [Sun et al., 2012]. Compared to PA01, both mucoviscidosis isolates had a stronger cytotoxic effect on the lung cells when applied at concentration $1x10^5$ cells/ml (Figs. 1 and 2).

Untreated cells appeared healthy with normal membrane and unstained nuclei. PA01 and Pa64 affected cell membrane permeability in a similar manner by causing membrane permeabilisation and nuclear staining of smaller or larger cell groups, while there were areas with relatively preserved permeability (Fig.1C, D and F). Pa48 co-cultivation caused significant changes in membrane permeability of alveolar cells (Fig.1E). These were similar to the dye penetration in the detergent-treated control cells (Fig. 1B). Such morphological changes are in accordance with these observed by other authors in strains with the ExoU effector mechanism [Hauser, 2009].

We further applied a quantitative analysis of the cytotoxicity by counting the number of cells with colored and not stained nuclei. The percentage of cells with trypan blue-stained nuclei was higher in samples co-cultured with Pa48 (88.9 ± 11.4%). The other two strains showed relatively lower cytotoxicity - respectively 81.1 ± 24.3% for Pa64 and 62.7 ± 26.3% for PAO1 (Fig. 2). It is noteworthy that the standard deviations of the results from cytotoxicity assessment of PAO1 and Pa64 are high. This is in accordance with the microscopic findings of local nature of the lesions in the monolayer of the A549 cells in the presence of these two strains, both showing areas with increased permeability of plasma membranes and with visibly intact cells. Co-cultivation with Pa48 resulted in large-scale membrane damage, which was manifested as prevalence of stained nuclei in the majority of cells in the monolayer (Fig. 1). Quantitative data for this strain are very close to the result of permeabilisation with Tween 20 (93.2 ± 5.9% stained cells; Fig. 2).

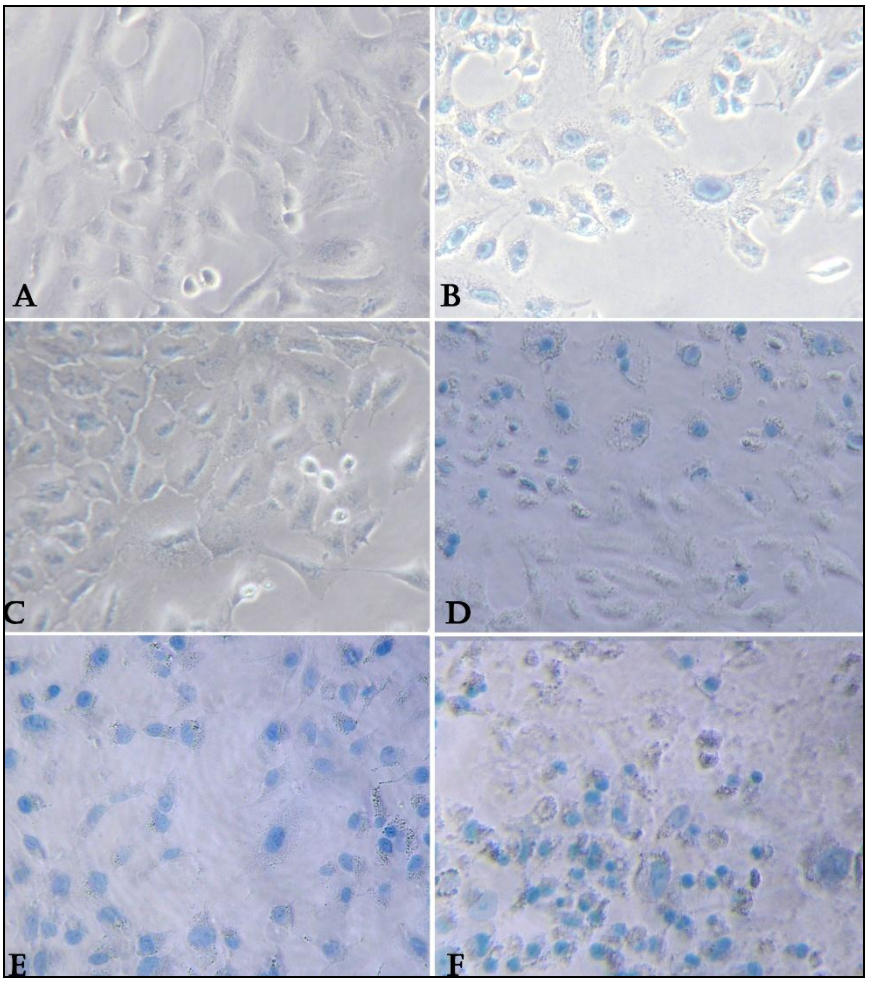

Fig.1 Changes in membrane permeability of A549 alveolar cells after co-cultivation with *Pseudomonas aeruginosa* (a modified trypan blue exclusion assay). (A) Monolayer of untreated control cells; (B) A549 cells permeabilised with 0.5% Tween 20; (C and D) A549 cells co-cultured with PA01; (E) A549 cells co-cultured with Pa48; (F) A549 cells co-cultured with Pa64. Magnification 200 x.

The observed differences could be explained by an expression of different virulence factors in the strains tested. The rapid increase of membrane permeability of A549 cells in monolayer by Pa48 shows similarity with the mechanisms associated with ExoU in literature. *In vitro* studies have indicated that ExoU has phospholipase A_2 activity, causes rapid loss of plasma membrane integrity and is generally associated with a highly cytotoxic phenotype, causing rapid and profound cell death [Bridge et al, 2012]. Unlike these, the identical events observed in A549 treated with of Pa64 and PAO1 show a local cell-bacteria interaction-based effects ascribed mainly to ExoS [Hauser, 2009]. ExoS is often associated with an invasive phenotype inducing changes in actin-associated proteins, increased permeability through cell-to-cell junctions, thereby facilitating bacterial invasion. There is generally an agreement in literature that ExoS and ExoU are mutually exclusive, and would not occur simultaneously in one and the same *P. aeruginosa* strain [Harmer et al., 2012]. If the two strains are compared, the pre-tobramycin treatment strain, Pa48, is obviously the more cytotoxic one.

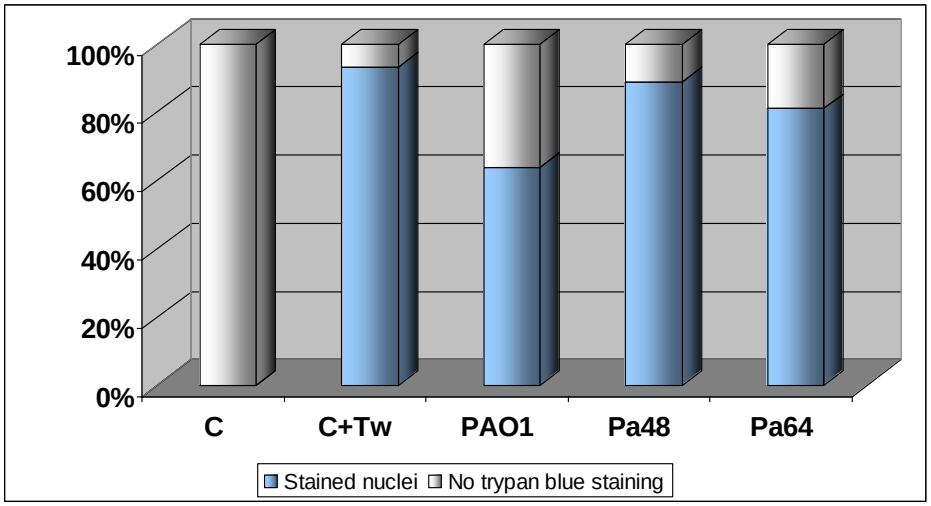

Fig.2 Cytotoxic effect of P. aeruginosa strains on A549 alveolar cells. C – control A549 cells; C+Tw – positive control: cells permeabilised with Tween 20; PA01 – alveolar cells co-cultured with the reference strain PA01; Pa48 – alveolar cells co-cultured with the clinical isolate strain Pa48; Pa64 – alveolar cells co-cultured with the clinical isolate strain Pa64;

To sum up, the morphological impairments and changes in the membrane permeability of A549 displayed similar mode of action of strain Pa64 and the reference strain PAO1. Strain Pa48 caused increased permeability of cell membrane and occurrence of necrotic characteristics of host cells. Both clinical isolates were more cytotoxic than the reference strain PAO1.

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