Brazilian Journal of Microbiology (2003) 34 (Suppl.1):25-26
ISSN 1517-8382

EXOU CONTRIBUTES TO LATE KILLING OF *PSEUDOMONAS AERUGINOSA* - INFECTED ENDOTHELIAL CELLS

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This paper corresponds to an "extended abstract" selected for oral presentation in the 22nd Brazilian Congress of Microbiology, held in Florianópolis, SC, Brazil, in November 17-20, 2003

ABSTRACT

To ascertain the role of ExoU in late *P. aeruginosa* cytotoxicity, endothelial cells (EC) were exposed to wild type PA103, PA103Δ*exoU* and PA103::*exsA* for 1h and to gentamicin in culture medium. After 24h, the viability of PA103-infected cells (33.7 ± 14.3%) was significantly lower than the viability of PA103Δ*exoU*- (77.7 ± 6.3%) or PA103::*exsA*- (79.5 ± 23.3%) infected EC. *P. aeruginosa* cytotoxicity did not depend on the bacterial ability to interact with EC because the percentage of cells with associated PA103 (35.9 ± 15.8%) was similar to the percentage in PA103Δ*exoU*- (34.2 ± 16.0%) and lower than the percentage in PA103::*exsA*-infected cultures (82.9 ± 18.9%). Cell treatment with cytochalasin D reduced the PA103 internalization by EC but did not interfere with its ability to kill host cells.

Key words: Type III secretion system; ExoU; endothelial cells; bacterial cytotoxicity.

INTRODUCTION

P. aeruginosa ExoU is a recently described lipase (6) translocated across host cell membranes by the type III secretion system (TTSS), usually associated with acute killing of *P.* aeruginosa-infected human cells (4). In this study, we investigated the role of ExoU in late death of endothelial cells by comparing the cytotoxic PA103 strain, known to produce ExoU and ExoT, with the isogenic mutants PA103Δ*exoU* and PA103::*exsA*, in which *exsA,* the transcriptional activator of the whole TTSS regulon, has been inactivated (5).

MATERIALS AND METHODS

PA103 and PA103Δ*exoU* (Saliba *et al.,* unpublished results) were cultured for 16h in Luria-Bertani (LB) broth whereas PA103::*exsA* was grown in LB containing carbenicilin at 300 µg/ml. Bacteria were harvested by centrifugation and resuspended in MCDB-131 medium at 10^8 CFU/mL. Endothelial cells (EC) from the HMEC-1 line (1) were cultured for 2 days in MCDB-131 medium supplemented with 10% fetal calf serum, EGF, hydrochortisone and antibiotics (complete medium). To assess bacteria-EC interaction, cells cultured on glass coverslips were exposed to bacterial suspensions for 3h, rinsed, fixed and stained with Giemsa stain. To assess cytotoxicity, bacterial suspensions were centrifuged at 1000 x g for 10 min onto cells cultured on 96-well tissue culture plates. After 1h at 37ºC, cells were rinsed and treated with complete medium containing gentamicin at 300 µg/ml, to kill extracellular bacteria. After 24h, the viability of control non-infected and infected cultures was assessed by the MTT assay (3). In other assays, EC were treated with cytochalasin D (CD) at 1.0 µg/ml for 30 min, prior to the bacterial centrifugation onto cells cultured in 96-well culture plates. Control untreated cells were also exposed to the bacterial suspensions. After 1h at 37ºC, cells were incubated with the gentamicin-containing culture medium for 1 or 24 hours. Cells treated with gentamicin

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for 1h were lyzed with Triton X 100 at 0.1% in PBS, diluted and plated, to assess the inhibition of bacterial internalization by CD treatment whereas cells exposed to gentamicin for 24h were rinsed and submitted to the MTT assay.

RESULTS AND DISCUSSION

By light microscopy, PA103 and PA103Dð*exoU* were seen to interact poorly with EC since the percentage of cells with associated bacteria were 35.9 ± 15.8% and 34.2 ± 16.0%, respectively. Most EC exhibited less than 5 bacteria per cell (93.3% and 100.0% for PA103::*exsA* and PA103Δ*exoU,* respectively). In contrast, 82.9 ± 19.8% of the cells exposed to PA103::*exsA* exhibited associated microorganisms, 48.7% of them with 6 to 10 bacteria and 15.4% with more than 10 bacteria. Light microscopic assays do not allow the distinction between extracellular adherent and intracellular microorganisms. However, based on our results obtained with endothelial cells in primary culture (5), we speculate that the difference between PA103 and PA103::*exsA* in their association with HMEC-1 cells may have resulted from the higher ability of the mutant to enter into host cells, secondary to inactivation of ExsA. In the absence of ExsA, PA103 does not produce ExoT, another TTSS effector protein known to interfere with mammalian cell cytoskeleton structure and with its ability to internalize bacteria (2). To circumvent the poor adhesiveness of PA103 and PA103Δ*exoU*, in the cytotoxicity assays, bacterial suspensions were centrifuged onto cultured EC. Infection by PA103 killed 66.7 ± 7.1% of the cells. The percentage of dead cells in cultures infected by PA103::*exsA* (20.5 ± 11.6) and PA103Δ*exoU* (22.9 ± 3.1) was significantly lower, as determined by ANOVA. We have previously shown that endothelial cells respond to IC *P. aeruginosa* by producing increased concentration of oxidant radicals and that host cells do not resist to the oxidative stress and die (7). Although PA103 is reported to be a non-invasive strain, by the gentamicin assay we detected low concentration of IC microorganisms ($2.7 \pm 1.3 \times 10^3$ CFU/ml) after centrifugation of bacteria onto HMEC-1cells (unpublished results). Therefore, we next investigated a relationship between the ability of PA103 to kill and to enter into EC by treating host cells with cytochalasin D, to inhibit actin polymerization. Although the cytochalasin D treatment reduced *P. aeruginosa* entry into HMEC-1cells in 98.1 ± 1.1%, no difference in the viability of PA103-infected treated (59.1 ± 4.9%) and untreated (57.7 ± 3.5%) EC was observed. Taken together, our results suggest that besides acute death of host cells, translocation of ExoU may account for late killing of human endothelial cells.

RESUMO

ExoU contribui para a morte tardia de células endoteliais infectadas por *Pseudomonas aeruginosa*

Para determinar o papel de ExoU na citotoxicidade tardia de *P. aeruginosa,* células endoteliais (CE) foram expostas às cepas PA103, PA103Δ*xoU* e PA103::*exsA* por 1h e à gentamicina em meio de cultura. Após 24h, a viabilidade das CE infectadas com PA103 (33.7 ± 14.3%) foi inferior à de CE infectadas com PA103Δ*exoU* (77.7 ± 6.3%) e PA103::*exsA* (79.5 ± 23.3%). A citotoxicidade não dependeu da capacidade de interagir com as CE porque o percentual de células com bactérias associadas em culturas expostas a PA103 foi semelhante ao percentual em culturas expostas a PA103Δ*exoU* e inferior em culturas expostas a PA103::*exsA.* O tratamento das CE com citocalasina D reduziu a internalização de PA103, mas não interferiu em sua citotoxicidade.

Palavras-chave: Sistema de secreção do tipo III; ExoU; células endoteliais, citotoxicidade bacteriana.

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