Pseudomonas aeruginosa exotoxin T induces potent cytotoxicity against a variety of murine and human cancer cell lines

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INTRODUCTION

Resistance to cancer chemotherapeutic drugs is a frequent cause of cancer treatment failure (Gonzalez-Angulo et al., 2007) and highlights the need for novel therapies. Tumour cells can utilize a number of mechanisms to develop resistance to cancer drugs (Gottesman, 2002; Calcagno & Ambudkar, 2010). One of the main mechanisms by which resistance to therapy develops in cancer is by modifications in the cellular drug target through mutations, deletions and/or gene amplification (Gottesman, 2002; Calcagno & Ambudkar, 2010). Development of drug resistance by this mechanism is achieved more easily if the therapy has limited cellular targets and/or processes. For instance, mutations in the epidermal growth factor receptor (EGFR) gene have been identified in specimens from patients with non-small-cell lung cancer treated with anilinoquinazoline EGFR inhibitors (Kobayashi et al., 2005). Similarly, resistance to the Abl tyrosine kinase inhibitor STI-571 in chronic myeloid leukaemia patients was shown to occur through either bcr–abl gene amplification or a single C→T nucleotide change within the abl locus at nt 944 (Gorre et al., 2001).

Bacterial toxins have been, and continue to be, evaluated in various clinical trials as potential cancer therapeutics (Kawakami et al., 2006; Kreitman, 2006, 2009). For example, Pseudomonas exotoxin A and diphtheria toxin are the most common bacterial toxins that have been or are currently under clinical evaluation against a variety of haematologic malignancies and solid tumours with promising results (reviewed by Becker & Benhar, 2012). Although these new recombinant immunotoxins have shown high potency in killing tumour cells with high specificity, they too share the limitation of targeting a single cellular substrate, eEF-2 (Jørgensen et al., 2008). In fact, mutations in eEF-2 have been shown to emerge easily, conferring resistance to these toxins in cancer cells (Foley et al., 1995; Jørgensen et al., 2008; Wei et al., 2012).

Pseudomonas aeruginosa exotoxin T (ExoT) is different from exotoxin A and diphtheria toxin in that instead of a single putative target (e.g. eEF-2), it has at least six cellular proteins (Krall et al., 2000; Kazmierczak & Engel, 2002; Sun...
& Barbieri, 2003; Garrity-Ryan et al., 2004), which play important roles in survival, proliferation, metastasis and angiogenesis in cancer. ExoT’s substrates include the small GTPase proteins, Ras homologue gene family, member A (RhoA), Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division control protein 42 homologue (Cdc42); which serve many important functions in cancer, such as regulation of actin cytoskeletal dynamics, activation of protein kinases, cell cycle progression, cytokinesis, metastasis and cellular survival (del Pesò et al., 1997; Gómez et al., 1997; Bagrodia et al., 1998; Muruga et al., 2002; Feng et al., 2010); the C10 regulator of kinases (Crkl, CrkII adaptor proteins), which are important for the formation and maintenance of cellular focal adhesions and cytokinesis (Cho & Klemke, 2000; Lamorte et al., 2002; Rodrigues et al., 2005; Heasman & Ridley, 2008); and the glycolytic enzyme phosphoglycerate kinase 1 (PGK1), which is important for angiogenesis in cancer (Lay et al., 2000; Hwang et al., 2006). Therefore, development of resistance in cancer cells to ExoT-induced cytotoxicity is highly unlikely.

We have shown previously that ExoT intoxication results in potent cell death in human cervical adenocarcinoma, HeLa cells (Shafikhani et al., 2008). In this report, we investigated ExoT’s cytotoxicity against a number of invasive and highly resistant skin, breast, lung and ovarian tumour cell lines in order to evaluate ExoT’s potential as a possible candidate for chemotherapy. We found that in vitro, ExoT was capable of causing potent cytotoxicity in all cell lines studied. We also found that ExoT was sufficient to induce cytotoxicity and to reduce tumour establishment and growth of B16 melanoma in vivo. Collectively, our data suggested that P. aeruginosa ExoT may be an attractive novel candidate as a cancer drug.

**METHODS**

**Transformed and non-transformed cell lines.** Tumour cell lines MCF-7 [human metastatic breast adenocarcinoma (Soule et al., 1973)], MDA-MB-231 [triple-negative human metastatic breast adenocarcinoma (Cailleau et al., 1974)], EMT6 [murine breast carcinoma (Rockwell et al., 1972)], 4T1 [murine metastatic breast cancer (Aslakson & Miller, 1992)], MCA-205 [murine-derived fibrosarcoma cell line (Korner & Routes, 2014)], B16 murine melanoma [WT BRAF (Wellbrock et al., 2008)], A375 human melanoma [with BRAF(V600E) mutation (Alczar et al., 2011)], Calu-3 [human lung adenocarcinoma (Fogh et al., 1977)], LLC1 [murine lung carcinoma (Bertram & Janik, 1980)], SK-OV-3 [human ovarian adenocarcinoma (Fogh et al., 1977)] and HeLa [human cervical adenocarcinoma (Scherer et al., 1953)] were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) with phenol red, sodium pyruvate and penicillin/streptomycin (Gibco). The non-transformed cell types used in these studies were mouse embryonic fibroblasts, isolated as described previously (Park et al., 2006), and peripheral blood mononuclear cells, cultured as described above. Cells were treated with bacteria when they were ~80–90 % confluent and transfected when they were ~60–70 % confluent (see below).

**Plasmids.** Expression vectors pIRE2-ExoT-EGFP (pExoT-GFP) and the empty expression vector (pGFP) were used in experiments involving transient transfection, and were described previously (Shafikhani & Engel, 2006).

**Cytotoxicity assessment by time-lapse immunofluorescent video microscopy.** ExoT was delivered into cancer cells either by P. aeruginosa bacteria or by transient transfection using pIRE2 mammalian expression vector and cytotoxicity was assessed as described previously (Shafikhani et al., 2008; Wood et al., 2013). Briefly, bacteria were grown overnight in LB media, adjusted to OD₆₅₀ 0.05, corresponding to m.o.i.–10. The bacterial strains used in these studies were an ExoT-expressing ExoU-deleted PA103 strain (AU) or its isogenic ExoT-defective type III secretion system (T3SS) mutant strain [PA103 pscL₆₇₋₋ (pscL)]. These strains were described previously (Shafikhani & Engel, 2006; Shafikhani et al., 2008; Mahmood et al., 2013). Cancer cells, infected with ExoT-expressing and ExoT-defective P. aeruginosa, were placed under 5 % CO₂ at 37 °C in an incubation chamber (Pecon) fitted to an Axiosvert Z1 (Zeiss) microscope using Axiovision version 4.2.2 software. Time-lapse video microscopy was performed at ×100 magnification (unless otherwise specified) and images were acquired at 15 min intervals, as described previously (Shafikhani & Engel, 2006; Shafikhani et al., 2008; Wood et al., 2013). Cytotoxicity associated with ExoT was assessed by the uptake of propidium iodide (PI) impermeant dye using time-lapse video microscopy as previously described (Wood et al., 2013). Briefly, the number of red pixels (PI) for each video frame was measured by thresholding the red fluorescence channel using ImageJ version 1.48 (http://imagej.nih.gov/ij/). For transfection experiments, cells were grown as described above and transfected with Effectene (Qiagen) as per the manufacturer’s protocol 1 h prior to the addition of PI and the start of time-lapse video microscopy. Time-lapse videos were assessed for cytotoxicity by determining the percentage of transfected GFP that became PI-positive, as described previously (Shafikhani & Engel, 2006; Shafikhani et al., 2008; Wood et al., 2013).

**Cytotoxicity assessment by flow cytometry.** Transformed cells were transfected with Effectene, whilst non-transformed cells were transfected using an electroporation kit (Lonza). At 24 h after transient transfection, cytotoxicity was also assessed using Fixable Viability Dye (Biosciences) stain to determine the per cent cytotoxicity in transfected cells and the data were analysed by flow cytometry (FACSCanto II; BD Biosciences) as per the manufacturer’s protocol. After 24 h, peripheral blood mononuclear cells were additionally stained with anti-CD3 antibody (Biosciences) in order to specifically gate on the expression of ExoT in T-lymphocytes alone.

**Cytotoxicity assessment by lactate dehydrogenase (LDH) release assay.** LDH release was measured using an LDH Cytotoxicity Detection kit (Clontech). In a 24-well plate, cancer cells were seeded at 8 × 10³ cells per well and cultured in 1 ml DMEM (Gibco) media without phenol red, sodium pyruvate and penicillin/streptomycin antibiotics, and supplemented with 1 % FBS. To further assess the cytotoxic effect ExoT had on cancer, tumour cell lines were infected with either ExoT-expressing ExoU-deleted PA103 strain (AU) or its isogenic ExoT-defective T3SS mutant strain (pscL) at m.o.i.–10 (Shafikhani & Engel, 2006; Shafikhani et al., 2008; Mahmood et al., 2013). To measure the amount of LDH release, the suspensions of uninfected and infected cells were collected and centrifuged at 2.5 g for 10 min to remove bacteria. Uninfected and infected samples, along with LDH-high control samples treated with 1 % Triton X-100 and LDH-low control samples with media alone, were added into a 96-well plate and treated with LDH detection reagents as outlined in the LDH Cytotoxicity Detection kit user manual. A₄₉₀ was measured using a Thermo Scientific Multiskan Spectrum spectrophotometer and SkanIt software. Per cent LDH release was calculated by substituting the appropriate absorbance values into the following formula:
**RESULTS**

**P. aeruginosa ExoT induces potent cytotoxicity in a variety of murine and human tumour cell lines**

ExoT has previously been shown to induce potent cytotoxicity in HeLa cells (Shafikhani et al., 2008). As ExoT targets six cellular proteins (Shafikhani & Engel, 2006; Shafikhani et al., 2008) that are essential for cancer cell survival and proliferation (del Peso et al., 1997; Gómez et al., 1997; Cho & Klenke, 2000; Lay et al., 2000; Lamorte et al., 2002; Murga et al., 2002; García et al., 2006; Hwang et al., 2006; Heasman & Ridley, 2008; Zhu et al., 2008; He et al., 2010), we hypothesized that resistance to ExoT-induced cytotoxicity would be highly unlikely and therefore it may be able to induce cytotoxicity in a wide range of tumour cell lines.

To gain insight into the efficacy of the ExoT-induced cytotoxicity, we first used *P. aeruginosa* to deliver ExoT into a number of highly resistant cancer cell lines, including B16, HeLa, EMT6, 4T1, MDA-MB-231, SK-OV-3, MCA-205 and Calu-3 (see Methods).

Tumour cells were treated with either an ExoT-expressing *P. aeruginosa* PA103 strain ΔU or ExoT-deficient *P. aeruginosa* PA103 strain ΔscJ, or left untreated (see Methods). We assessed ExoT-mediated cytotoxicity by measuring the extracellular release of the cytoplasmic protein LDH into the culture media. Treatment with the ExoT-expressing *P. aeruginosa* strain (ΔU) resulted in significantly higher LDH release in all the tumour cells, as early as 10 h post-infection (Fig. 1).

In order to home in on the kinetics of ExoT-induced cytotoxicity in these tumour cell lines, we assessed cytotoxicity at 15 min intervals using PI impermanent nuclear dye uptake as a marker for cell death. PI uptake and subsequent PI fluorescence is an established and irreversible marker for cell death (Shafikhani et al., 2008; Kroemer et al., 2009; Wood et al., 2013). ExoT-expressing *P. aeruginosa* was able to induce cytotoxicity in all the tumour cell lines we tested within 15–25 h post-infection (Fig. 2, Movies S1, S2 and S3, available in the online Supplementary Material; only the representative movie frames of B16 are shown in Fig. 2b).

**ExoT is sufficient to induce cytotoxicity in tumour cells**

Although LDH release is routinely used as a measure of cytotoxicity (Korzeniewski & Callewaert, 1983; Decker & Lohmann-Matthes, 1988; Arechabala et al., 1999), injured but viable cells can also release LDH as a result of damage to their membrane (Reddy et al., 2001). Moreover, LDH release could potentially underestimate cytotoxicity in apoptotic cells, because activated caspases, which are proteases that mediate apoptosis (Fiers et al., 1999; Lavrik et al., 2005), could degrade LDH peptides, thus reducing the amount of LDH released into the medium. ExoT is known to induce apoptosis (Shafikhani et al., 2008). In addition, the use of bacteria to deliver ExoT into tumour cells could introduce additional bacterial factors, which may contribute and/or synergize the ExoT-mediated killing of tumour cells. In fact, treatment with the ExoT-defective ΔscJ strain resulted in increased LDH release and cytotoxicity in some tumour cell lines, albeit at significantly lower levels than the LDH release or the cytotoxicity observed in the presence of the ExoT-expressing ΔU strain (Figs 1 and 2).

To determine if ExoT was sufficient to cause cytotoxicity in the aforementioned tumour cell lines and to account for the ExoT-independent cytotoxic effects of *P. aeruginosa* on tumour cells, we transfected the tumour cell lines with an expression vector harbouring either the *exoT* gene, C-terminally fused to *gfp* (pExoT-GFP), or the *gfp* empty vector control (pGFP) and assessed cytotoxicity by time-lapse fluorescence video microscopy, using PI uptake as a marker for cell death. This technique allowed us to quantify ExoT’s cytotoxic effect in tumour cells on a per cell basis. Some cancer cell lines were poorly transfectable and thus were not included in these analyses. However, in those tumour cell lines that we were able to transfect, ExoT expression resulted in significantly more killing than the GFP control vector, indicating that ExoT was sufficient to cause cytotoxicity in these cancer cells (Fig. 3, Movies S4 and S5; only selected frames from B16 melanoma cells are shown in Fig. 3b).
We further corroborated the cytotoxic effects of ExoT in transfected tumour cells by flow cytometry using a Viability Dye (eBiosciences), which, instead of using PI staining as a marker for cell death, works by binding to free amino acids in the cytosol (Perfetto et al., 2010). As expected, cells intoxicated with ExoT exhibited significantly higher cytotoxicities than the GFP control at 24 h post-transfection (Fig. 4). Depending on the techniques, there were some variations in the ExoT-induced cytotoxicities in these cell lines (compare Figs 1, 2 and 3). This is likely due to sensitivity of the techniques and/or the limitation of the use of peptides (i.e. LDH or Viability Dye) as markers for cell death in apoptotic cells as discussed above. Nevertheless, these data indicate that ExoT is sufficient to induce potent cytotoxicity in all tumour cell lines we tested.

Most cancer chemotherapeutics exert cytotoxicity in normal non-transformed cells, leading to well-chronicled side-effects associated with cancer therapy. To gain insight into potential side-effects of ExoT-based therapy, we transfected mouse embryonic fibroblasts and human T-lymphocytes with an ExoT expression vector as described above. Not surprisingly, ExoT also induced comparable cytotoxicity in these cell lines (Fig. S1). We were unable to evaluate ExoT’s cytotoxicity in human or mouse primary epithelial cell lines due to low transfection efficiencies as well as their sensitivity to bacterial infection as ExoT-deficient bacteria also caused massive cytotoxicity in these cell lines. Nevertheless, these results highlighted the need to target this toxin directly into tumour cells (see Discussion for possible approaches).

**ExoT is sufficient to induce cytotoxicity in vivo**

We next assessed the ability of ExoT to induce cytotoxicity in vivo, using the B16 melanoma tumour model (Overwijk...
& Restifo, 2001). We transplanted B16 melanoma cells subcutaneously in the flanks of C57BL/6 mice. Once the tumours reached 50 mm², the expression vector expressing ExoT-GFP was packaged within a lipid-based transfection reagent to facilitate its uptake and injected directly into the lesion. After 24 h, the animals were injected systemically (intravenously) with SR-FLIVO, a marker for apoptotic cell death in vivo (Riol-Blanco et al., 2009), and the tumours

Fig. 2. Kinetics of ExoT-induced cytotoxicity in cancer cell lines. (a) The indicated cancer cell lines were treated with ExoT-expressing (ΔU) or ExoT-defective (pscJ) P. aeruginosa strains at m.o.i.~10, or left untreated. Cells were observed by time-lapse fluorescence video microscopy in the presence of PI (×100 magnification, 15 min intervals). Cytotoxicity was assessed by total PI uptake at 15 min intervals (cytotoxicity is shown as the mean of three fields of view). (b) Cytotoxicity analysis of B16 melanoma cells as assessed by PI staining (red). (c) Representative frames from B16 time-lapse video microscopy.
were analysed by fluorescence microscopy. The data indicated that transfection with ExoT resulted in cell death in B16 cells in vivo as indicated by co-localization of ExoT-GFP (green) and SR-FLIVO (red) (Fig. S2).

As in situ transfection efficiencies into the tumours by this method were extremely low (~3%), ExoT failed to control overall tumour growth (data not shown). We modified our experimental approach to enhance transfection efficiencies in order to better evaluate the ability of ExoT to affect tumour establishment and growth in vivo. B16 cells were transfected with either pExoT-GFP or pGFP vector control ex vivo prior to subcutaneous injection in the mice. This approach increased the transfection efficiencies to ~20%. Next, pExoT-GFP- or pGFP-transfected B16 tumour cells were transplanted into mice. Despite these low transfection efficiencies, the mean size of the ExoT-transfected B16 tumours was significantly less than pGFP-transfected B16 tumours (Fig. 5a, n=5 per group, P<0.05; mean tumour size was analysed up to the first tumour end point). Tumour measurements for individual animals showed that tumours transfected with pExoT-GFP reached their end point significantly slower than pGFP tumours. Collectively, these data indicated that ExoT was sufficient to induce cytotoxicity in B16 tumour cells and delay tumour growth in vivo.

**DISCUSSION**

Bacteria have been and continue to be used to deliver toxins into tumours (Dang et al., 2001; Van Mellaert et al., 2006; Patyar et al., 2010; Karbach et al., 2012). This study is the first evaluation of ExoT as a potential candidate for cancer therapy. Our results demonstrate that ExoT is capable of causing potent cytotoxicity in all murine and human tumour cell lines that we have examined thus far (Figs 1, 2, 3 and 4, Movies S2 and S5, and data not shown). ExoT-induced cytotoxicity occurred across a wide range of tumour cell lines, including breast, lung, cervical and even melanoma that is largely refractory to current cytotoxic drugs in the clinical setting (Atkins et al., 2008; Flaherty et al., 2012; Homet & Ribas, 2013).

Our in vivo results are encouraging in that they demonstrate that ExoT can significantly slow B16 melanoma tumour growth in vivo (Fig. 5). Low transfection efficiency is likely a major reason why these tumours eventually grew out and reached their end point. Nevertheless, the ExoT-mediated reduction in tumour growth in vivo highlights the potential of ExoT in cancer therapy.

The primary challenge concerning the formulation of ExoT as a potential cancer therapy is the development of means to safely deliver this toxin and specifically target it to...
tumours *in vivo*. There are multiple approaches that have been or are currently being investigated in several clinical trials that may accomplish this task. One approach is conjugation with tumour-specific antibodies (reviewed by Becker & Benhar, 2012). Conjugating ExoT to tumour-specific antibodies or the immunoglobulin variable fragment regions can enhance the delivery of the toxin (e.g. intravenously) as well as improve the specificity of ExoT to target the tumour. Indeed, engineered toxins combined to a receptor ligand or the variable domain of a mAb can differentiate between normal and malignant cells by binding to tumour-associated cell surface receptors (e.g. CD22, CD25, claudin-4, glycoprotein-NMB and mesothelin) (Chaudhary et al., 1987, 1989; Pastan et al., 2004).

Another approach is the use of recombinant viral delivery systems, such as vaccinia virus, with inherent tropism toward tumour cells (Baguley, 2010). A viral-based delivery platform has a number of unique biological properties that make it ideally suited for delivery and amplification of transgenes within tumours. These include intravenous stability and the ability to spread to distant tissues (Vanderplasschen et al., 1998), preferential accumulation in solid tumours where neovasculature shows increased permeability (Kirn & Thorne, 2009), tropism toward cancerous cells (Park et al., 2008; Breitbach et al., 2011), and only minor side-effects in cancer patients even at extremely high infection titres (10⁷–10⁹ p.f.u.) (Park et al., 2008; Breitbach et al., 2011).

Another potential translational approach is the use of image-guided ultrasound-based microbubble technology to deliver and activate ExoT selectively in the tumour microenvironment. Gene therapy using this technology has gained significant momentum in recent years (Smith et al., 2011; Chen et al., 2012; Sirsi & Borden, 2012; Smith & Land, 2012).

**Fig. 4.** Assessment of ExoT-induced cytotoxicity in cancer by Viability Dye. Cancer cells were transfected with pGFP vector control or pExoT-GFP. At 17 h after transfection, cells were stained with Viability Dye and analysed by flow cytometry. (a) Representative FACS plots for B16 show ExoT was sufficient to cause cytotoxicity in transfected cells. SSC, side scatter; FSC, forward scatter. (b) FACS analysis of transfected B16 cells indicated that pExoT-GFP resulted in significantly more cytotoxicity compared with pGFP-transfected cells. *P < 0.0001; n = 7; Student’s two-tailed t-test.
In summary, we propose that *P. aeruginosa* ExoT possesses attractive anti-cancer properties that make it an attractive cancer drug candidate. ExoT’s attractive anti-cancer properties include: (1) its ability to induce potent cytotoxicity in all cancer cell lines, as we demonstrated in this report; (2) its anti-proliferative effect in cancer (Shafikhani & Engel, 2006); (3) its ability to inhibit cell migration (Garrity-Ryan *et al.*, 2004), thus potentially being able to interfere with metastasis; and (4) the low probability of tumour resistance to ExoT due to its multiple cellular targets.

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