FEBS Letters 580 (2006) 4172–4175

Quantitative analysis of the effect of cell type and cellular differentiation on protective antigen binding to human target cells

Alina Deshpande[a,b,1], Rebecca J. Hammon[b,1], Claire K. Sanders[b], Steven W. Graves[b,*]

[a] *Decisions Applications Division, Los Alamos National Laboratory, MS M888, Los Alamos, NM 87545, United States*
[b] *The National Flow Cytometry Resource, Bioscience Division, Los Alamos National Laboratory, MS M888, Los Alamos, NM 87545, United States*

Received 8 May 2006; revised 9 June 2006; accepted 23 June 2006

Available online 30 June 2006

Edited by Maurice Montal

Abstract **We quantitatively measured protective antigen (PA) binding to human cells targeted by anthrax lethal toxin (LT). Affinities were less than 50 nM for all cells, but differentiated cells (macrophages and neutrophils) had significantly increased PA binding and endothelial cells demonstrated the most binding. Combined with the function of such cells, this suggests that PA receptors interact with the extracellular matrix and that differentiation increases the number of PA-specific receptors, which supports previously observed differentiation-induced LT susceptibility. Our results quantifiably confirm that the generality of PA binding will complicate its use as a tumor targeting agent.**

Keywords: Protective antigen; Lethal toxin; Human cells; Macrophages; Endothelial cells; Receptors

1. Introduction

Lethal toxin (LT) and edema toxin (ET) of *Bacillus anthracis* use protective antigen (PA) to deliver toxic partner proteins, lethal factor (LF) and edema factor (EF), to the cytosol by binding to cellular receptors [1]. PA-mediated internalization of LF and EF occurs following PA heptamerization on the cell surface and pH-dependent pore formation within the endosome (reviewed in [2]). Quantification of the affinity and number of endogenous PA-specific receptors is important for understanding the role of PA in anthrax virulence and its potential as a pharmaceutical delivery agent [3–5]. Previous studies have measured the binding affinity of PA to non-human mammalian cells [6,7] and purified receptors [8], but information on PA binding to human cells is lacking. Although the known PA-specific receptors, tumor endothelial marker 8 (TEM8) and capillary morphogenesis gene 2 (CMG2) [9,10], were discovered in CHO-K1 cells and human endothelial cells [6,11], the susceptibility of mouse macrophages [12], activated monocytes [13], and platelets [14] to LT killing as well as LT induced paralysis of neutrophils [15] suggests that PA receptors are present on many cell types.

In this study, we quantified the affinity and number of PA-specific receptors on the surface of known target animal cell lines. These studies were extended to human cell lines which represent several phenotypes known to be targeted by anthrax; the effect of cell differentiation on the receptor parameters was also measured. Our results are considered in light of recent findings, which suggest a mechanism for cell type-specific susceptibility to LT based upon differential display of PA-specific receptors; the implications of these results for recent work on the use of PA as a mechanism for targeted drug delivery [4,5] are also discussed.

2. Materials and methods

2.1. Materials

All cell lines were obtained from the American Tissue Culture Collection (ATCC), Manassas, VA. Culture medium, supplements and phorbol 12-myristate 13-acetate (PMA) were from Sigma–Aldrich, St. Louis, MO. PA was from List Biological Laboratories, Campbell CA. Alexa Fluor 488 was from Molecular Probes, Eugene, OR. FITC-standard microspheres were from Bangs Laboratories, Fishers, IN.

2.2. Cell culture

Cells were maintained according to ATCC instructions with the exception of CHO-K1 cells (alpha minimal essential media and 10% FBS) and HL-60 cells (RPMI-1640 with L-glutamine and 10% FBS).

2.3. Fluorescent labeling of PA

PA was reconstituted according to manufacturer's instructions and labeled with 20-fold molar excess of Alexa Fluor 488 in sodium bicarbonate buffer (0.1 M, pH 8.3) for 1 h on ice in the dark, then purified by gel filtration. Fluor-to-protein ratio and relative quantum efficiency were obtained as described previously [16]. Fluor-to-protein ratios were between 1 and 2.5 to minimize steric effects due to labeling.

2.4. PA binding to cells

THP-1, HL-60, and U-937 cells were differentiated into macrophages with PMA (50 ng/ml for ~24 h) [17]. HL-60 cells were differentiated into neutrophils by incubation in 1.5% dimethyl sulfoxide for five days [18]. Adherent cells were harvested by Accutase treatment after rinsing with PBS and washed twice with their respective serum-free, phenol red-free medium with 1 mM $MgCl_2$, 1% BSA, penicillin, and streptomycin. Cells (1×10^6 cells/ml) were incubated with Alexa488-PA for 2 h on ice in the dark, washed twice and re-suspended in media with 1 μg/ml propidium iodide (PI) to identify non-viable cells. Surface localization of PA was confirmed by visualization of PA bound to CHO-K1 cells at 63× magnification on a Zeiss (Chester, VA) confocal microscope (data not shown).

*Corresponding author. Fax: +1 505 665 3024.
E-mail address: graves@lanl.gov (S.W. Graves).

[1] These authors contributed equally to this work.

Abbreviations: ET, edema toxin; LT, lethal toxin; PA, protective antigen; EF, edema factor; LF, lethal factor; CMG2, capillary morphogenesis gene 2; TEM8, tumor endothelial marker 8; PMA, phorbol 12-myristate 13-acetate; PI, propidium iodide; MESF, mean equivalent soluble fluorescein

0014-5793/$32.00
doi:10.1016/j.febslet.2006.06.070

Specific binding of PA was measured by the difference in bound Alexa488-PA fluorescence between cells pre-incubated with and without unlabeled PA (100-fold molar excess for 1 h on ice). Alternatively, specific binding was measured by washing the cells in media with 1% BSA after incubation with Alexa488-PA. The results for receptor affinity and number for the two methods were equivalent (data not shown); specific binding reported in this work was determined using washed cells.

2.5. Flow cytometry

Flow cytometric analysis was performed on a FACSCalibur (Becton-Dickinson, San Jose, CA). Alexa488 (530/30 nm band pass filter) and PI (670 nm long pass filter) fluorescence was collected following 488 nm excitation. Cells were gated based on light scatter, and PI-positive cells were excluded from analyzed cell populations (3000 cells).

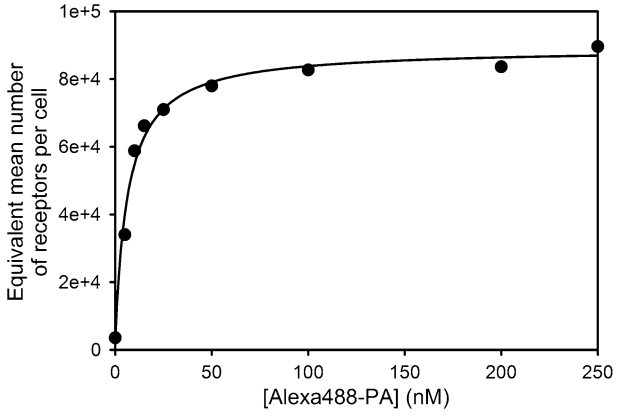

Fig. 1. Representative Alexa488-PA binding curve for CHO-K1 cells. Data from a single typical experiment were fit with a hyperbola; mean receptor number 86588, K_D 6.57 nM.

2.6. Data analysis

Mean fluorescence intensity of the cell population was converted to mean equivalent soluble fluorescein (MESF) using a standard curve generated with FITC-standard microspheres [19]. MESF was converted to equivalent mean number of Alexa488-PA molecules bound per cell as previously described [16] and plotted as a function of increasing Alexa488-PA concentration. Data were fit to a hyperbolic function $[y = (A \cdot x)/(K_D + x) + y_0]$ using SigmaPlot version 9 (Systat Software, Richmond, CA) to extract fitted values via non-linear regression. The amplitude of the curve, A, is an estimate of the amount of bound PA molecules per cell. Receptor number was estimated using the expected 1:1 stoichiometry of binding between PA and its known receptors [1]. The K_D is the equilibrium dissociation constant, which is also the value of x when $y = 1/2\,y_{max}$. In this curve, y_0 accounts for the initial cellular autofluorescence values prior to addition of fluorescent PA. Statistical analysis of the receptor number and K_D data was done by two-tailed Student's t test after verification of homoscedasticity using the F distribution (data not shown).

3. Results

3.1. Specific binding of PA to the surface of mammalian cells

Alexa488-PA (0–250 nM) was bound to CHO-K1 cells as described. Data from a typical experiment are shown in Fig. 1; the graph demonstrates specific and saturable binding of PA to these cells, as non-specific binding would be expected to show a linear trend with no saturation. The mean receptor number for all cell types studied is graphically represented in Fig. 2a and the mean K_D in Fig. 2b. The other non-human mammalian cell line analyzed, J774A.1 murine macrophages, also showed specific and saturable PA binding (data not shown).

Using the methods described above, we measured the receptor number and K_D for human target cell lines: HUV-EC-C, THP-1, HL-60, and U-937 (Fig. 2). The binding curves for

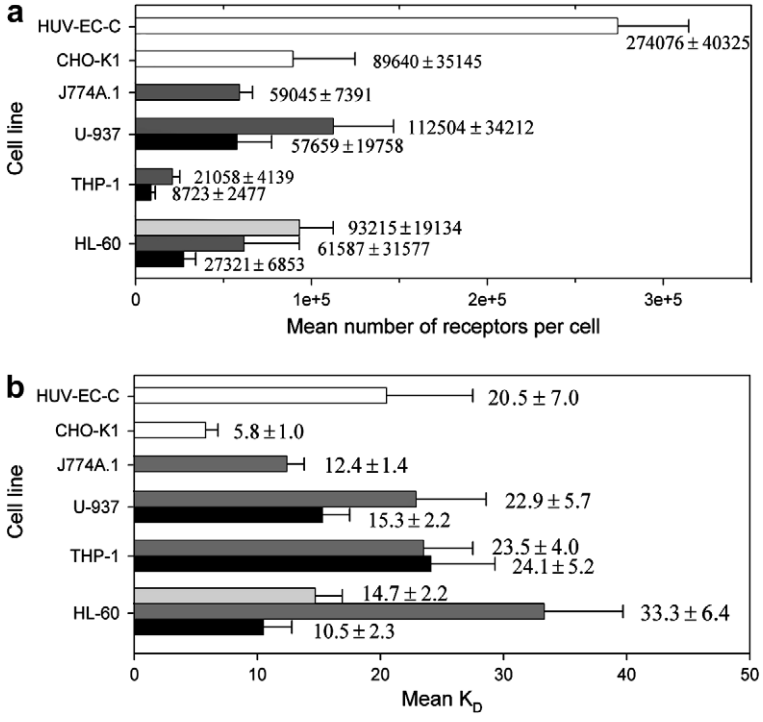

Fig. 2. (a) Mean receptor number per cell for all cell types studied. (b) Mean K_D for all cell types studied. Data shown are means ± S.D. Undifferentiated cells are shown in black; macrophages in dark gray; neutrophils in light gray; endothelial and epithelial cells in white.

all of these cells demonstrated specific and saturable binding (data not shown) with minimum R^2 values of 0.9839 (range 0.9839–0.9998, mean 0.9980). All human and non-human cell types showed mean K_D values in the low nanomolar range (range 5.8–33.3 nM). HUV-EC-C endothelial cells showed the highest amount of specific binding – with a high degree of statistical significance – of the cell lines studied here (P values < 0.006 for comparison to all other cells).

3.2. Cell differentiation increases the number of PA-specific receptors

We differentiated HL-60 promyelocytes into macrophage and neutrophil phenotypes and obtained PA binding curves as described above. Results from three typical experiments are shown in Fig. 3. Repeat experiments confirmed that neutrophils demonstrated a nearly 4-fold increase in receptor number from the undifferentiated promyelocytes while macrophages demonstrated a 2-fold increase (Fig. 2). A 2-fold increase in receptor number was also observed when THP-1 and U-937 monocytes were differentiated into their macrophage phenotypes (Fig. 2). Multiple experiments to measure both the number of receptors bound and the K_D of binding were used to provide estimates of the statistical significance of these measurements (Table 1). HL-60 cells also showed a statistically significant decrease in binding affinity upon differentiation into macrophages (Table 1), a trend which is not mirrored in the THP-1 cells but perhaps might be occurring in the U-937 monocytes (Fig. 2b).

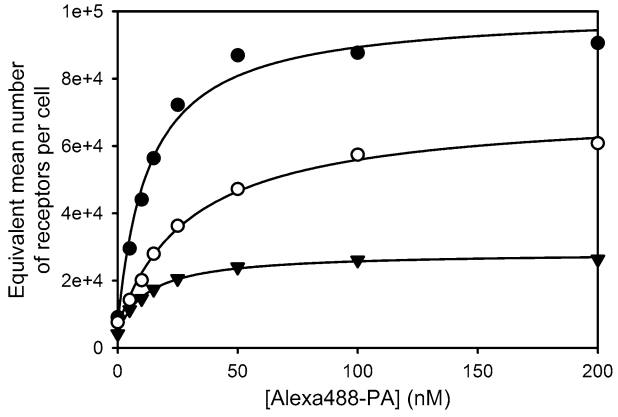

Fig. 3. Representative Alexa488-PA binding curves for undifferentiated and differentiated HL-60 cells. Specific binding of PA to HL-60 neutrophils (filled circles), HL-60 macrophages (open circles) and HL-60 promyelocytes (filled triangles) in three separate typical experiments.

4. Discussion

We have measured the affinity and number of PA-specific receptors on the surface of a variety of mammalian cell types. Binding of PA to all human target cell types was specific and most cell types had similar mean K_D values, suggesting the presence of a similar class of PA receptors. This work indicates that endogenous PA receptors on the tested cell types bind PA with affinities in the low nanomolar range in the presence of Mg^{2+} (Fig. 2b). This is in contrast to *in vitro* measurements showing that CMG2 has picomolar affinity for PA in the presence of Mg^{2+} ions [8], but is consistent with initial PA binding experiments conducted on CHO-K1 cells using radiolabeling techniques [6]. Our receptor numbers are higher for both CHO-K1 and J774A.1 cells as compared to other approaches [6,7]; this could be due to variations in cell culture conditions and experimental methods. Beyond experimental variability, variation in the number of PA-specific receptors could be due to increased expression of PA-specific receptors on certain cell types. Alternatively, it is also possible that PA receptors function in similar fashion to α integrins, which can alter their ligand binding affinities by many orders of magnitude via a conformational change in the I domain [20]. This is plausible, as the known receptors for PA (TEM8 and CMG2) have a high degree of homology to the I domains found in some α integrins [1]. Therefore, the change in PA-specific receptor number could be a result of conformational changes of very low affinity-state (effectively no affinity) receptors to high affinity-state PA-specific receptors. Regardless of the mechanism by which receptor number varies, among the human target cell lines studied, statistical analysis reveals several correlations between cell type and increased numbers of PA-specific receptors.

First, HUV-EC-C endothelial cells have the highest amount of specific binding of any cell type we studied. Recent studies suggest that LT attacks the endothelial barrier to achieve vascular permeability [21]. We postulate that the increased number of PA-specific receptors on endothelial cells provides a biochemical mechanism to support the theory that the vascular endothelium, which is continuously exposed to the blood stream, is a primary target of PA binding and subsequent toxin delivery [22,23]. Increased receptor number on endothelial cells also supports recent cell viability studies indicating that LT targets the microvascular endothelial cells of the human lung after anthrax spore inhalation [24].

Second, differentiation from monocytes to mature cell types such as neutrophils and macrophages results in a statistically significant increase in PA-specific binding (Table 1). The variance in the HL-60 macrophage population limits the P values to 0.0782 and 0.1375 for some cell-to-cell comparisons (promyelocytes vs. macrophages and macrophages vs. neutrophils,

Table 1
Statistical analysis of differences in receptor number and K_D within differentiated cell types

Compared cell lines	P value	
	Receptor #	K_D
HL-60 promyelocytes vs. HL-60 macrophages	0.0782	0.0005
HL-60 promyelocytes vs. HL-60 neutrophils	0.0006	0.0372
HL-60 macrophages vs. HL-60 neutrophils	0.1375	0.0015
THP-1 monocytes vs. THP-1 macrophages	0.0022	0.8505
U-937 monocytes vs. U-937 macrophages	0.0579	0.0850

P values are result of two-tailed Student's t test. Data are results of four independent experiments for each cell type, except U-937 monocytes (three experiments).

respectively), but the data still clearly follow the trend of increased binding upon differentiation. As neutrophils and macrophages interact with the extracellular matrix during extravasation and endothelial cells are themselves adherent, our data are consistent with previous observations that PA receptors have possible involvement in extracellular matrix interactions [11,25,26]. One study suggests that differentiation of monocytes to macrophages confers susceptibility to LT-mediated death despite internalization of LF by both cell types, and the authors provide several possibilities to explain the lack of cell killing in undifferentiated cells [27]. It is possible that such observations could arise from an increase in the number of PA-specific receptors present on differentiated cells, resulting in binding of larger amounts of PA, greater uptake of toxin, and consequently more intracellular LF to initiate multiple downstream effects. This model is further supported by studies demonstrating that CHO-K1 cells expressing TEM8 or CMG2 receptors at higher than endogenous levels were more susceptible to killing by N-terminal LF–diphtheria toxin A chain [10].

In summary, this is the first rigorous quantitative flow cytometry study to determine the prevalence of natural PA receptors across a range of potential target cell types for anthrax LT. Experiments are underway to determine the identity of the PA-specific receptors expressed on the human cell lines studied here. However, we have quantitatively confirmed that a variety of cell types are able to specifically bind PA and that cells targeted by LT have significantly increased PA binding, presumably resulting from greater display of PA-specific receptors. Furthermore, the generality of PA binding needs to be considered in studies that use PA as a cell specific delivery vehicle for cancer therapies [4], which supports the extensive efforts to create engineered PA molecules (bearing altered protease cleavage sites) to specifically target tumor cells with chemotherapeutic agents [5].

Acknowledgements: We thank Travis Woods for technical assistance and Dr. James Freyer for suggestions and critical reading of this manuscript. Funding was provided by the National Flow Cytometry Resource (NIH 2P41RR001315) and a LANL Director's Post-Doctoral Fellowship to Alina Deshpande.

References

[1] Scobie, H.M. and Young, J.A.T. (2005) Interactions between anthrax toxin receptors and protective antigen. Curr. Opin. Microbiol. 8, 106–112.
[2] Collier, R.J. and Young, J.A. (2003) Anthrax toxin. Annu. Rev. Cell. Dev. Biol. 19, 45–70.
[3] Moayeri, M. and Leppla, S.H. (2004) The roles of anthrax toxin in pathogenesis. Curr. Opin. Microbiol. 7, 19–24.
[4] Liu, S.H., Schubert, R.L., Bugge, T.H. and Leppla, S.H. (2003) Anthrax toxin: structures, functions and tumour targeting. Expert Opin. Biol. Ther. 3, 843–853.
[5] Liu, S., Redeye, V., Kuremsky, J.G., Kuhnen, M., Molinolo, A., Bugge, T.H. and Leppla, S.H. (2005) Intermolecular complementation achieves high-specificity tumor targeting by anthrax toxin. Nature Biotech. 23, 725–730.
[6] Escuyer, V. and Collier, R.J. (1991) Anthrax protective antigen interacts with a specific receptor on the surface of CHO-K1 cells. Infect. Immun. 59, 3381–3386.
[7] Singh, Y.S., Leppla, S.H., Bhatnagar, R. and Friedlander, A.M. (1989) Internalization and processing of *Bacillus anthracis* lethal toxin by toxin-sensitive and -resistant cells. J. Biol. Chem. 264, 11099–11102.
[8] Wigelsworth, D.J., Krantz, B.A., Christensen, K.A., Lacy, D.B., Juris, A.J. and Collier, R.J. (2004) Binding stoichiometry and kinetics of the interaction of a human anthrax toxin receptor, CMG2, with protective antigen. J. Biol. Chem. 279, 23329–23356.
[9] Bradley, K.A., Mogridge, J., Mourez, M., Collier, R.J. and Young, A.T. (2001) Identification of the cellular receptor for anthrax toxin. Nature 414, 225–229.
[10] Scobie, H.M., Rainey, J.A., Bradley, K.A. and Young, J.A.T. (2003) Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor. Proc. Natl. Acad. Sci. USA 100, 5170–5174.
[11] Bell, S.E., Mavila, A., Salazar, R., Bayless, K.J., Kanagala, S., Maxwell, S.A. and Davis, G.E. (2001) Differential gene expression during capillary morphogenesis in 3D collagen matrics: regulated expression of genes involved in basement membrane matrix assembly, cell cycle progression, cellular differentiation, and G-protein signaling. J. Cell Sci. 114, 2755–2773.
[12] Friedlander, A.M. (1986) Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. J. Biol. Chem. 261, 7123–7126.
[13] Popov, S.G., Villasmil, R., Bernardi, J., Grene, E., Cardwell, J., Popova, T., Wu, A., Alibek, D., Bailey, C. and Alibek, K. (2002) Effect of *Bacillus anthracis* lethal toxin on human peripheral blood mononuclear cells. FEBS Lett. 527, 211–215.
[14] Kau, J., Sun, D., Tsai, W., Shyu, H., Huang, H., Lin, H. and Chang, H. (2005) Antiplatelet activities of anthrax lethal toxin are associated with suppressed p42/44 and p38 mitogen-activated protein kinase pathways in the platelets. J. Infect. Dis. 192, 1465–1474.
[15] During, R.L., Wei, L., Hao, B., Koenig, J.M., Stephens, D.S., Quinn, C.P. and Southwick, F.S. (2005) Anthrax lethal toxin paralyzes neutrophil actin-based motility. J. Infect. Dis. 192, 837–845.
[16] Graves, S.W., Woods, T.A., Kim, H. and Nolan, J.P. (2005) Direct fluorescent staining and analysis of proteins on microspheres using CBQCA. Cytometry 65A, 50–58.
[17] Yang, M., Chen, T., Han, C., Li, N., Wan, T. and Cao, X. (2004) Rab7b, a novel lysosome-associated small GTPase, is involved in monocytic differentiation of human acute promyelocytic leukemia cells. Biochem. Biophys. Res. Commun. 318, 792–799.
[18] Collins, S.J., Ruscetti, F.W., Gallagher, R.E. and Gallo, R.C. (1978) Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. Proc. Natl. Acad. Sci. USA 75, 2458–2562.
[19] Schwartz, A., Fernandez Repollet, E., Vogt, R. and Gratama, J.W. (1996) Standardizing flow cytometry: construction of a standardized fluorescence calibration plot using matching spectral calibrators. Cytometry 26, 22–31.
[20] Shimaoka, M., Takagi, J. and Springer, T. (2002) Conformational regulation of integrin structure and function. Annu. Rev. Biophys. Biomol. Struct. 31, 485–516.
[21] Warfel, J.M., Steele, A.D. and D'Agnillo, F. (2005) Anthrax lethal toxin induces endothelial barrier dysfunction. Am. J. Pathol. 166, 1871–1881.
[22] Kirby, J.E. (2004) Anthrax lethal toxin induces human endothelial cell apoptosis. Infect. Immun. 72, 430–439.
[23] Pandey, J. and Warburton, D. (2004) Knock-on effect of anthrax lethal toxin on macrophages potentiates cytotoxicity to endothelial cells. Microb. Infect. 6, 835–843.
[24] Paddle, B.M., Wong, V.K. and Muller, B.D. (2006) The cytotoxic effect of anthrax lethal toxin on human lung cells *in vitro* and the protective action of bovine antibodies to PA and LF. J. Appl. Toxicol. 26, 162–168.
[25] Bradley, K.A., Mogridge, J., Rainey, G.J.A., Batty, S. and Young, J.A.T. (2003) Binding of anthrax toxin to its receptor is similar to alpha integrin–ligand interactions. J. Biol. Chem. 278, 49342–49347.
[26] Nanda, A., Carson-Walter, E.B., Seaman, S., Barber, T.D., Stampfl, J., Singh, S., Vogelstein, B., Kinzler, K.W. and St. Croix, B. (2004) TEM8 interacts with the cleaved C5 domain of collagen alpha 3(VI). Cancer Res. 64, 817–820.
[27] Kassam, A., Der, S.D. and Mogridge, J. (2005) Differentiation of human monocytic cell lines confers susceptibility to *Bacillus anthracis* lethal toxin. Cell. Microbiol. 7, 281–292.