Respiratory:
EOSINOPHIL - INFLAMMATION - ADHESION ASSAY

INTRODUCTION

Cellix Ltd. has developed a novel Microfluidic Platform consisting of a PC-controlled Nanopump with microfluidic biochips and DucoCell analysis software. The Nanopump enables very accurate flow rates to be achieved which are more reproducible and consistent compared to anything currently available. Importantly, flow rates are extremely low (5 pL min^{-1} to 10 µL min^{-1}) and the shear stress levels that the pump can mimic (up to 30 dyne cm^{-2}) are equivalent to those found in blood vessels *in vivo*. The Vena8 biochips are comprised of eight microcapillaries, and are manufactured such that the dimensions of the capillaries are similar to the blood vessels being assessed. Currently, the capillaries may be coated with recombinant human adhesion proteins for use in inflammation studies. The Nanopump is vital to the use of small diameter capillaries as standard syringe pumps are incapable of delivering the required low flow rates.

OVERVIEW

First of all, the cell type to be analysed must be determined. This is followed by establishing how to harvest such cells e.g. culturing in growth media, or isolation from *in vivo* fluids. Secondly, the assay itself should be outlined, including what proteins will be used to coat the capillaries of the biochip. Thirdly, the adhesion profile of the cells to be passed through the coated capillary should be determined. Next, if exogenous compounds are being analysed, these should then be introduced to the system and their effect on the adhesion profile assessed. This should include calculation of required concentrations and pre-incubation conditions, before introduction to the system. Finally, the images taken via the digital camera attached to the microscope should be masked and analysed using the Ducocell software.

ASSAY DEVELOPMENT STEPS

i) Choice of cell type and harvesting protocols

A microfluidic assay assessing the effect of levocetirizine on human eosinophil adhesion was developed. Human eosinophils were obtained from blood donated by normal volunteers, or individuals with mild allergic disease who were not taking any medication and who gave informed consent (eosinophilia < 0.5 x 10^6 eosinophils ml^{-1}).

Eosinophils were purified by a standard technique using dextran sedimentation and centrifugation on Percoll gradients, followed by CD16-dependent negative immunomagnetic selection as described (Blaylock *et al*, 1999). Using this method, eosinophils with a purity of at least 99% were obtained, with greater than 98% viability as assessed by trypan blue exclusion.

ii) Assay outline, including Vena8 biochip coating procedures

Each microcapillary was coated for one hour in humid conditions at ambient temperature with either rhVCAM-1 or BSA (both 10 µg mL^{-1} in HBSS containing Ca^{2+} and Mg^{2+}). All capillaries were then coated with BSA to occupy non-specific binding sites. Resting or GM-CSF–treated eosinophils were pre-incubated at 37°C in a water bath for 10 mins before incubation with/without levocetirizine (0.1 nM - 100nM), with anti-VLA-4 mAb as a positive control) for a further 20 mins.

iii) Adhesion profiles

Eosinophils were infused into the capillaries at stepwise increases in shear stress, from 0 to 5 dyne cm^{-2}, one minute per shear stress level. Images at each shear stress level were captured using the accompanying PixeLINK microscopy software. For experiments with GM-CSF-stimulated (1 ng mL^{-1}) eosinophils, the cytokine was added to the warmed cells at the same time as levocetirizine and incubated at 37°C for 20 mins prior to commencing the flow assay. Adhesion was evaluated by monitoring eosinophil migratory behaviour in real time with images captured via a digital camera connected to the microscope.

iv) Image analysis

Three images per shear stress level were captured (see figure 1 for examples), and adhered eosinophil numbers were recorded using Ducocell application software. Data was exported into Excel for interpretation. Statistical significance was determined by Students unpaired *t*-test, and $P>0.05$ was considered statistically significant. Data was presented as mean±s.e.mean.

cellix

Cellix Ltd., Unit 3.81,
Institute of Molecular Medicine,
Trinity Centre for Health Sciences,
James's Street, Dublin 8, Ireland.

Tel: +353 (0)1 896 3182
Fax: +353 (0)1 896 2771
URL: www.cellixltd.com
Email: info@cellixltd.com

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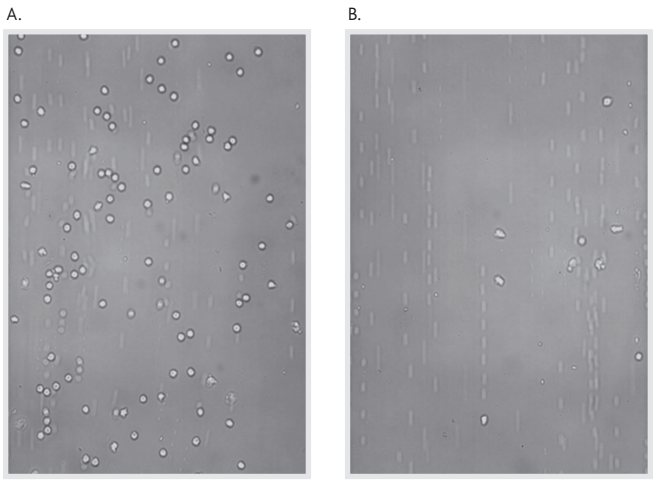

Figure 1: Microscope images (20x magnification) showing resting eosinophil adhesion to (a) rhVCAM-1 (10 µg ml^{-1}) and b) BSA (10 µg ml^{-1}), both at 1 dyne cm^{-2}. Cells were pre-incubated in Ca^{2+}/Mg^{2+}-containing HBSS for 30 mins at 37°C, including 20 mins pre-incubation with inhibitors where necessary, before being passed through the biochip capillary (400 µm wide, 100 µm deep).

RESULTS

We examined the effect of a concentration range of levocetirizine on GM-CSF-stimulated (10 ng mL^{-1} - optimal concentration established in preliminary experiments) eosinophil adhesion under flow to rhVCAM-1. Pre-incubation of eosinophils with GM-CSF increased the adhesion level by more than 50%, and also rapidly generated significantly more flattened cells at both the 5 and 15 min time-points. Levocetirizine significantly inhibited GM-CSF stimulated eosinophil adhesion to VCAM-1 and the ability of eosinophils to flatten on rhVCAM-1, in a dose-dependent manner, at both 5 min and 15 min. The data are summarized in Figure 2, with optimal inhibition of adhesion observed at a levocetirizine concentration of 10^{-8} M with an EC_{50} of 10^{-9} M. However, a greater effect on GM-CSF-dependent eosinophil flattening was observed at 15 min. Pre-treatment of GM-CSF-stimulated eosinophils with a CD11b mAb had no measurable effect (data not shown).

ASSAY CONDITIONS

The following solutions were used during the above procedure:

Eosinophil Isolation Media	Eosinophil Adhesion Media
HBSS without Ca^{2+} and Mg^{2+}	HBSS without Ca^{2+} and Mg^{2+}
10% FCS	10% FCS
100U penicillin/0.1mg streptomycin	100U penicillin/0.1mg streptomycin

Note: Incubate eosinophils at 5 x 10^6 ml^{-1} in the adhesion media for at least 30 mins before commencing the assay. Also, dissolve eosinophil stimulants/inhibitors in this media.

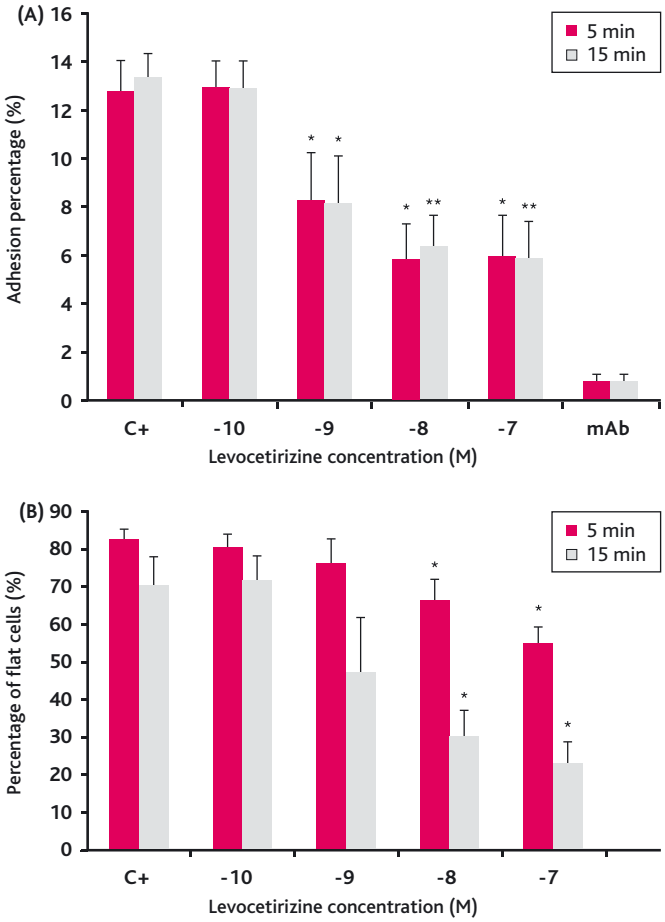

Figure 2: (a) Dose response of the effect of levocetirizine on granulocyte macrophage-colony stimulating factor (GM-CSF)-stimulated eosinophil adhesion to rhVCAM-1 under flow at 5 and 15 min after the start of the flow assay. Data are from cell counts of 50 fields of view for each microchannel; (b) effect of increasing concentrations of levocetirizine on GM-CSF-dependent eosinophil flattening between the 5 and 15 min time-points of the flow assay. In a and b, each bar represents the mean±SEM (standard error of mean) of at least four independent experiments. mAb denotes anti-VLA-4. *P<0.05, **P<0.005 vs. GM-CSF-stimulated cells (C+).

REFERENCES

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Cellix Ltd., Unit 3.81,
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