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Application Note I200

Inflammation: Leukocyte Adhesion; Quantifying Adhesion; Inhibition, Stimulation

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Objectives

To analyse the role of different adhesion molecules and chemokines involved in various stages of inflammation under physiological flow conditions using Cellix's VenaFlux microfluidic platform.

Introduction

Inflammation is an adaptive response that is triggered by noxious stimuli and conditions, such as infection and tissue injury. Inflammation is traditionally defined by the four Latin words *calor*, *dolor*, *rubor* and *tumor*, as firstly described by the Roman Aulus Cornelius Celsus in its *De Medicina*, meaning heat, pain, redness and swelling, as effects if the action of cytokines and other inflammatory mediators on the local blood vessels.

Cytokines have important effects on the adhesive properties of the endothelium, causing circulating leukocytes to adhere to the endothelial lining of the blood vessel wall before transmigrating to the site of inflammation.

The traditional three-step leukocyte adhesion cascade involves selectin-mediated rolling, chemokine-triggered activation and integrin-dependent arrest; followed by intraluminal crawling and paracellular and transcellular migration, and finally migration through the basement membrane.

Selectins are a family of single-chain transmembrane glycoproteins, expressed on the surface of leukocytes, platelets and activated endothelial cells. E-selectin, also known as CD62E, is a cell adhesion molecule expressed only on endothelial cells activated by cytokines. E-selectin recognizes and binds with low affinity to sialylated carbohydrates present on the surface proteins of certain leukocytes, causing the cells to roll along the endothelial surface of the blood vessel, as temporary interactions with exceptionally high on- and off-rates are established.

As the inflammatory response progresses, chemokines released by injured tissue enter the blood vessels and activate the rolling leukocytes, which are now able to tightly bind to the

endothelial surface, an essential step prior to transmigration towards the inflamed tissue.

Chemokines cause surface integrins to switch from a low-affinity state to a high-affinity state. In the activated state, integrins bind tightly to complementary receptors expressed on endothelial cells. This promotes the firm adhesion of the leukocytes through integrin-mediated binding, such as VLA-4 that binds to VCAM-1, or LFA-1 to ICAM-1.

Monocyte chemoattractant protein (MCP-1) is an essential chemokine involved in monocyte rrafficking, together with its receptor CCR2, and a major chemoattractant for monocytes to inflamed endothelial cells.

Cellix's VenaFlux platform enables in vitro cell-based flow assays to study adhesion systems under physiological shear stress conditions, given the importance of shear stress in many steps of the adhesion cascade.

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Keywords

Inflammation, cell-based flow assay, cell adhesion, rolling, adhesion molecule, chemokine.

Materials

Proteins and antibodies:

- rhVCAM-1
- rhICAM-1
- rhE-selectin
- rhTNFα
- rhCCL2/MCP-1
- Anti human VCAM-1 mAb
- Anti human ICAM-1 mAb
- Anti human E-selectin mAb all from R&D;

Cells:

- THP1-monocytic cell line, from ECACC.
- Monocytes, PBMC, isolated from healthy donors.
- HUVEC, isolated from patients giving written consent.

Methods

1. Vena8 Fluoro+ Biochip Experiments

Biochips were coated with 10 µg/ml of recombinant protein overnight, at 4°C. Incubation with monoclonal antibodies was for 1 h, RT. Flow assays were performed at 0.5 dyne/cm^2 for 3 min.

2. VenaEC Experiments

HUVECs were seeded at 4 x 10^5 cell/cm^2 and grown in static conditions for 48 h prior to flow experiments.

Stimulation with TNFα (50 ng/ml) was for 4 h, 37°C. Incubation with monoclonal antibodies was for 1 h, 37°C: anti-VCAM-1 mAb, 10 µg/ml; anti-E-selectin mAb, 10µg/ml; all Abs, 15 µg/ml (anti-VCAM-1, anti-ICAM-1, anti-E-selectin mAbs, 5 µg/ml each). Flow assays were performed at 0.5 dyne/cm^2 for 5 min. Cell suspensions were always at a density of 2 x 10^6 cells/ml.

3. Image Analysis

All images acquired during flow assays were analysed with Image Pro Premier analysis software.

4. Statistical Analysis

All data are represented as mean±SEM (n=3÷5). Statistical analysis was performed with GraphPad Prism®5. ns: $p>0.05$; *: $0.01<p<0.05$; **: $0.001<p<0.01$; ***: $p<0.001$.

Results and Discussion

Adhesion to rhVCAM-1 under physiological flow conditions was studied with Vena8 Fluoro+ biochips, with respect to THP1, monocytes and PBMCs. The level of cell adhesion and the effect of PMA stimulation (50 µg/ml, 3h) are shown in Figure 1.
Figure 2 refers to the same three cell types with regard to rolling on rhE-selectin.
Adhesion of THP1 to rhVCAM-1 was studied further assessing the specificity of the binding and blocking the adhesion with an anti-VCAM-1 mAb (Figure 3). The same study was carried out in relation to E-selectin (Figure 4), and data show a specific interaction between cell receptors and purified proteins, with a complete inhibition at a concentration of 50 µg/ml of

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antibody in the case of E-selectin. Controls were performed coating the channels with the antibodies, and no adhesion was detected.

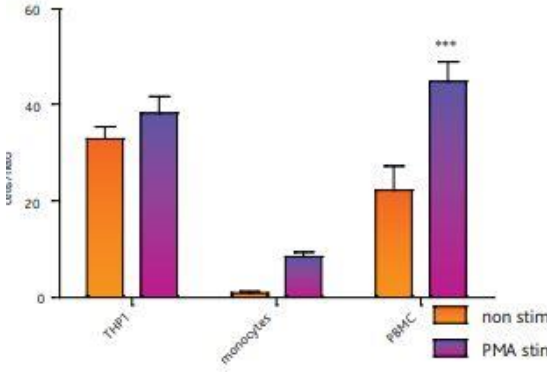

Figure 1

Figure 1: THP1, monocytes and PBMC adhesion to VCAM-1. Adhesion of non-stimulated and PMA stimulated (50ng/ml) THP1, monocytes and PBMC was studied on rhVCAM-1 (10µg/ml).
To assess the statistical difference, two-way ANOVA was performed, followed by Bonferroni posttest to look at the effect of PMA stimulation for each of the cell types.

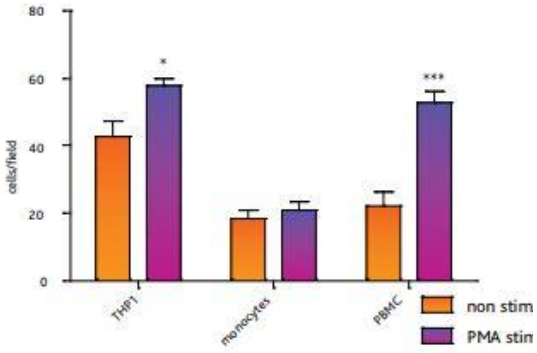

Figure 2

Figure 2: THP1, monocytes and PBMC rolling on E-selectin. Rolling of non-stimulated and PMA stimulated (50 ng/ml) THP1, monocytes and PBMC on rhE-selectin (10 µg/ml).

To assess the statistical difference, two-way ANOVA was performed, followed by Bonferroni posttest to look at the effect of PMA stimulation for each of the cell types.

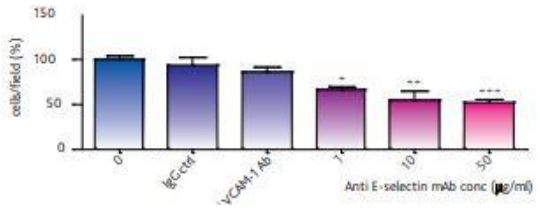

Figure 3

Figure 3: Adhesion blockade to VCAM-1. The inhibition profile of THP1 was measured using an anti-VCAM-1 mAb to block the binding site on VCAM-1, which coated the microchannels.

To assess the statistical difference, one-way ANOVA was performed, followed by Dunnett's posttest to compare all columns to IgG control.

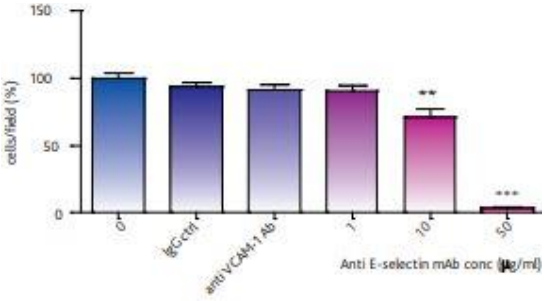

Figure 4

Figure 4: Adhesion blockade to E-selectin. The inhibition profile of THP1 rolling on rhE-selectin was measured using an anti-E-selectin mAb.

To assess the statistical difference, one-way ANOVA was performed, followed by

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Dunnett's posttest to compare all columns to IgG control.

Cell–cell interaction can be studied with VenaEC biochips, which allow for the culture of endothelial cells on the bottom plane of the microchannel. All the experiments presented here used HUVECs ranging between passage 1 and 4. An initial characterization of this cell type through flow cytometry analysis was performed, showing a very low basal level of adhesion molecule expression, which can be greatly increased with TNFα stimulation (Figure 5). This difference in adhesion molecule expression levels is reflected in a different degree of adhesion of THP1 to HUVECs under flow conditions (Figure 6).

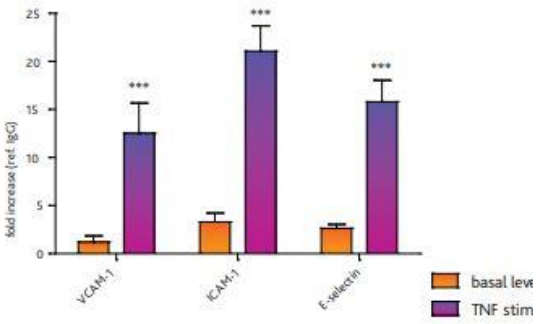

Figure 5

Figure 5: Adhesion molecule expression on HUVECs. Flow cytometry analysis was performed to assess the level of VCAM-1, ICAM-1 and E-selectin expression on endothelial cells, in non-stimulated and TNFα activated HUVECs. To assess the statistical difference between data from non-stimulated and TNFα treated samples for each adhesion molecule, unpaired t-test was performed.

The relative importance of various adhesion molecules was then studied Application Note I200 blocking them with specific antibodies (Figures 7 & 8). The sum of the individual contributions of single antibodies doesn't equal the effect of all the antibodies used together, suggesting a compensatory effect when only one ligand is blocked, or the presence of a critical ligand cluster size for cell adhesion. MCP-1 stimulation was also investigated, which demonstrated to have a much stronger effect in the case of PBMCs isolated from blood compared to THP1 cell line. These data support the hypothesis of MCP-1 priming PBMCs for increased adhesion to inflamed endothelium under physiological flow conditions. Isolation of the different cell populations within PBMCs would be required to assess at which extent their adhesion is affected by MCP-1 stimulation. The VenaFlux platform, with its accurate control over shear stress and integrated image acquisition, together with Vena8 Fluoro+ and VenaEC biochips, proves to be an important tool for researchers to investigate inflammation related processes under physiological flow conditions, closely mimicking the in vivo microenvironment.

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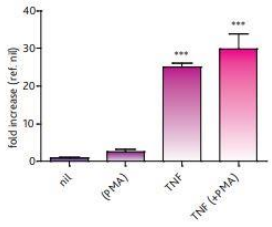

Figure 6A

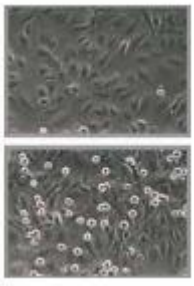

Figure 6B

Figure 6: THP1 adhesion to HUVECs. Non-stimulated and PMA stimulated (50 ng/ml) THP1 adhesion to HUVECs, either treated or not with TNFα (50 ng/ml), was measured under physiological flow conditions.

To assess the statistical difference, one-way ANOVA was performed, followed by Dunnett's posttest to compare all columns to control (nil) (Fig.6A).

Fig.6B shows THP1 on non-treated HUVECs (top), and THP1 on TNFα stimulated HUVECs (bottom).

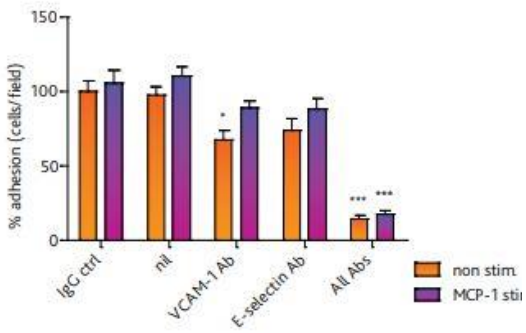

Figure 7

Figure 7: THP1 adhesion to TNFα stimulated HUVECs. Different adhesion molecules were blocked using specific antibodies: anti-VCAM-1 mAb (10 µg/ml), antiE-selectin mAb (10 µg/ml), anti ICAM-1 mAb (10 µg/ml, data not shown: no effect was detected), all Abs (anti-VCAM-1, antiE-selectin, anti-ICAM-1 Abs, 5 µg/ml each). The effect of MCP-1 stimulation (50 ng/ml) is also shown for each condition.

Two-way ANOVA assessed a non-significant effect of MCP-1 stimulation ($p>0.05$).

With regard to adhesion blockade, one-way ANOVA was performed to statistically analyse the effect of the different antibodies. For the non-stimulated series, anti-VCAM-1 mAb and all Abs showed a significant difference compared to IgG control. For the MCP-1 stimulated series, only the all Abs treatment exhibited a statistically significant difference compared to IgG control.

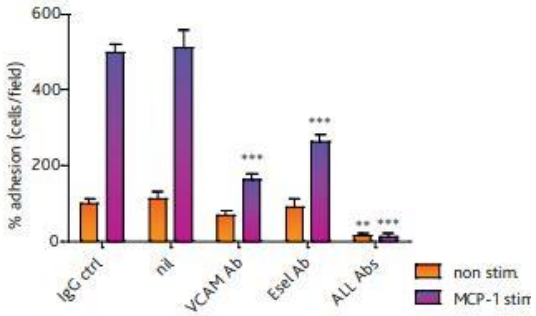

Figure 8

Figure 8: PBMC adhesion to TNFα stimulated HUVECs. Different adhesion molecules were blocked using specific antibodies: anti-VCAM-1 mAb (10 µg/ml), antiE-selectin mAb (10 µg/ml), anti ICAM-1 mAb (10 µg/ml, data not shown: no effect was detected), all abs (anti-VCAM-1, antiE-selectin, anti-ICAM-1 Abs, 5 µg/ml each). The effect of MCP-1 stimulation (50 ng/ml) is also shown for each condition.

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Two-way ANOVA assessed an extremely significant effect of MCP-1 stimulation ($p<0.0001$).

With regard to adhesion blockade, one-way ANOVA was performed to statistically analyse the effect of the different antibodies. For the non-stimulated series, only all Abs treatment showed a significant difference compared to IgG control.

For the MCP-1 stimulated series, anti-VCAM-1 mAb, anti-E-selectin mAb and all Abs exhibited a statistically significant difference compared to IgG control.

Acknowledgements

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References

1. R Medzhitov. Origin and physiological roles of inflammation. Nature, 454, 428-35 (2008). 2. K Ley, C Laudanna, MI Cybulsky, S Nourshargh. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol, 7(9), 678-89 (2007).