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Application Note C300

Cardiovascular: Atherosclerosis; Monocyte Adhesion; Chemokines; Oxidized LDL

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Objectives

To investigate the role of different stimuli recognized to play an important part in atherosclerosis development (chemokines, cytokines, oxidized lipoproteins) on leukocyte adhesion to endothelial cells or purified adhesion molecules, under physiological flow conditions using Cellix's VenaFlux platform and biochips.

Introduction

Atherosclerosis is a chronic inflammatory disease that constitutes the primary cause of heart disease and stroke. It is a progressive condition characterized by the gradual accumulation of lipid material in the artery wall.[1]

Traditionally atherosclerosis was considered a simple lipid disorder, but now inflammation is recognized as an important factor in all stages of the disease, from its genesis to plaque rupture and associated thrombotic complications.

Fatty streaks, the hallmark of early atherosclerotic lesions, are composed of lipid-laden macrophages called foam cells, which originate from circulating blood monocytes.

Therefore, the recruitment of leukocytes, their adhesion to the arterial endothelium and subsequent migration into the intima are central events in the pathogenesis of atherosclerosis.

This process is triggered by local production of chemokines: endothelial cells that are activated by inflammatory cytokines express adhesion molecules ex novo and synthesise chemokines and lipid chemo-attractants.

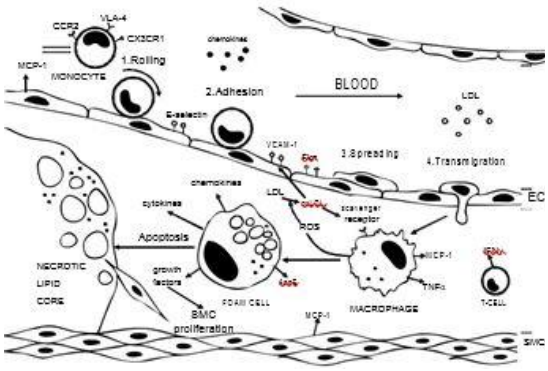

Figure 1

Figure 1: Monocytes leave the blood stream to enter the sub-endothelial tissue (transmigration), after rolling, adhering and spreading on the endothelium. This multistep process involves the joint action of numerous chemokines and adhesion molecules. Of interest in atherosclerosis are MCP-1, a potent chemo-attractant for monocytes, and Fractalkine, which may act both as an adhesion molecule and a soluble chemokine. After transmigration, monocytes differentiate into macrophages, and the continuous uptake of oxidized LDL via scavenger receptors leads to the formation of foam cells, which will gradually build up the necrotic lipid core typical of atherosclerotic lesions.

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The responsiveness of a leukocyte to a chemokine is determined by its set of chemokine receptors. Chemokine binding

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activates a signal transduction cascade that leads to various effects, such as integrin activation and subsequent cell adhesion.

Monocyte chemoattractant protein-1 (MCP-1/CCL2) has been indicated as a key player in the recruitment of monocytes from the blood stream into early atherosclerotic lesions and may be involved in other processes leading to thrombotic events.

MCP-1 has been shown to be present in macrophage-rich atherosclerotic plaques in humans. Furthermore, studies reported that MCP-1 receptor CCR2 expression is greatly enhanced in monocytes from hypercholesterolemic patients compared with normal controls. Inducers of MCP-1 expression include oxidized low-density lipoprotein and shear stress, two well documented atherogenic stimuli.

Increasing relevance in different steps of atherogenesis is being directed to Fractalkine (Fkn/CX3CL1), the only known member of the CX3C chemokine family. It consists of a transmembrane domain and an extended mucin-like stalk with a chemokine domain on top. Fkn is bound directly to the cell membrane, but a soluble form also exists through cleavage with an endogenous protease, and this form exhibits chemoattractant activity for T cells, monocytes and NK cells. The full-length cell-bound chemokine promotes strong adhesion of leukocytes to activated endothelial cells under physiological flow conditions.

High levels of Fkn expression have been observed in human arteries with atherosclerotic lesions, adding evidence to its potential role in atherogenesis and cardiovascular pathophysiology.

Another important factor to be considered is oxidative stress, which is caused by an excess of reactive oxygen species (ROS) or diminished antioxidant ability against them. Oxidative stress increases with atherosclerosis risk factors such as obesity, diabetes, hypertension, hyperlipidaemia, smoking, and is considered to play a key role in the pathogenesis of atherosclerosis.

An initial event in atherosclerosis is LDL (low density lipoprotein) conversion into modified LDL or OxLDL (oxidized LDL) by factors including radicals, transition metals and lipoxygenases, in the sub-endothelial microenvironment. OxLDL has been shown to upregulate adhesion molecule expression on endothelial cells, increasing the recruitment of leukocytes at the site of the atherosclerotic lesion.

It has also been reported that oxLDL, but not native LDL, induces MCP-1 production in vascular endothelial cells and smooth muscle cells.

Keywords

Atherosclerosis, inflammation, MCP-1, fractalkine, oxLDL.

Materials

Proteins and antibodies:

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- rhVCAM-1
- rhCX3CL1/Fractalkine
- rhCCL2/MCP-1
- rhTNFα
- Monoclonal anti human integrin α4
- Fluorescein-conjugated mouse anti-human VCAM-1, ICAM-1, E-selectin mAbs
- Fluorescein-conjugated mouse IgG matched isotype control all from R&D
- rhIFNγ from Sigma
- oxLDL, oxidized with copper II sulphate (LDL from human plasma) from Kalen Biomedicals.

Cells:

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

Methods

1. Vena8 Fluoro+ experiments

Vena8 Fluoro+ biochips were coated with 10 µg/ml of recombinant protein overnight, at 4°C. THP1 incubation with anti-integrin α4 mAb was for 15 min, 37°C. PBMC incubation with Fractakine (chemokine domain) or MCP-1 was for 30 min, 37°C. Flow assays were performed at 0.5 dyn/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. Incubation with oxLDL (150 µg/ml) was for 16 h, 37°C. Stimulation with TNFα (50 µg/ml) or IFNγ (500 units/ml) was for 4 h, 37°C. 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 analyzed with Image Pro Premier analysis software.

4. Statistical analysis

All data are represented as mean ± SEM (n=3 or 4). 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 & Discussion

Atherosclerosis is a complex multifactorial inflammatory disease, with signalling cascades involving different adhesion molecules, chemokines, lipids etc. However, their interactions, their independent or complimentary and synergistic roles are still unclear. Cellix's technology offers the possibility to perform a simple and physiologically relevant functional assay to study individual mechanisms or the combined effects of more stimuli, in relation to the initial steps in atherogenesis of cell recruitment and adhesion to the vascular wall.

Adhesion to VCAM-1, a major adhesion molecule expressed on inflamed endothelium, was completely inhibited when blocking the α4 integrin on THP1

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cells (Figure 2). Interestingly, adhesion was not significantly reduced when looking at adhesion to endothelial cells, even with a concentration of anti-α4 mAb 100 times higher (Figure 3). This suggests the idea that blocking adhesion to VCAM-1 only might not be enough to achieve a satisfactory decrease in leukocyte infiltration. Adhesion blockade to full-length Fractalkine was also studied using the soluble form of the chemokine to block CX3CR1 receptors on PBMCs (Figure 4).

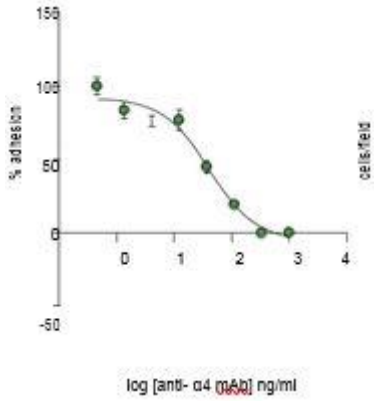

Figure 2

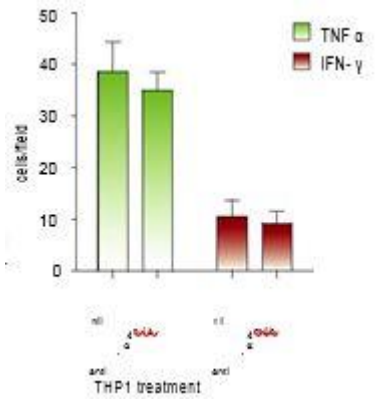

Figure 3

Figure 2: Adhesion blockade to VCAM-1. The inhibition profile of THP1 adhesion to VCAM-1 was measured using an anti-α4 mAb to block the α4β1 (VLA-4) receptor for VCAM-1. A non-linear regression was used to interpolate the data, with a hill slope of -1.076. Best fit IC50 value: 41.09 ng/ml.

Figure 3: THP1 adhesion to HUVECs. Endothelial cells were stimulated with inflammatory cytokines, either TNFα or IFNγ, and adhesion of THP1 was measured. THP1 were also treated with an anti-α4 mAb to block the integrin, but a non-significant inhibition of adhesion was shown.

To assess the statistical difference, unpaired t-test (two-tailed p value) was performed, showing a non-significant difference between the samples treated or not with the anti-α4 mAb, for both TNFα and IFNγ stimulated endothelial cells.

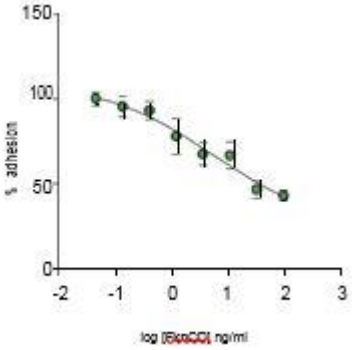

Figure 4

Figure 4: Adhesion blockade to Fractalkine (full-length). The inhibition profile of PBMCs adhesion to Fkn was measured using the soluble form of Fkn (chemokine domain) to block the CX3CR1 receptor for Fkn. A non-linear regression was used to

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interpolate the data, with a hill slope of -0.4595. Best fit IC50 value: 5.543 ng/ml.

To verify if a synergistic effect existed between VCAM-1 and the chemo-tactic cytokine MCP-1 (CCL2), we studied the adhesion of PBMCs to purified human VCAM-1 using different concentrations of the chemo-kine. An increased affinity for the binding was manifested from a more consistent adhesion at the higher concentrations of MCP-1 (Figure 5).

Different considerations should be made for Fractalkine since MCP-1 was shown to cause no effect in relation to PBMC adhesion to the immobilized chemokine (Figure 6). Consistently with studies on ApoE$^{-/-}$ mice, MCP-1 and Fkn, two important players in atherosclerosis initiation and development, were proved to have independent roles in promoting monocyte recruitment. [2] It was shown that genetic deletions of MCP-1, Fkn, or their associated receptors, CCR2 and CX3CR1 respectively, considerably reduced lesion size in murine models, but the combined genetic deletion of both MCP-1 and Fkn, on an ApoE$^{-/-}$ background, resulted in a much greater reduction of atherosclerotic lesions. All these data suggest that, from a clinical perspective, a double blocking could lead to a significant improvement in atheroprotection.

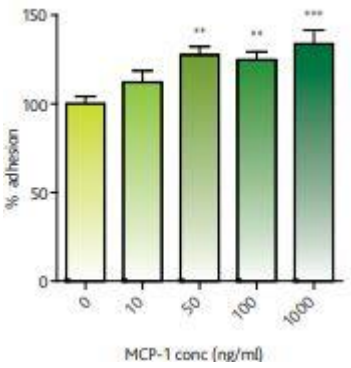

Figure 5

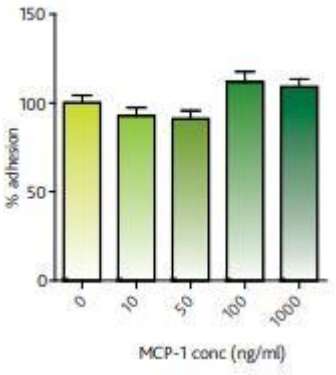

Figure 6

Figure 5: Effect of MCP-1 on adhesion to VCAM-1. A significant increase in PBMC adhesion to VCAM-1 was shown upon stimulation with MCP-1, which therefore may be involved in integrin activation and enhanced affinity in the VCAM-1/VLA-4 axis.

To assess the statistical difference, one-way ANOVA was performed, followed by Dunnett's post-test to compare all columns to control (MCP-1 concentration=0).

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Figure 6: Effect of MCP-1 on adhesion to Fractalkine. No significant effect of MCP-1 stimulation was detected in relation to PBMC adhesion to Fkn, suggesting that MCP-1 and Fkn don't act in concert, but independently promote monocyte recruitment and atherogenesis. To assess the statistical difference, one-way ANOVA was performed, followed by Dunnett's post-test to compare all columns to control (MCP-1 concentration=0).

We also found that Fkn increased PBMC adhesion to VCAM-1 (data not shown), adding evidence to the importance of this chemokine in leukocyte recruitment and lesion initiation.

In relation to oxidative stress, a central factor in atherosclerosis development, we studied the effects of oxidized LDL on adhesion molecule expression on endothelial cells and subsequent leukocyte adhesion. Three different levels of oxidation were considered, and the result of oxLDL incubation regarding adhesion molecule upregulation on HUVECs was assessed via flow cytometric analysis (Figure 7). Only the highly oxidized LDL (Hox) was shown to cause a significant increase in VCAM-1, ICAM-1 and E-selectin expression. When endothelial cells were incubated with TNFα on top of oxLDL this result was not seen.

A small but significant increase in THP1 adhesion was seen on Hox stimulated HUVECs compared to the non-treated control (Figure 8) once the monocytic cells were perfused over the endothelial layer at physiological flow conditions.

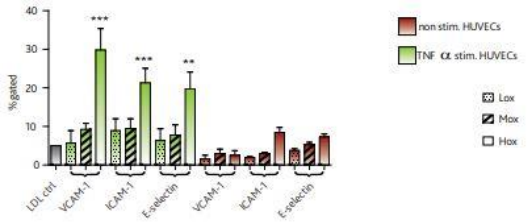

Figure 7

Figure 7: Adhesion molecule expression on endothelial cells upon stimulation with oxidized LDL. Flow cytometry analysis was performed to assess the level of VCAM-1, ICAM-1 and E-selectin expression on HUVECs, after a 16 h incubation with low-oxidized LDL (Lox), standard (medium) oxidized LDL (Mox), and high-oxidized LDL (Hox), in the presence or not of TNF α. Only Hox stimulation without TNF α induced a significant upregulation of adhesion molecule expression. The reference is HUVECs treated with native LDL, or native LDL plus TNF α. To assess the statistical difference, two-way ANOVA was performed, followed by Dunnett's post-test to compare all columns to LDL control.

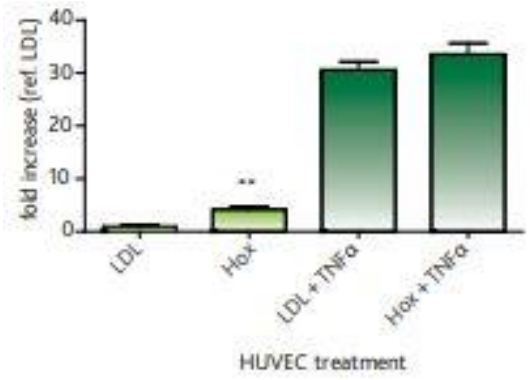

Figure 8

Figure 8: THP1 adhesion to endothelial cells: effect of oxLDL. HUVECs were treated with native LDL or high-oxidized LDL (Hox), with or without co-stimulation with TNF α. The upregulation of adhesion molecule expression in endothelial cells due to oxLDL is much lower

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in comparison to TNF α effect, but anyway it corresponded to a small but significant increase in THP1 adhesion. To assess the statistical difference, unpaired t test (two-tailed p value) was performed.

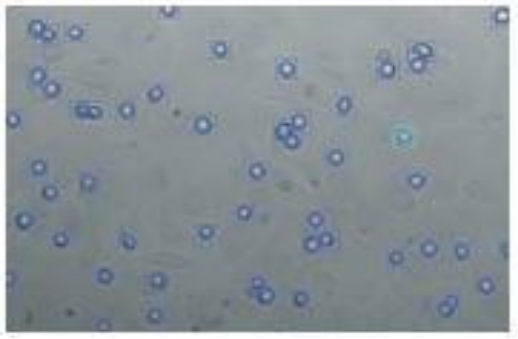

Figure 9

Figure 9: Detection of THP1 adhesion to TNF α stimulated endothelial cells. HUVECs were seeded on a VenaEC biochip, cultured for 48h in static conditions and then treated with TNF α 4 h prior to flow assay. THP1 (2 x 10^6 cells/ml) were perfused over the endothelial layer for 5 min at 0.5 dyn/cm^2. Detection was performed with Image Pro Premier analysis software, which returned the cell count and other relevant shape parameters.

Conclusion

These in vitro studies, closely mimicking the human in vivo vascular microenvironment, represent a useful model to dissect different mechanisms involved in atherogenesis. This allows for a better design of more focused experiments involving animal models, and for a more realistic prediction of the responses in humans, considering the difficulty in engaging into clinical trials, due to the dramatic end-points of the disease (such as myocardial infarction, stroke or death), and the lack of specific markers.

Acknowledgements

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References

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