Laminin-332 Regulates Expression of CC chemokine ligand 7 and 20 in Human Umbilical Vein Endothelial Cells

Laminin-332 Reglerar Uttryck av CC-kemokinligand 7 och 20 i Humana Venösa Endotelceller från Navelsträng

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ABSTRAKT

Celler som täcker kroppens inre och yttre ytor kallas för epitelceller. Endotelceller är specialiserade epitelceller som bland annat bekläder insidan av blodkärlen. Endotelet är förankrat till basalmembranet via molekyler kallade lamininer. Vid en akut inflammation kan lamininer binda till leukocyter för att de ska kunna ta sig ut till den inflammerade vävnaden. Kemokiner är molekyler som attraherar leukocyter och som kan produceras av endotelcellerna. I denna rapport utforskas vilken påverkan som laminin-332 har på endotelcellers genuttryck för kemokinerna CCL7, CCL8, CCL20, CXCL6 och CXCL10. En tidigare utförd analys för proteinuttryck som gjorts under samma förhållanden visade en uppreglering av samtliga kemokiner, med undantag för CCL8 som blev nedreglerad. Analysen för proteinuttryck var utförd med Olinks Proximity Extension Assay och analys för genuttryck utfördes med qRT-PCR. Resultaten visade att genuttrycket för CCL8, CXCL6 och CXCL10 var för lågt för att detekteras med den valda metoden. Genuttryck för CCL7 och CCL20 var detekterbart och visade båda en uppreglering av genuttryck vilket överensstämde med resultatet från studien som analyserat proteinuttrycket. Detta ledde till slutsatsen att stimulans med laminin-332 uppreglerar uttryck av mRNA, proteinproduktion och proteinsekretion i humana venösa endotelceller från navelsträng för kemokinerna CCL7 och CCL20. Slutligen, utforskas involveringen av kemokinerna CCL7 och CCL20 vid inflammation och cancerassocierade sjukdomar samt vilken roll de kan spela som biomarkörer vid behandling.

ABSTRACT

Cells that cover the body's inner and outer surfaces are called epithelial cells. Endothelial cells are specialised epithelial cells which, among other things, line the inside of blood vessels. The endothelium is anchored to the basement membrane through molecules called laminins. In an acute inflammation laminins can bind to leukocytes so that they can reach the inflamed tissue. Chemokines are molecules that attract leukocytes and can be synthesized by endothelial cells. This report will investigate what impact stimulation with laminin-332 on endothelial cells has on their gene expression for the chemokines CCL7, CCL8, CCL20, CXCL6 and CXCL10. A previously performed analysis for protein expression which had been performed under the same conditions revealed an upregulation of all chemokines except for CCL8, which was downregulated. The analysis for protein expression was executed with Olink's Proximity Extension Assay and analysis of gene expression was carried out with qRT-PCR. The results revealed that gene expression for CCL8, CXCL6 and CXCL10 was under the detection limit for the chosen method. Gene expression for CCL7 and CCL20 was detectable and revealed an upregulation of gene expression for both genes, which was consistent with the results from the study that analysed protein expression. This led to the conclusion that stimulus with laminin-332 upregulates mRNA expression, protein production and protein secretion in human umbilical vein endothelial cells for chemokines CCL7 and CCL20. Lastly, the involvement of the chemokines CCL7 and CCL20 in inflammation and cancer diseases is explored as well as their potential role as a biomarker for clinical treatment.

Key words: Laminin-332, chemokines, endothelial cells, gene expression, protein expression.

BACKGROUND

Endothelial cells, laminins and inflammation

Endothelial cells are specialized cells which, among other things, line the inside of blood vessels and regulate the transportation of macromolecules and blood components [1]. In blood vessels, the endothelial cells are bound to the basement membrane by laminins. Laminins are molecules which are involved in cell adhesion that consist of a family of glycoproteins with a molecular weight ranging from 400 to 900 kDa. Laminins can be produced by endothelial cells and are cross- or T-shaped heterotrimers which are made up by one α, one β and one γ chain. The different types of laminins are named after their composition of the α, β and γ chain. The name laminin-332 therefore implies that the trimer is composed of the chains α3, β3 and γ2 [2, 3].

Features following an acute inflammation is, among others, vasodilation and increased permeability of the blood vessels in the area. When vasodilation occurs the blood flow at the site slows down and becomes turbulent. Consequently, the cells in the blood flow closer to the vessel's wall, a condition required for them to be able to bind to the endothelium and migrate to the inflammation site [4]. In order for leukocyte recruitment to occur they must interact with both the endothelial junctions in the vessel's wall and with the vascular basement membrane. At this occasion, laminins on the basement membrane can act as ligands that bind to integrins on the leukocyte and thereby initiate integrin-mediated signalling [2]. The endothelial cells in blood vessels express, among others, integrin α3β1 as one of the receptors for laminins. Laminin-332 can bind to integrin α3β1 and it has been demonstrated that this binding is required for gap junctional intercellular communication between epithelial cells. Furthermore, all endothelial integrins that can bind to laminin are dynamically regulated by cytokines [5].

Proximity extension assay, PEA

Proximity extension assay (PEA) was used in a master thesis to measure relative quantities of proteins such as the chemokines CCL7, CCL8, CCL20, CXCL6 and CXCL10 following stimulation of human umbilical vein endothelial cells with laminin-332 for 48 hours [6][figure 1]. It was discovered that protein expression of the chemokines CCL7, CCL20, CXCL6 and CXCL10 was upregulated, whereas chemokine CCL8 was downregulated. The chosen method, PEA, is a multiplex targeted protein biomarker immunoassay developed by

Olink Proteomics AB (Sweden, Uppsala). With Olink Target 96, 92 proteins can be measured across 96 different samples simultaneously and yield a quantitative polymerase chain reaction (qPCR) readout. Antibodies labelled with unique DNA-sequences for each type of antibody bind pairwise to their target protein. When pair-wise binding has occurred the DNA-sequences will hybridize and be extended by a DNA-polymerase to a PCR-target sequence. The obtained sequence is then amplified using standard qPCR where the C_T-value is related to the concentration of the protein in the sample. Data is then processed and reports the relative concentration of protein in each sample [7][figure 2].

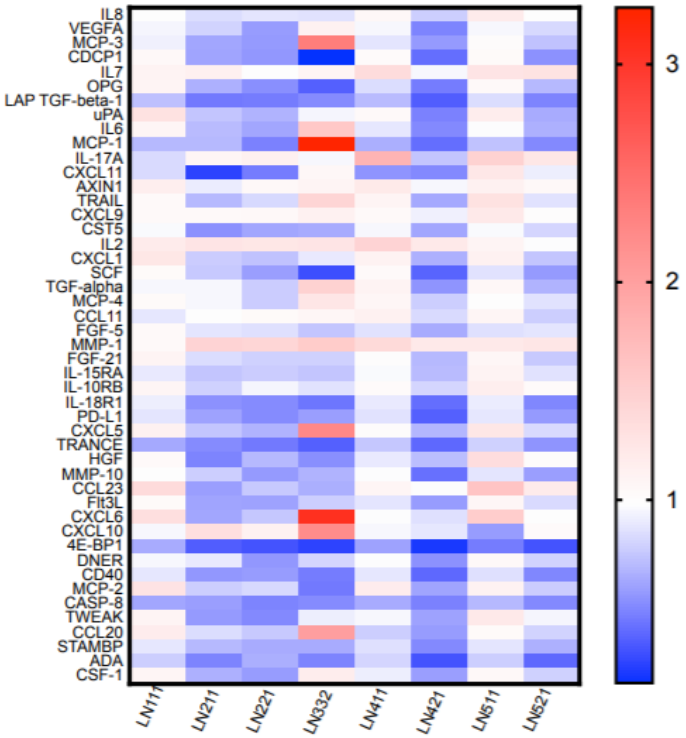

Figure 1. Relative amount of proteins, measured by OLINK's proximity extension assay (PEA), in supernatants of human umbilical vein endothelial cells (HUVECs) cultured on different laminin isoforms for 48h (n=2) [6]. Note that CCL7 and CCL8 are referred to by their alternate names, MCP-3 and MCP-2, respectively.

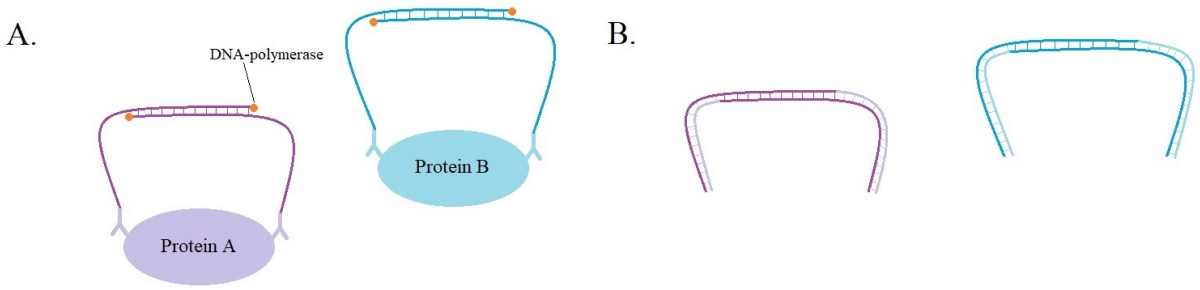

Figure 2. Principle of PEA (Proximity Extension Assay). DNA-sequence labelled antibodies bind pairwise to their complementary protein antigen. The DNA-sequences hybridize and are extended by a DNA-polymerase (A.). The extended DNA-sequences serve as a template for, and are detected with, qPCR (quantitative polymerase chain reaction) (B.).

Cytokines and chemokines

Cytokines are small proteins (usually <20 kDa) which act as carriers of molecules in the inflammation and immune system as well as enable communication between immunologic cells. There are many different cytokines and these can be divided into families which are: interleukins, colony stimulating factors, interferons, tumour necrosis factors, growth factors and chemokines. Chemokines are chemotactic cytokines that can attract and activate different types of leukocytes and have great importance regarding the migration of cells, both within and in between tissues. When inflammation occurs leukocytes are lured out of the blood vessels to the inflammation site and since there are about 50 different chemokines and about 15 chemokine receptors, a very selective recruitment of leukocytes can be carried out. Since chemokines are chemotactic they can control cell movement, a phenomenon referred to as chemotaxis where the cell movement usually follows an increasing concentration gradient of a chemotactic agent. The chemokines can be divided into, at least, three structural classes: C-chemokines, CC-chemokines and CXC-chemokines [8, 9].

Relevant chemokines in this report includes chemokines from the CC and CXC family. Namely, CCL7, CCL8, CCL20, CXCL6 and CXCL10. Chemokine CCL7 and CCL8 both attract monocytes, whereas CCL8 also attracts lymphocytes, basophils and eosinophils [10, 11]. Like CCL8, CCL20 has activity towards lymphocytes but beyond that it also attracts dendritic cells [12]. CXCL6 has stimulatory and chemotactic activity towards neutrophils, whereas CXCL10 is chemotactic towards monocytes and T-lymphocytes [13, 14].

qPCR

Real time-polymerase chain reaction or quantitative polymerase chain reaction (qPCR) is, as the name implies, a quantitative analysis in which the concentration of a target sequence in a specimen can be calculated. The technique is based on the generation and detection of fluorescence when the target sequence is amplified. The fluorescence increases with an increased amount of PCR-product and allows the detection of the PCR-product as it is made, hence the alternate name real-time PCR. Detection of fluorescence in early cycles of the PCR run indicates a higher amount of starting template than detectable fluorescence in later cycles. The generation of fluorescence in real time can be accomplished in several ways, one of which is the usage of TaqMan probes. These consist of single-stranded DNA complementary to a specific sequence in the targeted region of the PCR-template with a reporter dye covalently attached to its 5′ end and a quencher covalently attached to its 3′ end. The reporter dye fluoresces and the quencher, which can either be fluorescent or not, pulls the fluorescent energy from the reporter dye so that its fluorescent signal cannot be detected. The probe will hybridize to its target sequence which is located between the primer binding sites and Taq-polymerase will begin to extend the primer. The quencher is chemically modified so that it cannot be extended by the polymerase resulting in digestion of the probe into single and oligonucleotides with separation of the reporter dye and quencher as a consequence. Since the reporter dye and quencher are no longer in close proximity to each other the quencher can no longer pull the fluorescent energy from the reporter dye resulting in a detectable fluorescence [15][figure 3].

By running the analysis with serial fold dilutions containing known positive standards a relationship between the amount of starting template and cycle number at which fluorescence reaches a threshold amount can be established. The cycle at which the fluorescence increases over a set amount is called the threshold cycle, C_T. The produced relationship between fluorescence and C_T then allows the calculation of the amount of starting template in an unknown specimen [15].

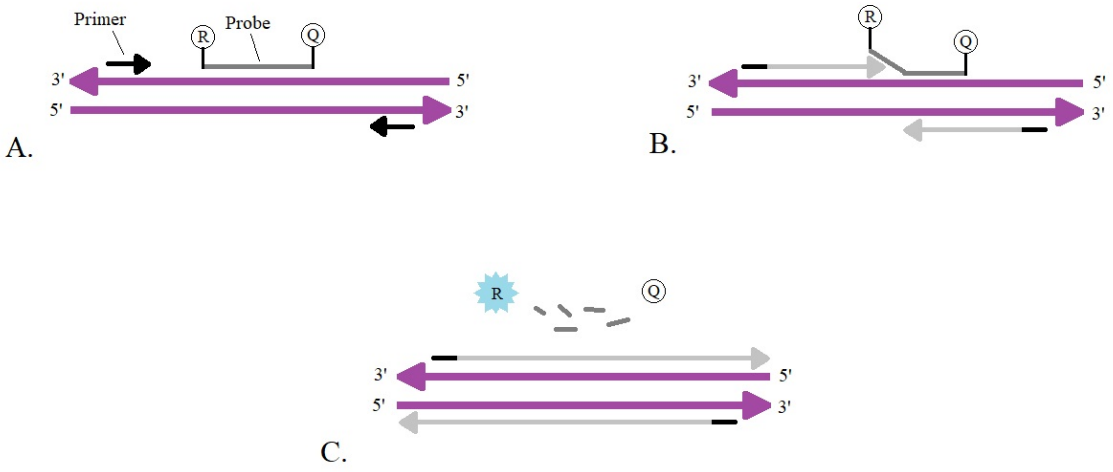

Figure 3. Principle of qPCR (quantitative polymerase chain reaction) using TaqMan probes. The TaqMan probe hybridizes to the target region in between primer-binding sites (A.). Polymerase extends the primers and uses its exonuclease activity to remove and degrade the probe (B.). As a result of the degradation of the probe, the reporter dye and quencher are now separated, allowing detection of fluorescence (C.).

RT-PCR

When using RNA as starting material for a laboratory procedure, such as a quantitative polymerase chain reaction (qPCR), it may first be converted to complementary double stranded DNA (cDNA) since this is a better starting template for amplification than single stranded RNA. This is accomplished by performing a reverse transcriptase-PCR (RT-PCR). Reverse transcriptase is an enzyme isolated from RNA-viruses which can use single stranded RNA as a starting material for synthesis of a DNA-strand and thereby produce an RNA:DNA hybrid. By denaturing the hybrid DNA-polymerase can synthesize a complementary DNA-strand resulting in a double stranded cDNA-molecule [15][figure 4].

The RT-PCR can be performed in one or two steps. When using the one-step method the reaction tube contains both reverse transcriptase and DNA-polymerase, meaning both cDNA synthesis and PCR will be performed in the same tube. In contrast, when using the two-step method one reaction tube will contain reverse transcriptase where synthesis of the RNA:DNA hybrid will take place and a separate tube will contain DNA-polymerase which will be used for ds cDNA synthesis and PCR. The method can be used for detection of microorganisms

with RNA genomes, measurement of gene expression profiles and analysing gene regions which have been interrupted by long introns, amongst other things [15].

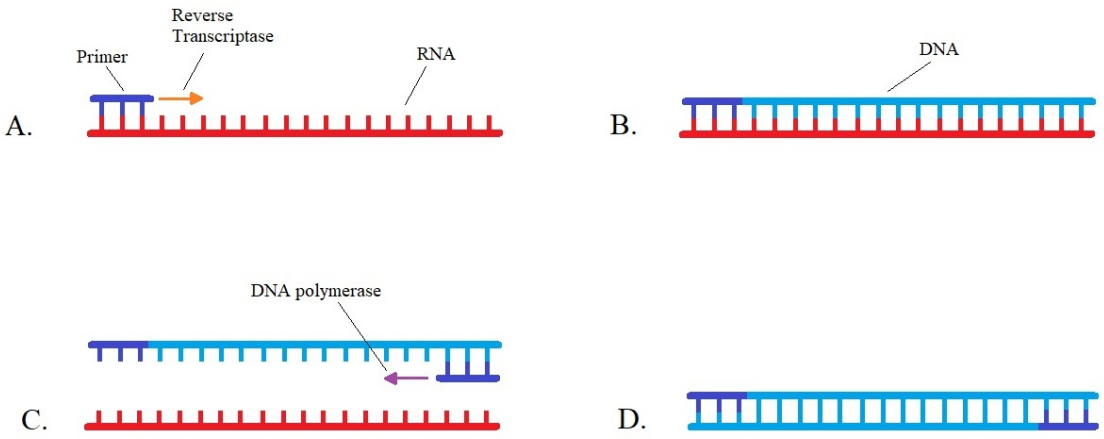

Figure 4. Principle of RT-PCR (reverse transcriptase-polymerase chain reaction). Random primers are used which hybridize to complementary RNA-sequences allowing for them to be extended by reverse transcriptase (A.). The extension of the primers by reverse transcriptase the yields an RNA:DNA hybrid (B.). Denaturation of the RNA:DNA hybrid allows binding of primers to the DNA-strand which can be extended by DNA-polymerase (C.). The extension by DNA-polymerase yields double stranded DNA as a final result (D.).

Aim

The aim of this study is to examine what impact stimulation of human umbilical vein endothelial cells with laminin-332 has on their gene expression of the chemokines CCL7, CCL8, CCL20, CXCL6 and CXCL10.

METHODS AND MATERIALS

cDNA synthesis

Starting material consisted of RNA extracted from Human Umbilical Vein Endothelial Cells (HUVECs) which had been stimulated with laminin-332 (3 µg/ml) for 2, 4, 8, 24, 48 and 72 hours. The cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, USA). Before preparation of the 2X reverse transcription master mix the kit components were allowed to thaw on ice. Master mix was prepared according to the recipe (n=1) 2.0 µl 10X RT Buffer, 0.8 µl 25X dNTP mix (100 mM), 2.0 µl 10X RT Random Primers and 1.0 µl Reverse Transcriptase to a total volume of 5.8 µl. 5.8 µl of the master mix was added to RNase/DNase free microcentrifuge tubes followed by adding 14.2 µl of RNA-sample mixed with ultra-purified water from Biopak Polisher (Sigma-Aldrich, Saint Louis, USA) to a total reaction volume of 20 µl. The RNA-samples and water were mixed so that each added sample for cDNA synthesis had an RNA concentration of 70 ng/µl. The microcentrifuge tubes were briefly centrifuged in order to spin down contents and eliminate possible air bubbles followed by being loaded onto thermal cycler UNO-Thermoblock (Analytik Jena, Jena, Germany). The cycling conditions for the samples were as follows: 25 ℃ for 10 minutes, 37 ℃ for 120 minutes, 85 ℃ for 5 minutes and then kept at 4 ℃ until storage at -20 ℃.

qPCR

The 20X TaqMan Gene Expression Assay (FAM) primer/probe used to analyse gene expression for CCL7, CCL8, CCL20, CXCL6, CXCL10 and GAPDH (Applied Biosystems, Foster City, USA) were allowed to thaw on ice. GAPDH served as a housekeeping gene used to normalize relative quantities recorded for each well. The 100X Reference Dye for Quantitative PCR (ROX) (Sigma-Aldrich, Saint Louis, USA) was diluted 1:25 with ultra-purified water from Biopak Polisher (Sigma-Aldrich, Saint Louis, USA) to a concentration of 4X. Master mix was prepared according to the recipe (n=1) 5 µl LuminoCt qPCR ReadyMix (Sigma-Aldrich, Saint Louis, USA), 0.3 µl 4X Reference Dye for Quantitative PCR (ROX), 0.25 µl 20X TaqMan Gene Expression Assay primer/probe and 3.45 µl ultra purified water from Biopak Polisher yielding a total volume of 9 µl. 9 µl of the qPCR master mix was added to a MicroAmp Fast Optical 96-Well Reaction Plate 0.1 mL (Applied Biosystems, Foster City, USA) followed by adding 1 µl of sample material. Sample material for the unknown samples came from three different experiments and were run in duplicates. Six standards,

consisting of pooled cells from the separate experiments, were run in duplicate with a serial fold dilution of 1:2 where the first dilution had a value of 1.0. Illogical values of standards were removed which for CCL7 included values for standards with dilution 0.125, 0.0625 and 0.03125, and for CCL20 included values for the standard with dilution 1.0. Negative control was run in single and consisted of ultra-purified water from Biopak Polisher. The plate was then sealed using MicroAmp Optical Adhesive Film (Applied Biosystems, Foster City, USA). Before placing the reaction plate in the qPCR machine it was briefly centrifuged in order to spin down contents and eliminate possible air bubbles. The qPCR was performed in QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, USA) with cycling conditions: 95℃ for one sec and 60℃ for 20 sec for 40 cycles with one step initialization at 95℃ for 20 sec.

Statistical analysis

The data is presented as mean±SD for three sets of experiments run in duplicate. For comparison between groups a two-sided t-test was used where a p-value <0.05 was considered to be statistically significant.

Ethical considerations

Human umbilical vein endothelial cells (HUVECs) are commercially bought. No other ethical conflicts were identified.

RESULTS

CCL8, CXCL6 and CXCL10

The qPCR runs for CCL8, CXCL6 and CXCL10 had a gene expression below the detection limit and were therefore deemed as negative due to the high C_T-values.

CCL7

A statistically significant ($p < 0.005$) upregulation of gene expression for CCL7 was seen after 48 hours of stimulation with laminin-332. No up- or downregulation of gene expression was seen after 24 hours of stimulation and no statistically significant upregulation was seen after 72 hours of stimulation [figure 5]. Data collected from samples stimulated for 2, 4 and 8 hours are not reported since no evident difference was seen in comparison to the 24 hour control.

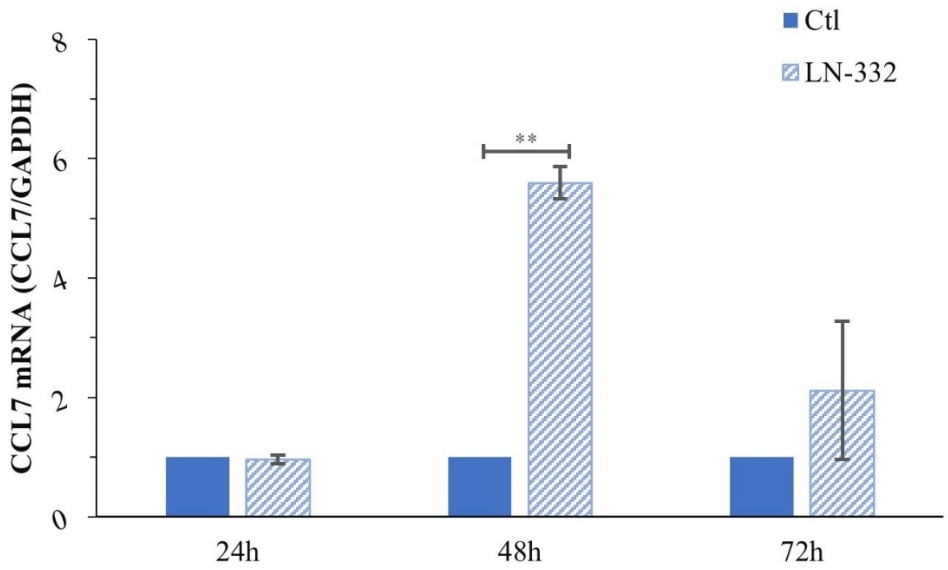

Figure 5. Relative amount of mRNA for CCL7 in human umbilical vein endothelial cells (HUVECs) exposed to laminin-332 (3 µg/ml) for 24, 48 and 72 hours (n=3). Data is shown as mean±SD. The upregulation seen after 48h was considered to be statistically significant.
**p <0.005

CCL20

A statistically significant ($p < 0.01$) upregulation of gene expression for CCL20 was seen after 48 hours of stimulation with laminin-332. No statistically significant up- or

downregulation of gene expression was seen after 24 and 72 hours of stimulation [figure 6]. Data collected from samples stimulated for 2, 4 and 8 hours are not reported since no evident difference was seen in comparison to the 24 hour control.

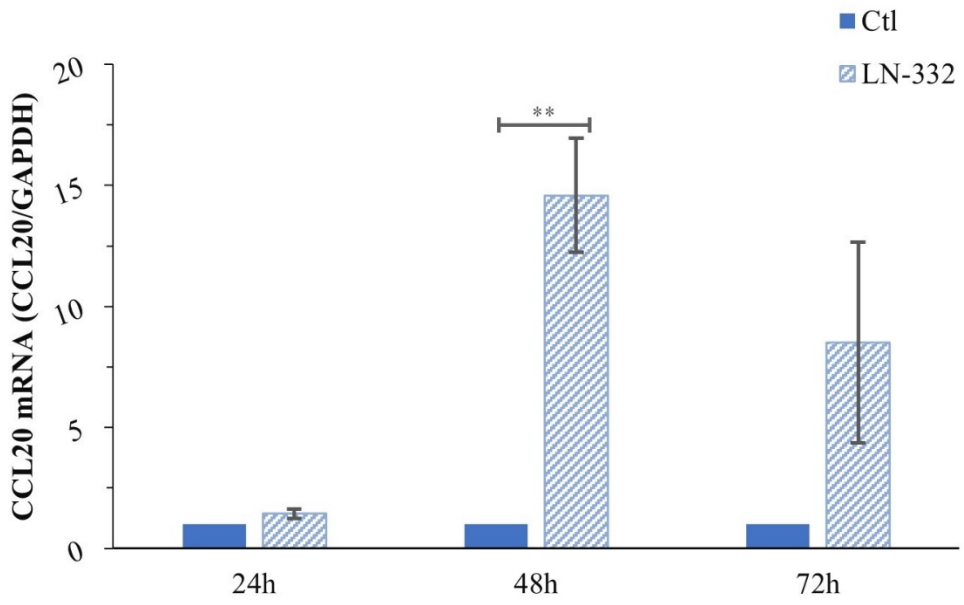

Figure 6. Relative amount of mRNA for CCL20 in human umbilical vein endothelial cells (HUVECs) exposed to laminin-332 (3 µg/ml) for 24, 48 and 72 hours (n=3). Data is shown as mean±SD. The upregulation seen after 48h was considered to be statistically significant.

**p <0.01

DISCUSSION

CCL8, CXCL6 and CXCL10

The qPCR performed with primer/probes for CCL8, CXCL6 and CXCL10 all had too high C_T-values to yield a reliable result, naming the qPCR as negative. This result does not rule out a possible up- or downregulation of the mRNA gene expression. The produced mRNA in the endothelial cells may have been produced to such a small extent for the genes in question that it cannot be detected by qPCR, even though an up- or downregulation following the stimulation may be present. To see whether an up- or downregulation is present for the genes a new qPCR may be performed where the cDNA used has been synthesized with a higher concentration of extracted RNA. Another possible explanation for the negative results is that the qPCR was unsuccessful. Something that cannot be determined since the qPCR was run without a positive control.

CCL7

Results from the qPCR run for gene expression of chemokine CCL7 in endothelial cells revealed that an upregulation was present after 48 hours of stimulation with laminin-332. When looking at the previously obtained results from the proximity extension assay an upregulation on protein level after 48 hours of stimulation with laminin-332 is also present. Since the protein levels were measured in the supernatant not in the cells this suggests that laminin-332 regulates mRNA expression and protein production, as well as protein secretion for the chemokine CCL7 [7].

CCL7 is expressed by a wide number of cell types, such as endothelial cells, and can be involved in anti-inflammatory responses by mediating recruitment of immune cells [16]. Laminins regulate both phenotype and function of endothelial cells and therefore have an indirect influence on the interactions between endothelial cells and leukocytes. It has been discovered that CCL7 is involved in tumorigenesis and is highly expressed in squamous cancer cells, which endothelial cells are a subcategory of [17, 18]. Since CCL7 is a monocyte chemoattractant an increased level of CCL7 in cancer cells can recruit monocytes to the periphery of the tumour. The recruitment of monocytes promotes their differentiation to macrophages and assists in the formation of an environment suitable for progression of carcinoma [17]. Macrophages that are recruited to the tumour microenvironment can be referred to as tumour associated macrophages and it has been found that monocytes who

differentiate to tumour associated macrophages through interaction with tumour cells are involved in the clinical prognosis. This comes as a result of their involvement in immunosuppression, migration and metastasis [17]. Since laminin-332 upregulates both gene and protein expression for CCL7 it may be a biomarker and possible therapeutic target in clinical applications to avert tumorigenesis.

CCL20

Results from the qPCR run for gene expression of chemokine CCL20 in endothelial cells following stimulation with laminin-332 revealed that an upregulation was present after 48 hours of stimulus. The previously obtained results of protein expression measured in the supernatant following 48 hours of stimulation with laminin-332 also revealed an upregulation. This suggests that laminin-332 regulates mRNA expression, protein production and protein secretion of the chemokine CCL20 [7].

Unlike many of the other CC-chemokines, CCL20 only has specificity for one receptor and the receptor only has specificity for the chemokine CCL20 [4]. The receptor is named CCR6 and is highly expressed on dendritic cells, T- and B-lymphocytes [19]. CCL20 is involved in the normal immune defence and is upregulated during inflammation [20]. Interaction with the CCR6 receptor in mice has shown to promote adhesion of monocytes to inflamed endothelium as well as regulate mobilisation, adhesion and recruitment of monocytes or macrophages to inflamed blood vessels. By inhabiting these properties CCR6 promotes atherosclerosis and targeting either CCR6 or CCL20 could serve a therapeutic purpose to ease atherosclerosis [21]. The interaction between CCL20 and CCR6 has also been suggested to play a role in tumour progression and metastasis in colorectal cancer. CCR6 has a significantly upregulated expression in colorectal cancers and in vitro studies have shown that stimulation with CCL20 promotes tumour cell proliferation. Colorectal cancer is prone to metastatic spread, especially to the liver, and colorectal cancer patients with liver metastasis express significantly higher amounts of CCL20 in the liver. These findings give the opportunity to investigate whether the interactions between CCL20 and CCR6 can be used as a target for prevention of liver metastasis in colorectal cancer [22]. Since an upregulation of CCL20 can be induced by laminin-332, a potential upregulation of laminin-332 in both atherosclerosis and colorectal cancer may be investigated and, if present, possibly be used as a target in clinical applications.

Conclusion

Stimulus with laminin-332 upregulates mRNA expression, protein production and protein secretion of chemokines CCL7 and CCL20 in human umbilical vein endothelial cells. An increased amount of CCL7 and CCL20 are suggested to play a role in pathological conditions such as tumorigenesis, atherosclerosis and colorectal cancer. Since laminin-332 upregulates both CCL7 and CCL20, it could also play a key role in the mentioned pathological conditions.

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