The Effect of *Aggregatibacter actinomycetemcomitans* Leukotoxin on Cell-to-Cell Communication in Human Monocytes

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ABSTRACT

Periodontitis is a complex and multifactorial dental disease and without proper treatment it eventually leads to the loss of teeth. Individuals with a higher number of Aggregatibacter actinomycetemcomitans in their micro-flora have a greater risk of developing periodontitis and the bacterium is associated with local aggressive periodontitis that affects younger populations. Aa produces a toxic virulent factor named Leukotoxin-A that can activate monocytes by a cellular chain reaction resulting in activation and secretion of IL-1β. This cytokine is an important pro-inflammatory key player for maintaining the metabolic balance in tissue homeostasis, which is also of great importance for the pathogenicity of periodontitis. The aim of this study is to investigate if the effect of LtxA on human monocytes involves activation of cell-to-cell (gap-junction) communication trough opening of the Connexin-43 channels (i.e. Connexons) on the target cell membrane. A human monocyte cell line, THP-1, were exposed to LtxA in combination with and without Carbenoxalene, which is an inhibitor of gap junction communication. The activity of GJC was studied by using FACS (fluorescence activated cell scanner) with the Parachute-technique. The results showed that the THP-1 cells do express Cx43 on their membrane both with and without exposure to lipopolysaccharide. LtxA induces a dose and time dependent increased activity of GJC, which was significantly reduced in presence of Cbx. These effects of LtxA and Cbx indicate a specific induction of GJC through Cx43 channels by LtxA. In conclusion, our results show that the activation of human monocytes by LtxA involves stimulation of increased GJC through connexon channels.
INTRODUCTION

The virulence mechanisms of Leukotoxin (LtxA) are of great importance in the understanding of pathogenesis of periodontitis and other inflammatory disorders. This exotoxin is one of two toxins produced by the oral bacteria Aggregatibacter actinomycetemcomitans (Aa), the other is cytolethal-distending toxin (Cdt) (Belibasakis et al., 2004; Johansson, 2011). It has recently been shown that individuals carrying Aa with high leukotoxicity have increased risk for disease progression (Höglund Åberg et al., 2014). LtxA has the ability to eliminate all subgroups of cells with hematopoietic origin and trigger a pro-inflammatory reaction by facilitating the activation and release of cytokines such as interleukin (IL)-1β from monocytes/macrophages and proteolytic enzymes from PMNs (Johansson, 2011; Kelk et al., 2011). IL-1β is a key substance for many biological processes that upholds the equilibrium in the formation and degeneration of tissue and is strongly connected to the pathogenesis of periodontitis which makes this mechanism an interesting goal for future therapeutic approaches (Dinarello, 2011). Therefore it is of great importance to fully understand the cellular and molecular mechanisms that are involved on the LtxA induced cell death.

An important mechanism for inflammatory reaction is communication between cells via gap junction, where signals transfers from one cell to another that leads to a faster and greater reaction. One of the most common gap junction proteins is Cx43 that forms connexon channels on the surface of cell membrane (Ilvesaro et al., 2000; Ransjö et al., 2003). There are five types of intercellular junctions, tight junction, adherence junctions, desmosome, hemi-desmosome and gap junctions and in the present paper the focus will be on gap junctions. Some trans-membrane proteins called connexon, which are composed of six connexin (Cx) units, join together to create tiny channels that form gaps between adjacent cell called gap junction (Ceelen et al., 2011). There are two types of channels that are similar, connexon and pannexon. They maintain cellular haemostasis and can also dysregulate this critical balance (Ceelen et al., 2011). The connexin family has about 20 isoforms on mammals. They are built identically containing four membrane-spanning domains, two extracellular loops, one intercellular loop, one cytoplasmic N-terminal tail and one cytoplasmic C-terminal tail.
Connexins are named after their weight. The most common type in human body is about 43 kDa and is called Cx43. Connexin metabolism and its function depends on interaction with other cellular proteins. These channels are responsible for the transport of ions and small hydrophilic molecules between cells. The ability of substances to migrate depends on the type of connexin that the gap junctions are made of. Some molecules such as ATP are more likely to migrate through gap junctions composed of the Cx43 type than those made up by the Cx32 type (Ceelen et al., 2011).

To precisely measure the number of cells involved in gap junctional coupling a method called the Parachute technique can be used in which the cells are stained with fluorescent dye and analysed by using Fluorescence-activated cell sorting (FACS) (Czyz et al., 2000). The green fluorescent dye Calcein-AM can be conserved within the cells but is also transferable from donor to recipient cells via gap junction interaction.

The donor cells were coloured with the dye Dil (red) to extinguish them from recipient cells. The donor cells were subsequently impregnated with Calcein-AM (green dye), in order to monitor the gap junction activity by measuring the fluorescence that emanates from each cell in a population (FACS-analyses) (Czyz et al., 2000).

It has been shown in previous studies that when Leukotoxin-A binds to the cell surface it leads to the release of ATP through membrane channels, which activates the nucleotide-receptors (P2X7R) at the surface of target cell. The involvement of P2X7R is a prerequisite for the initiation of the Caspase-1 cascade reaction that triggers the pro-inflammatory cell death (Kelk et al., 2011).

The aim of this study is to investigate if the effect of LtxA on human monocytes involves activation of cell-to-cell (gap-junction) communication through opening of the Connexin-43 channels on the target cell membrane. Our hypothesis is that Leukotoxin increases gap-junction communication by activating Cx43 channels (i.e. Connexons).
MATERIALS & METHODS

Literature

To find articles about the bacteria used in our study we used the MeSH terms; ("aggregatibacter actinomycetemcomitans"[MeSH Terms] OR ("aggregatibacter"[All Fields] AND "actinomycetemcomitans"[All Fields]) OR "aggregatibacter actinomycetemcomitans"[All Fields]) AND ("toxins, biological"[MeSH Terms] OR ("toxins"[All Fields] AND "biological"[All Fields]) OR "biological toxins"[All Fields] OR "toxin"[All Fields]) AND “periodontal” [All Fields].

Searching articles concerning cell-cell communication we used MeSH terms; ("osteoclasts"[MeSH Terms] OR "osteoclasts"[All Fields] OR "osteoclast"[All Fields]) AND ("connexins"[MeSH Terms] OR "connexins"[All Fields] OR "connexin"[All Fields]) AND ("gap junctions"[MeSH Terms] OR ("gap"[All Fields] AND "junctions"[All Fields]) OR "gap junctions"[All Fields] OR ("gap"[All Fields] AND "junction"[All Fields]) OR "gap junction"[All Fields]).

Ethical consideration

The THP-1 cell line that was used is derived from patients with AML and is commercially available for research and cannot be traced. Aa samples were previously collected from adolescents in Ghana for another study (Johansson et al., 2000) and consent was given by the parents, also ethical permission had been given by Ghana University (IRB 000 1276) and Umea University (Dnr 2010-188-31M). We had no access to personal data for any of the samples that were used and all experimental data will be used according to good scientifically praxis. Our study will hopefully lead to a better understanding and treatment of periodontitis caused by Aa-JP2 thus the advantage is greater than the impairment.
Cell culture

In the method that was selected in the series of experiment the human monocyte cell line THP-1 (TIB-202™, ATCC®, Manassas, USA) was used and quantified in Bürker’s chamber and cultured in medium RPMI1640 with 10% FBS (30-2001™, ATCC®) and was further diluted to get $0.5 \times 10^5$ cells/ml.

Cx43 expression on THP-1 cell membrane

An identification of Cx43 expression in human monocyte cell line (THP-1) was conducted in presence and absence of *Escherichia coli* lipopolysaccharides (LPS) (Sigma Aldrich, St. Louis, USA). The cells were divided into two separate tubes. LPS (100 ng/ml, stock 10 µg/ml) was added to only one of them before they were incubated in $37^\circ$C and 5% CO$_2$ for 24h.

Continuously the cells were centrifuged to a pellet by placing them into 15 ml Saarstedt-tube for 5 min at 300x g in room temperature. The pellets were diluted in 500 µl PBS with 2% fetal bovine serum (BS, Sigma Aldrich) and divided into two (2 ml) Eppendorf tubes, 100 µl cells/tube. Each tube was then divided into three smaller tubes and lastly conjugated antibody against humane Cx43 (APC mouse IgG$_{2A}$, R&D systems, Minneapolis, USA) was added tother with an isotype-antibody that acted as a negative control against Cx43. The final outcome was three tubes with added LPS and same number of tubes without LPS; both sets of tubes had the combination of 1) Cx43 antibody, 2) Isotype control and 3) No additives.

The samples were incubated in RT for 60 min, and later 1 ml PBS were put in each tube and centrifuged. The supernatant was removed and the procedure was repeated three times. The cells were suspended in 500 µl PBS for later analyses with FACS with the objective to determine if LPS had any influence on the expression and quantity of Cx43.
Analysis of gap junction communication by parachute technique

Some of THP-1 cells were divided into ten FACS-tubes, 500 µl each and were put on ice for later use as recipient cells. The remaining THP-1 cells were used as donor cells which was centrifuged to a pellet that dissolved in approximately 2 ml PBS, 18 µl DiI “red dye” (Vybrant® DiI Cell-Labeling Solution Molecular Probes®, Life Technologies, Waltham, USA) and 10 µl Calcein AM “green dye” (Calcein AM Molecular Probes®, Life Technologies) and incubated for 30 min at 37°C and 5% CO₂ in an isolated compartment.

Donor cells were washed with PBS and FBS 10% for three consecutive rounds at 200 x g, 5 min, 4°C and were diluted with 1 ml RPMI1640 (Sigma Aldrich). Later, 10 µl of the cells that were impregnated with dye (donor cells) and transferred to each of the twelve FACS-tubes (recipient cells) while on ice at a ratio of one donor cell per 25 recipient cells (Donor: Recipient 1:25). Leukotoxin (Johansson et al., 2000) was added in different concentrations at 0, 30, 100, 300 ng/ml and the tubes were incubated for 5, 15 and 30 minutes to determine the optimal setting for the induction of gap junction communication. At the end of the incubation period, the sample was put on ice in order to break the biological processes. The samples were later analysed by FACS-calibur for quantification of gap junction activity.

Effect of the gap junction inhibitor Carbenoxalene on the leukotoxin induced cell-cell communication (GJC)

A batch of ten FACS tubes was prepared with 500 µl of THP-1 cells in each used as recipient cells as described earlier. Likewise an amount of 10 µl double stained donor cells were added to each of the tubes together with different concentrations of Carbenoxalene (Sigma Aldrich) 120 µM and 240 µM while placed on ice. Lastly different concentrations of LtxA 30 ng/µl and 100 ng/µl were added. The samples were incubated at 37°C for 30 min. They were later analysed by FACS to determine if Cbx had an effect on GJC.
The experiment was repeated in replicates with constant concentration of Cbx and leukotoxin. As previously the double stained donor cells where added to 10 x FACS tubes with recipient cells waiting on ice at a ratio of Donor: Recipient 1:25. The tubes where then divided into three separate groups and a concentration of Cbx at 120 µM was added to all tubes except the controls. Subsequently they were incubated for 30 min at 37°C before the Leukotoxin 30 ng/ml was added to the same tubes. The final combination of Cbx and LtxA where left to incubate a second time for 30 min at 37°C before being analysed through FACS.

**Statistical analyses**

To reach a statistical conclusion an analysis was done in the Microsoft Excel software (Microsoft, Redmond, WA, USA) by using a one-tailed paired student t-test. The confidence interval was set at 95% (p-value <0.05). The values that were used in the t-test consisted of the results that were obtained from the last triplicate experiment. This made it possible to determine if there was a significant difference in gap-junction communication between the cells exposed to LtxA and Cbx and the non-treated cells (control).

**RESULTS**

**The expression of Cx43 on THP-1 cells**

The objectives of the first experiment were to determine if the protein Cx43 was expressed on cell membrane of THP-1 cells and whether LPS had any influence on their expression and quantity.

To acquire the accurate results a Cx43 specific antibody and an isotype (that shows if the antibody binds randomly to cell surface even if not specifically on Cx43 protein) were added to two different samples, with and without LPS.

There were no differences in cell membrane protein adhesion when comparing the THP-1 cells (control) with a sample with isotype and a one with LPS and isotype.
This assured that Cx43 specific antibody wouldn’t bind to cell membrane if there were no Cx43 proteins. The quantities of Cx43 were the same in both samples with (Fig. 1, pink line) and without LPS (Fig. 1, green line), indicating that LPS has no effect on expression of Cx43.

**The effect of different concentration and duration of Leukotoxin exposure on THP-1 cells**

The influence of different concentrations and exposure times of Leukotoxin on cell-cell communication through Connexon channels were conducted by Parachute technique with double stained THP-1 cells, which was analysed and processed in FACS-calibur. The results showed a relation between Leukotoxin and activity of Connexon channels, which transferred dyes from the donor cells to the recipient cells. Both the concentration of Leukotoxin and exposure time had an impact on the amount of dyes that were transferred between cells. It is clearly shown in Fig. 2A-B, that the effect which Leukotoxin has on the THP-1 cells over time leads to a steady increase of gap junction activity when increasing the concentration of Leukotoxin and the time of exposure. Fig. 2A illustrates the coupling between the donor cells at the upper right corner and the recipient cells at the bottom left corner that were selected by attractors analysing software. The statistical data was collected from the region close to the recipient cells (R1) where time and concentration of LtxA determines the degree of change in activity (GJC positive cells) Fig. 2B.

**Inhibition of GJC by Cbx on THP-1 cells exposed to LtxA**

Different concentration of LtxA and an inhibitor of cell-cell communication, Carbenoxaline (Cbx) were used to examine the activity of gap junction communication (GJC). Increasing the concentration of LtxA causes a steady increase in communication between cells and using a concentration of LtxA 100 ng/ml had the greatest effect, which is about four times higher compared to the control. The results show that Cbx inhibited LtxA-induced GJC, but that higher concentration of Cbx is needed at higher concentrations of LtxA (Fig. 3).
**Triplicate experiment with Cbx inhibition of LtxA-induced GJC**

To confirm previous results a new experiment was conducted with triplicate samples of a constant concentration of Ltx and Cbx. The result of this experiment confirmed that Cbx (120 µM) significantly inhibited LtxA induced (30 ng/ml) GJC which is shown in Fig. 4A. The figure shows how the GJC positive cells (black dots) increases in the analyzed area of R1 when using LtxA and how the same activity is reduced when Cbx is added. The statistical analysis (student t-test) show that the samples with LtxA and Cbx are significantly different from the control with an observed p-values of Cbx = 0.00139 and LtxA = 0.00002. Also LtxA is significantly different from LtxA + Cbx with a p-value of p=0.00033. However the LtxA + Cbx sample is not significantly different from the control with an observed p-value LtxA + Cbx = 0.11128. These results show that LtxA significantly stimulates GJC positive cells and that Cbx significantly inhibits the increased stimulation of GJC by LtxA. The inhibition of the control by CBX is also significant compared to the control (Fig 4B).

**DISCUSSION**

Previous studies have shown that Aa LtxA induces a pro-inflammatory cell death in human macrophages (Kelk et al., 2011). The cellular processes in these interactions involve ATP-release, which indicate involvements of the connexon channels. A clear observation in the present study is that gap junction communication between human immune cells can be stimulated when they are exposed to the Aa LtxA. Our observations are made in cultures of a human monocyte cell line (THP-1 cells) with documented Cx43 expression. The presence of Cx43 on inflammatory cells has been proven previously in other studies and even confirmed in our research (Ilvesaro et al., 2000; Ransjö et al., 2003). The sets of experiments that have been presented answer our hypothesis that Leukotoxin A increases gap-junction communication by activating Cx-43 channels (i.e. Connexons). The connexon channels were then blocked with Cbx, which was confirmed by the reduction of the gap junction communication that was measured through FACS analysis using the Parachute technique.
Our statistical data from the t-test also confirms our findings, however a more comprehensive research with more samples would further support our study.

This study clarifies the impact of LtxA on human monocytes, which is presented in the results and indicates that there are many factors that have an influence on the gap junction communication between the cells. We found that concentration and time of exposure of LtxA had an important effect on GJC in THP-1 cells. Our assumption is that the presence of highly leukotoxic Aa, with a confirmed pathogenic potential, involves GJC as a mechanism promoting an enhanced pro-inflammatory response. However we don’t know the exact mechanism behind this potential inflammatory reaction via GJC and more research is needed. In the present study it was possible to block the signalling between cells by Cbx and thereby stopping part of the response induced by LtxA, which is the activation of Connexon channels. It can therefore be assumed that Cbx could be a potential drug candidate for severe cases of periodontitis where Aa is involved in the pathogenesis of the disease. Whether the blockage of the cell-to-cell communication of cells has a positive or negative overall effect is yet to see. The THP-1 cells are derived from patients with acute monocytic leukemia (AML) and our findings of their communication might help us to further understand the disease.

Conclusion

LtxA significantly increases GJC in cultures of human monocytes (THP-1). The Cbx proves to be an efficient inhibitor of LtxA induced GJC however more studies are needed with healthy human cells to confirm this theory.

ACKNOWLEDGMENTS

Our gratitude’s to tutors Anders Johansson and Peyman Kelk for their supervision and contributions to this study.
REFERENCES


Fig. 1: THP-1 frequently expresses Cx-43 irrespective of exposure to E. coli LPS (100 ng/ml 24hr). Analyzed by FACS; black line shows isotype that doesn’t bind to cells. Pink line and green line shows no difference on Cx43 expression on cells stimulated with LPS (pink) and without LPS (green).
Fig. 2 A-B: The effect of different concentration and duration of Leukotoxin exposure on THP-1 cells. (A) Flow cytometric results on activity of gap junction communication. The figures illustrates FACS analysis plots of data that were processed from the second experiment where the parachute technique was used with double stained THP-1 cells which was analyzed in FACS-calibur (Calcein & DiI 530/30-nm (FL1), 575/26-nm (FL2). The region R1 represents the recipient cells and R2 the donor cells. By measuring the region R1 the activity of the recipient cells could be monitored when absorbing green color from the donor cells when stimulated with different concentrations of LtxA (0, 30, 100, 300 ng/ml) at varying times (5, 15, 30 min). (B) Comparison of data from region close to the recipient cells (R1) where time and concentration of Leukotoxin determines the degree of change in activity (GJC+ cells %).
Fig. 3: Inhibition of GJC by Cbx on THP-1 cells exposed to LtxA. Different concentrations of LtxA 30 ng/ml and 100 ng/ml together with Cbx 0, 120 and 240 µM were used and the relative results indicates that the inhibiting effect of Cbx is proportional to LtxA concentration when compared to the control. In addition higher concentration of Cbx 240 µM is needed to block about 50% effect of the induced GJC when higher concentration of LtxA 100 ng/ml is used.
Fig. 4 A-B: Cbx inhibition of LtxA-induced GJC.

Figure A: Plots showing FACS-analysis of activity of cell-to-cell communication: 
A1: Control A2: Addition of LtxA 30 ng/ml A3: Inhibition with Cbx 120 µM A4: Stimulated by LtxA 30 ng/ml and inhibited by Cbx 120 µM. All samples were incubated for 30 min in 37°C. Figure B: Showing the statistical data (student t-test) of the FACS-analysis of triplicate samples. P-values < 0.001 = ***. Mean values: LtxA 20.2, LtxA & Cbx 4.27. SD: Control 0.24, CBX 0.14, LtxA 4.20, LtxA & Cbx 4.38.