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EXPRESSION OF DEFA1-3 AND RELATIONS WITH RISK FACTORSOF CARDIOVASCULAR DISEASES

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Abstract

The infiltration of immuno-inflammatory cells is one of the earliest and durable steps that lead to atherosclerosis. These cells produce many immune components that act as a double-edged sword in this inflammatory disease. Among these components, we note antimicrobial peptides, including defenses. Defenses are natural cationic peptides of the innate immune system. In Humans, these small peptides have a large antimicrobial spectrum. In addition, they play an important role in both infectious and inflammatory diseases. Our objective was to study the relation between alpha-defenses (DEFA) 1-3 genes expression and cardiovascular risk factors. This objective was built on the hypothesis that defenses may be involved in cardiovascular pathologies, and may serve as a new generation of biomarkers. To verify this hypothesis, we treated HL-60 differentiated cells with glucose decreased alphadefenses 1, 2 and 3 gene expression levels, while insulin treatment restored its expression. These findings suggest that DEFA1-3 are involved in the complex glucose-insulin metabolic pathway. In summary, DEFA1-3 genes expression is significantly correlated with glucose. These findings we suggest that DEFA1-3 could be involved in the evolution of cardiovascular complications.

Keywords

Antimicrobial peptides, alpha-defences 1, 2 and 3, inflammation, atherosclerosis, cardiovascular diseases

EXPRESSION OF *DEFA1-3* AND RELATIONS WITH RISK FACTORS OF CARDIOVASCULAR DISEASES.

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ABSTRACT: *The infiltration of immuno-inflammatory cells is one of the earliest and durable steps that lead to atherosclerosis. These cells produce many immune components that act as a double-edged sword in this inflammatory disease. Among these components, we note antimicrobial peptides, including defenses. Defenses are natural cationic peptides of the innate immune system. In Humans, these small peptides have a large antimicrobial spectrum. In addition, they play an important role in both infectious and inflammatory diseases. Our objective was to study the relation between alpha-defenses (DEFA) 1-3 genes expression and cardiovascular risk factors. This objective was built on the hypothesis that defenses may be involved in cardiovascular pathologies, and may serve as a new generation of biomarkers. To verify this hypothesis, we treated HL-60 differentiated cells with glucose decreased alpha-defenses 1, 2 and 3 gene expression levels, while insulin treatment restored its expression. These findings suggest that DEFA1-3 are involved in the complex glucose-insulin metabolic pathway. In summary, DEFA1-3 genes expression is significantly correlated with glucose. These findings we suggest that DEFA1-3 could be involved in the evolution of cardiovascular complications.*

KEYWORDS: *Antimicrobial peptides, alpha-defences 1, 2 and 3, inflammation, atherosclerosis, cardiovascular diseases.*

1. INTRODUCTION

Antimicrobial peptides (AMPs) are considered key components of the innate immune defense system in multicellular organisms (1). They have a broad spectrum of antibacterial and antifungal activity, even antiviral. Currently, in multicellular organisms, there are more than 1000 AMPs (M. Zaiou, 2007). Among many classes of AMPs in humans, two classes are well characterized: defences and cathelicidin (M. Zaiou, 2007). The cathelicidin is a major class of AMPs in mammals. They are synthesized as *human* precursor *cathelicidin antimicrobial protein* -18 KDa (hCAP-8) (M. Zaiou, 2007). Defensins are produced in abundance by the tissues involved in body defense, such as the skin, the intestinal mucosa, and the mucous membranes of the respiratory (M. Zaiou, 2007). In humans, these peptides are divided into two groups: alpha and β-defensins (M. Zaiou, 2007).

The – defences (DEFA) are abundant in atherosclerotic plaques: a study has shown that elevated levels of DEFA deposited in the skin are associated with a high risk of cardiovascular morbidity and mortality in diabetic patients (G. Joseph, 2008). This suggests that DEFA may act as risk markers for cardiovascular diseases (CVDs) (G. Joseph, 2008). Similarly, the expression of LL-37 has been shown to be a key peptide favoured cardiovascular risk (Ciornei, 2006). Its abundance at the level of atheromatous plaques and its apoptotic activity on smooth muscle cells could lead to the weakening of the cell cluster, and facilitating the formation of thrombi under the action of blood pressure (Ciornei, 2006). A study published by Benachour et al., showed that the expression of LL-37 was associated with cardiovascular risks, such as the Body Mass Index (BMI), waist circumference, Glucose level, Blood pressure, High-Density rates Lipoprotein-Cholesterol (Benachour et al., 2009). All these results suggest that the expression of AMPs could be associated with the syndrome metabolic, which is complicated by CVDs (Benachour et al., 2009).

Since the immune system and inflammation are part of the processes that contribute to the development of CVDs, and defensins seem to be involved in the regulation of lipid metabolism, a better understanding of the functions of these peptides in this context would be possible. Thus, we intended to the association between

the expression of the genes of *DEFA1-3* and the cardiovascular risk factors; mainly glucose through establishing a cellular model for the study of the regulation of *DEFA1-3* expression.

2. METHODS

2.1 Cell Models

2.1.1 The culture of U-937 and HL-60 lines

The cells are stored at -196°C. in liquid nitrogen, in RPMI 1640 medium supplemented with 10% of the Fetal Bovine Serum (FBS) (lineage U-937) and 15% FCS (lineage HL-60) with 10% DMSO. After rapid thawing in a water bath at 37°C, the cell suspension is introduced into a 15ml flask containing RPMI 1640 medium prepared beforehand, then centrifuged for 5 min at 200g to remove traces of DMSO. The cell pellet obtained is re-suspended in the medium complete RPMI 1640 then put back in culture in flasks of 25cm^2 with a final volume 8ml of the corresponding medium. The U-937 cell line is cultivated in medium RPMI 1640 supplemented with 10%, in contrast, the HL-60 cell line is cultured in RPMI 1640 medium supplemented with 15%. Once confluent, cultured cells are sub-cultured approximately every 2 days (d) then incubated at 37°C with 5% CO_2.

2.1.2 Semi-quantitative RT-PCR analysis
a- Reverse transcription of mRNAs

200ng of total RNA is mixed with the enzyme buffer and 500ng/µl of oligodT are added.

b- PCR amplification semi-quantitative

To quantify the expression of *DEFA1-3*, the PCR is carried out in a reaction mixture containing: 0.25µM sense and antisense primer, buffer 1x $MgCl_2$, dNTPs 0.2µM, and Taq polymerase 2U/µl. The PCR was carried out in a thermocycler I- cycler and lasts 30 cycles. PCR conditions include DNA denaturation step at 95°C for 30 sec, hybridization at 63°C, and an elongation stage at 72°C for 30 sec. This protocol was used to quantify *Cluster of Differentiation* genes (*CD14), 11b* and the *18S* gene.

2.3 Statistical analyzes

Statistical analyzes were performed with the SAS software (*SAS Institute Inc*). The verification of the normality of the distribution of values of the studied parameters was realized by the calculation of the dissymmetry coefficients (Skewness). To analyze the difference between *DEFA1-3* in HL-60 cells when treated with glucose we used student t test. For all analyzes, the threshold significance was set at $P<0.05$.

3. RESULTS

In order to identify the cell line expressing *DEFA1-3*, total RNA was extracted from HL-60 and U-937 cells. Semi-quantitative RT-PCR analysis showed that *DEFA1-3* are expressed only in the HL-60 cell line and not in line U-937. Since macrophages and neutrophils contribute to the development of the inflammatory process of atherosclerosis, we have differentiated HL-60 cells into macrophages and neutrophils. We chose to treat HL-60 cells with 1, 2 and 13% DMSO to induce differentiation of pro-myeloblasts HL-60 in neutrophils (Figure 1).

A- Schematization of HL-60 cells differentiation to neutrophils when treated with DMSO. B- Morphological changes detected by May-Grunwald Giemsa staining after 4 days of treatment with 1.2% DMSO. Cells differentiated into neutrophils are smaller in size and have a segmented nucleus. C-RT-PCR analysis of the expression of the CD11b gene in HL-60 cells treated with 1.2% DMSO for 4 and 5 days, shows induction as early as 4 days to reach a maximum at 5 days after treatment. D- The RT-PCR analysis of the expression of the CD11b gene in the HL-60 cells treated with 1.3% of DMSO, shows a maximum of induction of the expression of the CD11b gene from 3 to 4 days later.
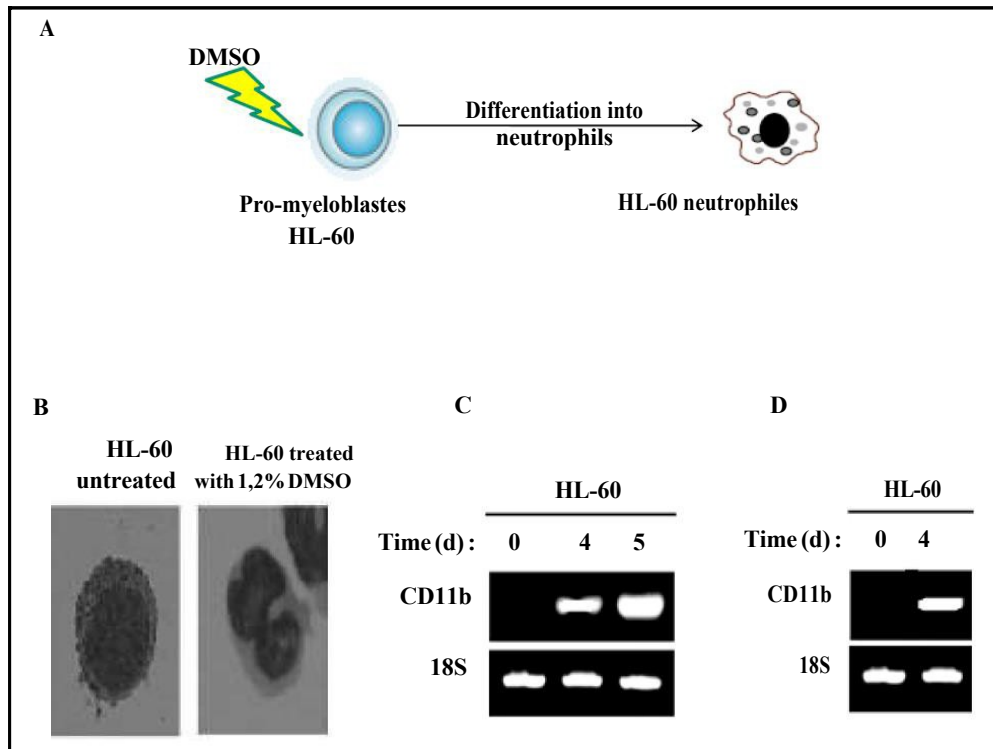
Fig 1. Analysis of the differentiation of HL-60 cells into neutrophils after treatment with dimethylsulfoxide.

To initiate the differentiation of HL-60 pro-myeloblasts into macrophages, we treated these cells with PMA, a compound known for its ability to differentiate U-937, and HL-60 into macrophages. The analysis of *DEFA1-3* expression by semi-quantitative RT-PCR showed that undifferentiated HL-60 cells consistently expressed this gene. On the other hand, we found a complete inhibition of DEFA during the differentiation of HL-60 into macrophages (not shown). As for the differentiation of HL-60 in neutrophils, it did not affect the expression of *DEFA1-3*. Expression levels similar to those found in pro-myeloblasts have been detected (Figure 2).
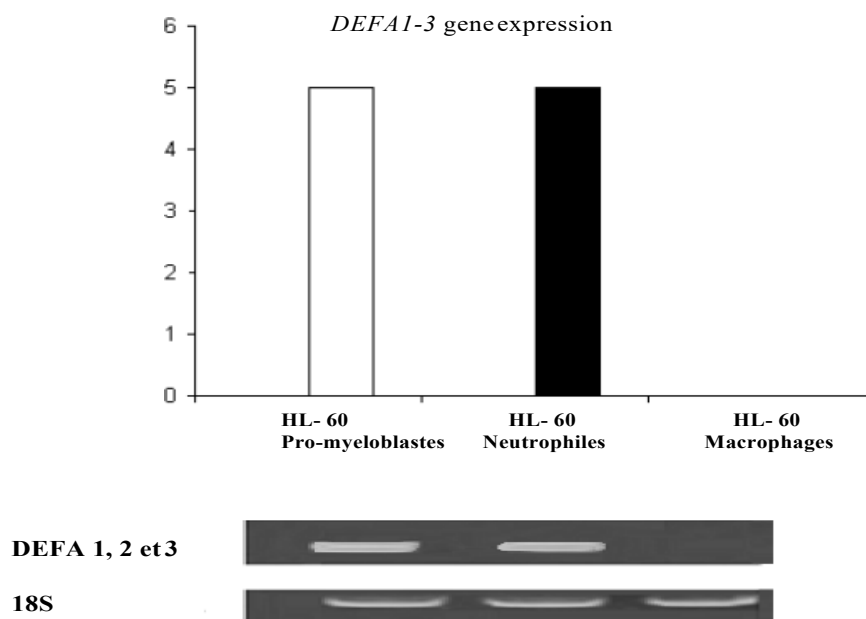

Fig 2. Analysis of the expression of alpha-defensin genes 1-3 in HL-60 cells differentiated into neutrophils and macrophages.

The HL-60 cells were differentiated into neutrophils and macrophages following treatment with 1.2% DMSO for 5 days and with 100 nM PMA for 24 hours respectively.

HL-60 differentiated into neutrophil cells were treated with concentrations of 4.5mM and 10mM glucose. The duration of treatment with these different concentrations of glucose was 6 h. This duration of treatment mimics the post-meal stage of the human organism, following the administration of the meal. It is characterized by a minimal secretion of insulin and therefore by the presence of high levels of glucose in the body. Treatment of HL-60 differentiated into neutrophils with 10 mM glucose during 6 hours decreased the levels of *DEFA1-3* expression by comparison to control (P<0.001) (Figure 3).
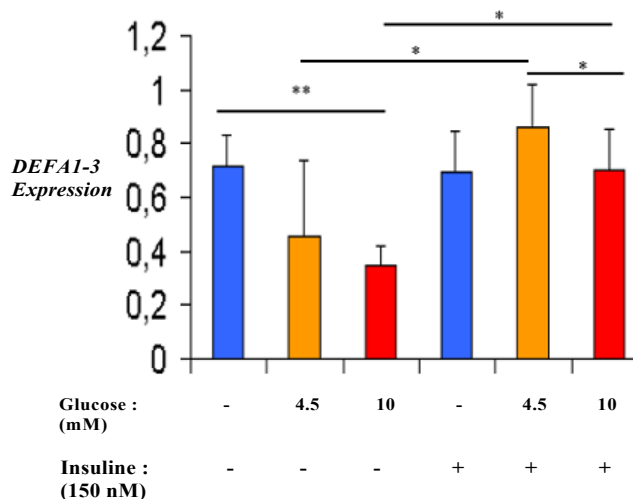

Fig3. Effect of glucose and insulin treatment on alpha-defensin 1-3 gene expression in HL-60 cells differentiated into neutrophils.
HL-60 cells differentiated into neutrophils were treated with increasing concentrations of glucose (4.5mM and 10mM), in the absence or in the presence of 150nM insulin. Cells previously incubated with the three increasing glucose concentrations were treated with 150nM insulin for 6 h. *DEFA1-3* gene values were normalized to the 18S gene. Bilateral correlation coefficients were calculated with P* <0.05 and P** <0.001.

After treatment of the cells with the same concentrations of glucose and insulin at a concentration of 150nM, semi-quantitative PCR analysis was showed that insulin has an opposite effect to that of glucose on the expression of *DEFA1-3*. Indeed, a doubling of the level of mRNA of *DEFA1-3* was observed in insulin-treated cells compared to cells grown under hyperglycemic conditions only (Figure 3). We thus demonstrate an *in vitro* regulation of *DEFA1-3* in HL-60 cells differentiated into neutrophils by glucose and insulin.

4. DISCUSSION

Our observations are consistent with other studies showing that defensins are overexpressed in human atherosclerotic lesions and that their level of expression is correlated with the degree of severity of CVDs (Lopez-Bermejo, 2007). Unlike the associations we have described, a recent study shows that obesity and smoking decrease the concentration of *DEFA1-3* in plasma and high concentrations of these peptides are associated with a reduction in LDL cholesterol and total cholesterol (P. Libby, 2002). The differences between the two studies could be explained by the fact that these authors assayed defensins at the protein level in the plasma, while in our case, we measured the mRNA expression of defensins in specific cells, PBMCs. For these reasons, additional studies either on larger populations as well as subjects suffering from well-defined pathologies (hypertension, diabetes, obesity) on an animal or cellular model, will be necessary to allow access to mechanisms for the release and action of AMPs and to understand their role that could be atherogenic or pro-atherogenic. In this perspective and to understand the relationship between defensins and studied clinical parameters, we initiated the study of the regulation of these peptides in the HL-60 cell line in response to different stimuli including glucose. The HL-60 cells are pro-myeloblasts that express *DEFA1-3*. They

are immature cells that normally mature in the bone marrow before gaining blood circulation, their differentiation into neutrophils and macrophages is necessary (P. Libby, 2002).

Differentiation of HL-60 cells into neutrophils did not affect the expression of *DEFA1-3* since similar levels of expression those observed at the pro-myeloblast stage were detected. For these reasons, we have chosen the HL-60 neutrophil model as the leukocyte type valid for study the possible regulation of *DEFA1-3* during the inflammatory process linked to atherosclerosis.

In order to understand the relationship between glucose and *DEFA1-3* expression levels, we differentiated HL-60 cells into neutrophils. We found that glucose significantly decreases the expression of *DEFA1-3* mRNA. This decrease was restored when insulin was added in the culture medium. These observations suggest a role for *DEFA1-3* in the metabolic pathways of glucose and insulin. Our preliminary results are consistent with a recently published study (Froy, 2007). Using a mouse model of diabetes, Froy et al., observed low levels of β - defensin 1 at the renal level and that were re-standardized by treatment with insulin (Froy, 2007).

5. CONCLUSIONS

To conclude, our results demonstrated that glucose might change *DEFA1-3* gene expression in HL-60 cells. .

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