Regulation of TSH Receptor Autoantibodies by a long Non-Coding RNA (Heg) and Cdk1- A Review

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Authors’ contributions

The original work was carried out in collaboration between all authors. Author NJC wrote the first draft of the review. All authors read and approved the final manuscript.

ABSTRACT

Aims: A substantial part of the genome is transcribed in non-coding RNAs. We review our finding of a long non-coding RNA (designated Heg) in mononuclear cells (MNC) and regulation of TSH receptor autoantibodies (TRAb).

Results: The Heg RNA transcript in MNC is negatively correlated with TRAb in patients with early and untreated Graves’ disease. In treated patients and in controls Heg correlated negatively with CD14 mRNA. Transfection studies with fragments of Heg added to MNC (exogenous Heg) decreased CD14 mRNA in MNC and increased gene expression of RIG-I, TLR7 and IFN-γ. Heg is likely to activate TLR7 receptors. CD14 is a co-receptor of TLR7. Decrease in gene expression of CD14 after Heg is a sign of differentiation of MNC to dendritic cells. This may reduce surface expression of CD14, cytokine responses and the responsiveness to TSH receptor antigens. Thus the relationship between TRAb and Inc Heg RNA is most likely explained by receptor cross-interference. Cdk1 mRNA (an index of cell cycle activity) is positively related with TRAb. Cdk1 mRNA and TRAb but not Heg decreased significantly during antithyroid treatment. Cdk1 decreased to values below normal.

Conclusion: Thus both Heg RNA and Cdk1 may regulate the level of TRAb but by two different mechanisms.
Keywords: Antithyroid drugs; autoimmunity; CD14; Cdk1; receptor cross-interference; long non-coding Heg RNA; TSH receptor autoantibodies.

ABBREVIATIONS

RIG-I: Retinoic-acid-inducible gene 1; IFIT: Interferon-induced protein with tetratricopeptide repeats; IFN: interferon; α,β,γ; Amol: Attomol; Zmol: Zeptomol.

1. INTRODUCTION

A substantial part of the genome is transcribed in non-coding RNAs. Many studies have focused on miRNAs, which are important for cell proliferation and cancer. Recently two studies of miRNA profiles have been reported in thyroid diseases [1-2]. We review our finding of a lnc (long non-coding) RNA (designated Heg) in peripheral blood mononuclear cells (MNC) and regulation of TSH receptor autoantibodies (TRAb). We have not been able to find information about other lnc RNAs related to the physiology or pathophysiology of TRAb, but the function and regulatory principles of lnc RNAs have recently been summarized [3-6]. Some lnc RNAs are rapidly degraded and important for activation of inducible genes. Other lnc RNAs are very stable [7-8]).

In our laboratory quantification of RNA was performed by RT-PCR-HPLC [9-10]. HPLC was applied to separate the peak value of the specific standard and the RNA to be measured. All chromatograms were examined graphically on a computer screen. During a study of Foxp3 mRNA in MNC we observed on the chromatogram a RNA fragment without annotation. The area of the peak correlated with gene expression of CD14 mRNA as measured in a small group of subjects. The sequence was localized by a BLAST search to a clone from the HUGO project on chromosome 1 and designated Heg (4002 bases; GenBank EU137727). Heg RNA is a single stranded RNA fragment and antisense to and overlapping a major part of exon 7 of the Nucks mRNA (GenBank NM_022731.4). Nucks, nuclear ubiquitous casein kinase substrate, is known to play a major role in transcription regulation and is a substrate for Cdk1 [11]. Heg is considered to be a lnc RNA, and we have not been able to transcribe Heg by oligo (dT) priming. Heg includes an open reading frame (ORF) of 97 amino acids. A lnc RNA may contain such an ORF by chance and many well-characterized lnc RNAs do indeed contain relatively long ORFs. A protein corresponding to the 97 amino acids has not been isolated. Furthermore the relationship between Heg RNA and CD14 mRNA may be imitated by fragments of Heg RNA.

To examine the possible role of Heg in the development of autoimmunity we studied TRAb in patients with Graves’ disease [12-14]. Our studies included 17 patients with early, untreated Graves’ disease, 20 patients who had been treated with antithyroid drugs for several months and 18 normal subjects. Additional samples were obtained from normal subjects for incubation studies. We also analyzed different types of non-activated MNC. Information about subjects included in the study and a description of methods applied for quantification of RNA have been presented earlier [9,15].

2. TRAb AND RELATIONSHIP WITH LONG NON-CODING HEG RNA AND CD14 mRNA

In the first part of the study we examined, if TRAb was correlated with lnc Heg RNA. There was a negative and significant relationship in patients with untreated Graves’ disease...
between TRAb and Inc Heg RNA amol/μg DNA [9]. We have not found any other factor, which correlated with TRAb in untreated patients except Cdk1 mRNA. Cdk1 is an index of cell cycle activity [16] and will be discussed later. Including Cdk1 mRNA zmol/μg DNA in the regression analysis increased the r value (numerically) from -0.61 to -0.83. Fig. 1 shows the negative relationship observed between log TRAb and the ratio Heg RNA/Log Cdk1 mRNA [15]. There was no significant relationship between TRAb and Heg RNA in treated patients with Graves’ disease. A negative relationship was observed, however, between CD14 mRNA and Inc Heg RNA. Cd14 is a co-receptor of toll-like receptor 4 (TLR4). It has recently been reported, that CD14 is also a co-receptor of TLR7 and is required for TLR7 dependent cytokine responses [17-18]. Results were approximately similar in treated patients and in controls. In the combined group of subjects we observed a strong negative correlation between Cd14 mRNA and Inc Heg RNA. We also included Nucks mRNA in the analysis, to see if Inc Heg was dependent on the transcription rate of Nucks. Nucks mRNA was positively related to Heg RNA but not to CD14. The best description of the relationship between CD14 mRNA and Heg RNA was obtained, if a correction was made for the influence of Nucks on the Heg level. Thus subjects with a high Inc Heg RNA/Nucks mRNA ratio had low levels of CD14 mRNA [9].

3. TRANSFECTIONS STUDIES

These relationships do not necessarily imply any causal relationship. We did therefore a number of experiments, where MNC were incubated with a single-stranded fragment of Heg RNA. One of these experiments is shown in Fig. 2 [9]. No significant change was observed in the control experiment, but in the experiment with Heg RNA CD14 mRNA decreased from 23±1.2 to 1±0 amol/μg DNA at 24 hours. Exogenous RNA is not taken up by MNC unless a transfection agent is added. No effect of fragments of Heg was observed unless a transfection agent was added to the incubation medium. We applied lipofectamine, which alters the cellular plasma membrane allowing nucleic acids to cross into the cytoplasm. Lipofectamine had no effect on basal levels of Heg demonstrating that it was of endogenous origin. Transfection studies with fragments of Heg and lipofectamine added to MNC clearly increased Heg RNA in MNC and decreased CD14 mRNA. A similar decrease in CD14 mRNA was also observed after incubation with antisense Heg RNA derived from the Nucks sequence demonstrating that the response was not dependent on the specificity of the sequence.
TSH receptor autoantibodies and gene expression of Heg and Cdk1

![Graph](image1)

Fig. 1. The relationship between log_{10} TSH receptor autoantibodies (TRAb) IU/Liter and the ratio Heg RNA amol per µg DNA/Log_{10} Cdk1 mRNA zmol per µg DNA. R=-0.82; (P<.001). Patients with early and untreated Graves’ disease. (Reproduced with permission from Christensen et al. [15])

Effects of Heg RNA on gene expression of CD14

![Graph](image2)

Fig. 2. CD14 mRNA amol per µg DNA plotted on the ordinate. Results are basal values and values after 24 h incubation without (-) and with (+) addition of a Heg fragment (P<.001). (Reproduced with permission from Christensen et al. [9])
4. TOLL-LIKE RECEPTOR 7

The TLR7 protein is a member of the Toll-like receptor family. It recognizes single stranded RNA in the endosome (viral genomes) and activates cytokines. It is important for innate immunity. RIG-I, IFIT and IFN proteins are all important for activity against viral infections. Rig-I is a cytoplasmic receptor and function in the same way as TLR7 as a sensor for recognition of viral RNA. Both RIG-I and TLR7 activate gene expression of IFN. Heg RNA in patients with untreated Graves’ disease showed a weak but significant positive correlation with gene expression of TLR7 and RIG-I (P<0.01 and 0.02, respectively). Heg RNA fragments increased gene expression of both TLR7 and RIG-I approximately five fold. Both endogenous Heg (coming from inside the cell) and exogenous Heg (added to cells with lipofectamine) were probably detected by RIG-I and other factors like IFIT and activated TLR7 in the endolysosome (see below). TLR8 is also considered to be active against single stranded RNA, but TLR8 mRNA did not increase. We also measured TLR7 mRNA in different non-activated MNC types obtained from healthy subjects. CD14+ cells had high levels of TLR7 mRNA as compared with other cell types (CD14+cells 2838±34; dendritic cells 346±14; CD8 cells 0±0 expressed as the peak area/µg RNA). Decrease in CD14 mRNA after exogenous Heg (meaning Heg coming from outside the cell in transfection studies with lipofectamine added) is a sign of differentiation of blood monocytes to dendritic cells.

IFN-α mRNA increased significantly in response to exogenous Heg, and correlation was observed between IFN-α mRNA and the corresponding IFN-α protein. IFN-α mRNA was not detectable in the basal state. IFN-γ mRNA increased 22 and 137 fold 6 and 24 hours after addition of exogenous Heg. There was also a positive relationship in normal subjects between endogenous levels of IFN-γ mRNA amol/µg DNA and endogenous Heg. IFN-γ mRNA values ranged from 0.02 to 0.18 amol/µg DNA.

5. MECHANISMS

What is the explanation of the negative relationship observed between TRAb and Inc Heg RNA? Lnc Heg RNA is negatively correlated with TRAb or CD14 mRNA in untreated patients with Graves’ disease and in treated patients and controls, respectively. Transfection studies with fragments of Heg also decreased CD14 mRNA and increased gene expression of TLR7, RIG-I and IFN-γ.

Recent studies have shown that some Lnc RNAs are stable, but half-lives may vary [8]. Some Lnc RNAs rapidly broken down in the nucleus may represent noise. Other Lnc RNAs are expressed proximal to inducible genes and may regulate the chromatin state. The clearance of these genes by decapping results in gene activation [7]. Exogenous Heg (+ lipofectamine) added to MNC was probably fused with the cell membrane and transported to the endosome, where it activated TLR7. The marked increase in RIG-I mRNA suggests that exogenous Heg was also detected in the cytoplasm [19-20]. It is not clear at present how endogenous Heg (coming from inside the cell), activated RIG-I and TLR7, because there are several RNA degrading pathways in the cytoplasm [21-22]. The response pattern to endogenous and exogenous Heg was, however, rather similar and both RNAs were associated with a decrease in CD14 mRNA. The effect of Heg was not dependent on its specific sequence but more on its molecular pattern as a single stranded RNA molecule. This is also so with viral RNA. Pattern recognition receptors sense molecular signatures associated with viral RNA.
It has recently been reported that CD14 is a co-receptor not only of TLR4 but also of TLR7 and is required for TLR7 dependent cytokine responses [17-18]. Control of TLR7 expression is important to restrict autoimmunity and dendritic cell expansion [23]. The negative relationship between Heg and CD14 mRNA and the decrease in CD14 mRNA after transfection with Heg RNA is likely to be a sign of differentiation of monocytes to dendritic cells. Upon differentiation the cell surface expression of CD14 is lost, whilst CD209 expression is increased [24]. There is likely to be a continuously small production of Heg RNA in MNC, which decreases gene expression of CD14 mRNA, reduces cytokine secretion and cytokine responses and this may reduce responsiveness to TSH receptor antigens. Our findings are most likely explained by receptor cross-interference. Small increments in the flow of Inc Heg RNA to the endolysosome may also reduce autoantibody production for instance in early juvenile diabetes. Receptor cross-interference has recently been reported by Negishi et al. [25]. These authors showed that recognition of double-stranded RNA by RIG-I-like receptors suppresses TLR induced expression of interleukins 12 and 23 and antibacterial responses.

6. CDK1 mRNA AND ANTITHYROID TREATMENT

It is well known that TRAb decreases during treatment with antithyroid drugs. As mentioned previously 20 patients were studied after treatment had been initiated. Heg RNA concentrations in MNC were not measured before treatment, but their TRAb levels were available. Expectedly TRAb had decreased approximately 50% (from a median level of 13.5 to 6.5 IU/l; P < .004). This decrease in TRAb during treatment cannot be explained by Heg, which remained unchanged.

We have previously shown that Cdk1 was positively related to TRAb (see above) and we wanted to see if gene expression of Cdk1 changed during treatment. Cdk1 is a cyclin-dependent kinase, which is necessary to drive cell division. Furthermore, the Nucks protein plays a major role in transcription regulation and is a substrate for Cdk1. Concentrations of Cdk1 mRNA were significantly reduced in the group of treated patients to 43% as compared with untreated patients and normal subjects (Table 1; ANOVA (P < .001) [15]. Calculated TRAb values obtained from the regression line (relating TRAb to Heg RNA and Cdk1 mRNA) after an assumed reduction in Cdk1 mRNA values of 50% also resulted in a decrease in TRAb of 50%. Note that Cdk1 mRNA decreased to levels significantly below levels observed in normal subjects. Concentrations of Cdk1 mRNA were not significantly different in untreated patients and in normal subjects. This suggests that the decrease in TRAb during treatment with antithyroid drugs may be due to a reduction in cell cycle activity. The decrease in Cdk1 during antithyroid treatment was in all probability a pharmacological effect of antithyroid treatment. Clearly further studies may be of interest especially in vitro studies to examine, if addition of antithyroid drugs to MNC in vitro decreases Cdk1 mRNA. It is unclear at present, if the effect of antithyroid drugs on Cdk1 mRNA is specific for Graves’ disease.

Table 1. Cdk1 mRNA concentrations expressed in zmol/μg DNA (median and 25% and 75% ranges) in untreated and treated patients with Graves’ disease and in controls. (reproduced with permission from Christensen et al. [15])

| Untreated patients | Treated patients | Normal subjects |
|--------------------|-----------------|----------------|
| 33 (22 to 39)      | 13 (10-17)*     | 27 (18-34)     |

*Significantly different from the two other groups (ANOVA; P < .001).
7. LONG NON-CODING HEG RNA AND SUSCEPTIBILITY GENES

A number of genes may contribute to the development of Graves’ disease 1) genes from the HLA-DR gene locus 2) immune-regulatory genes (CD40, CTLA-4 and PTPN22) and 3) thyroid specific genes. Autoantigens may bind to receptors on T-cells, which have escaped tolerance [12-14]. CTLA-4 and PTPN22 genes are both negative regulators of T-cell activation and CD40 is important for activating of B-cells. Polymorphism of these genes may influence TRAb production. Genetic variations in TLR receptors may also contribute to disease [26]. There is no evidence that lnc Heg RNA has any specific effect on the development of Graves’ disease. Lnc Heg RNA is related to the Nucks gene, but it is not the Nucks mRNA. Lnc Heg RNA may perhaps influence the early inflammatory response during the development of Graves’ disease. Cdk1 is proinflammatory and may activate B-cells. Lnc Heg RNA is likely together with gene expression of Cdk1 and other factors to regulate the level of TRAb and to some extent disease activity. Relationships between lnc Heg and the above mentioned susceptibility genes deserve further investigations. Our results suggest that decrease in TRAb during treatment with antithyroid drugs may be due to a decrease in Cdk1 mRNA to levels below normal. Clearly further studies are necessary to confirm this hypothesis.

8. CONCLUSIONS

The present study indicates that two different factors, a lnc Heg RNA and Cdk1 mRNA may regulate TRAb. Heg may activate TLR7 in the endolysosome and decrease gene expression of CD14 mRNA. It is likely to be a sign of differentiation of monocytes to dendritic cells. This change may reduce the surface expression of CD14, decrease cytokine secretion and the responsiveness to TSH receptor antigens. Decrease in TRAb during treatment with antithyroid drugs cannot be explained by Heg. Cdk1 mRNA, which is an index of cell cycle activity, decreased significantly during treatment to values below normal. Gene expression of lnc Heg RNA and Cdk1 mRNA may both regulate the level of TSH receptor autoantibodies but by two different mechanisms. The correlations observed about the decrease in TRAb were not a direct immunologic mechanism to regulate the particular autoantibody production, but an indirect cellular mechanism(s) resulted in the autoantibody decrease. Therefore it may be of interest to study the same mechanisms for example in subjects at risk of developing Type 1 diabetes.

CONSENT

Written Informed consent was obtained from all subjects, who participated in the original studies. The study protocols were approved by the Ethics Committee of Copenhagen County.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the Ethics Committee of Copenhagen County and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

None. No competing financial interests exist.

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