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ORIGINAL ARTICLE

T-helper-related cytokines in gingival crevicular fluid from adolescents with Down syndrome

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Abstract Subjects with Down syndrome have a high prevalence of periodontal disease. The aim was to investigate the level of Th1-, Th2- and Th17-related cytokines in the gingival crevicular fluid (GCF) of subjects with Down syndrome. Subjects with Down syndrome (n=24) and controls (n=29) with a mean age of 16.4 years were clinically examined with respect to periodontal probing depth (PD) and gingival inflammation in terms of bleeding on probing (BOP%). The controls were matched to subjects with Down syndrome regarding age and gingival inflammation (BOP%). All subjects answered a questionnaire regarding oral hygiene, medical history and socioeconomic background. GCF was collected and the concentration of the cytokines, IFN-γ, TNF-α, IL-1β, IL-4, IL-6, IL-10, IL-12 and IL-17 were determined using Bio-Plex cytokine multiplex assays. The volume of GCF (microliters) was significantly higher in subjects with Down syndrome (P< 0.001) compared with controls. The mean concentrations (picogrammes per millilitre) of IL-1β (P<0.001), IL-4 (P= 0.002), IL-6 (P=0.005), IL-10 (P=0.001), IL-12 (P=0.003), IFN-γ (P=0.002), and TNF-α (P=0.002) in GCF, respectively, were significantly higher in subjects with Down syndrome compared with controls. The regression line of the relationship between IFN-γ and IL-4 in GCF differed significantly (P=0.006) in subjects with Down syndrome compared to controls. Subjects with Down syndrome demonstrated higher concentration of Th1-, Th2- and Th17-related cytokines with an altered relationship between Th1 cytokine, IFN-γ and Th2 cytokine, IL-4, in volume GCF compared to controls.

Keywords Down syndrome · Th-subsets · Cytokine · Gingivitis · Adolescents

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Introduction

Down syndrome, caused by a chromosomal aberration in chromosome 21 [1], is characterised by several functional and physical disorders including mental retardation, dementia, congenital heart defects, leukaemia and increased infection susceptibility as well as periodontal disease [2, 3]. Subjects with Down syndrome are more prone to periodontal disease with early signs of periodontitis, often diagnosed during adolescence [3]. Numerous etiological factors have been suggested to explain the enhanced risk for periodontal disease among subjects with Down syndrome including poor oral hygiene, tongue pressure and the occurrence of periodontal pathogens such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* [4]. However, the impaired host response in the Down syndrome group is a significant contributing factor to periodontal disease [4], even though the mechanism(s) is still unclear.

The impaired host response in subjects with Down syndrome is characterised by reduced chemotactic ability and impaired phagocytosis of polymorphonuclear leukocytes as well as disturbances of the T and B lymphocyte subsets [5–7]. In subjects with Down syndrome, it has been reported that the function of the thymus is altered, with a decreased number of mature thymocytes expressing high

levels of the α and β forms of the T cell receptor and associated CD-3 molecules as well as an overexpression of TNF-α and IFN-γ. The overexpression of these cytokines suggests a dysregulation in cytokine production in the Down syndrome group and provides an explanation for the abnormal thymic anatomy and thymocyte maturation [8, 9]. Although, the absolute numbers of T lymphocyte populations in the Down syndrome group gradually approach those of normal children over time [10], it is doubtful whether these cells in subjects with Down syndrome have a normal phenotype and function.

T-helper cells (Th) play a crucial role in the host response and through the influence of specific cytokines differentiate into subsets of Th1, Th2, Th17 and regulatory T (T-reg) cells mediating inflammation, tissue damage and autoimmunity [11–13]. Th1 cells produce cytokines such as IFN-γ, which stimulates cytotoxic T lymphocyte responses and immunoglobulin (Ig)G1 and IgG3 production, whereas Th2 cells produce cytokines such as IL-4 and IL-10, which stimulate antibody responses by B lymphocytes and the formation of IgG2 and IgG4. It is reported that subjects with Down syndrome have hypergammaglobulinaemia of IgG characterised by low levels of IgG2 and IgG4 and high levels of IgG1 and IgG3 [14]. Furthermore, de Hingh et al. [10] have described profound B lymphocytopenia in the Down syndrome group caused by either an intrinsic B lymphocyte defect or as a consequence of a deficient T-helper lymphocyte function.

The inflammatory response in periodontal tissue is enhanced in subjects with Down syndrome [4], and we previously reported higher levels of prostaglandin E_2 (PGE_2) in gingival crevicular fluid (GCF) [15, 16]. In healthy adults, an association between the level of PGE_2 in GCF and progression of periodontal disease [17–19] has been demonstrated, indicating that PGE_2 is an important mediator in the development of periodontal disease. In light of the immunomodulatory effect of PGE_2, suppressing Th1 differentiation by raising intracellular cyclic AMP [20–22] and amplifying IL-23-mediated Th17 cell expansion in vitro [23], it would be of interest to investigate T-helper-related cytokines in subjects with Down syndrome.

The aim was to investigate Th1-, Th2- and Th17-related cytokines in subjects with Down syndrome and healthy controls with similar degree of gingival inflammation.

Methods

Subjects

The study design was cross-sectional and approved by the local ethics committee at Karolinska Institutet, Huddinge University Hospital. The study population comprised subjects with Down syndrome that were consecutively referred from the Public Dental Health Services, Stockholm to the Department of Pediatric Dentistry, Eastmaninstitutet, Stockholm. The control subjects were randomly selected from the Public Dental Health Services, Eastman Dental Institute, Stockholm with respect to age.

All subjects and/or their parents provided oral consent before participating in the study. Inclusion criteria were age (13–20 years) and degree of gingival inflammation expressed as bleeding on probing (BOP) less than 50%. Exclusion criteria were previous and ongoing smoking habits, ongoing orthodontic treatment, one or more sites with a periodontal probing depth over 3 mm or the occurrence of marginal alveolar bone loss on radiographs.

A power analysis was performed before the start of the study. Based on a previous report, enhanced level of the inflammatory mediator prostaglandin E_2 in GCF in subjects with Down syndrome [15], a 5% significance level and 80% power required a sample size of 48 subjects to detect difference in cytokine concentration (pg/ml) between subjects with Down syndrome and controls. To perform the power analysis we used simple interactive statistical analysis (SISA).

Of the Down syndrome group (n=50), six were excluded because of the occurrence of periodontitis, eight because of BOP>50% and 12 because of a lack of compliance during clinical examination. The final group of subjects with Down syndrome (n=24) were between the ages of 13 and 20.5 years, with a mean age of 16.4 years. The mean percentage of BOP in subjects with Down syndrome was 32% and varied between 15% and 49%. Of the subjects with Down syndrome, six had congenital cardiac malformations, three had increased susceptibility to upper respiratory tract infections, one had epilepsy, one had autism, two had asthma and five had thyroid deficiencies treated with levothyroxine (Levaxin®; Table 1).

Of the control group (n=78), 12 were excluded due to undergoing orthodontic treatment, 15 because of a missed appointment, 20 declined participation in the study and two exhibited a medical disorder. The final control group (n=29) were between the ages of 13.5 and 19 years, with a mean age of 16.4 years. The mean percentage of BOP was 28% and varied between 18% and 46% (Table 1).

Questionnaire

All the parents/subjects answered a questionnaire regarding socioeconomic background, level of education, oral hygiene habits, smoking habits and medical conditions. In case the subjects did not understand the Swedish language, an interpreter assisted. Place of birth was categorised into ‘Europe’ or ‘outside of Europe’. The educational level of

Table 1 Characteristics of the subjects

Variables	Down syndrome (n=24) mean (SD)	Controls (n=29) mean (SD)	P value
Female/male	11/13	15/14	
Age (yrs)	16.4 (2.0)	16.4 (1.7)	0.940
Range	13–20.5	13.5–19	
Age interval			
13–14	5	6	
15–17	13	17	0.932
18–21	6	6	
Clinical condition			
PD (0–3 mm)	24	29	
BOP (%)	32 (9.4)	28 (8.2)	0.114
Range	15–49	18–46	
Chronic disease			
Heart disorder	6	0	
Asthma	2	0	
Upper airway	3	0	NA
Autism	1	0	
Epilepsy	1	0	
Thyroid deficiencies	5	0	
Birth region			
Europe	23	27	1.000
Outside of Europe	1	2	
Mother's education (yrs)			
<9	1	0	
9–13	11	9	0.201
>13	12	20	
Housing			
Inner city	6	26	
Suburbia	18	3	<0.001
Rural	0	0	
Oral hygiene habits			
Twice a day with assist.	22	0	
Twice a day without assist.	1	25	
Once a day with assist.	0	0	<0.001
Once a day without assist.	1	3	
Never	0	1	

Student's t test or Chi-square exact test was used as the statistical method

NA not available

the mother was categorised into <9, 9–13 and >13 years of education. Housing was categorised into 'inner city', 'suburbia' and 'rural' areas (Table 1).

Clinical examination

Gingival inflammation Gingival inflammation was based on BOP of the gingival sulcus at four sites of all teeth (wisdom teeth excluded). The percentage of surfaces with BOP was calculated for each individual and expressed as BOP%, shown to be a reliable indicator for gingival inflammation [24].

Periodontal probing depth Periodontal probing depth (PD) was recorded using a graded periodontal probe (Hu-Friedy, Chicago, IL, USA) and measured to the nearest millimetre (mm) at four sites of all teeth (wisdom teeth excluded). The occurrence of pathological PD was classified when the subject exhibited one or more sites with a periodontal probing depth >3 mm.

Marginal alveolar bone loss Radiographical examination was conducted by taking bitewing and periapical radiographs, both digital and conventional. A lack of cooperation in some subjects with Down syndrome meant panoramic radiographs had to be taken. Alveolar bone loss was classified when the distance from the cementoenamel junction to the alveolar crest on the radiograph exceeded 2 mm of the first molars and incisors in both jaws [25].

The characteristics of the subjects with respect to gender, age, general health, place of birth, housing, educational level of the mother, oral hygiene habits and clinical condition in terms of BOP% and PD are shown in Table 1.

Collection of GCF samples

GCF samples were collected from each patient from the mesial surfaces of 16, 26, 36, 46, 41 and the distal surface of 11 prior to the clinical examination. Both GCF sampling and the clinical examination were conducted at the same appointment. Before GCF collection, the tooth surface was carefully cleaned and supragingival plaque was eliminated using a cotton pellet and curette. The tooth surface was gently dried with air. A paper strip (Periopaper, Pro Flow, Amityville, NY, USA) was inserted into each sulcus and left for 15 s. Paper strips contaminated with blood during the GCF sampling were excluded. GCF was collected during 15 s and determined by using a Periotron 8000 (Pro Flow) and calculated by interpolation from a standard curve and expressed as volume GCF (μL). The periopaper was placed in 120 μl assay buffer containing 0.9% Na Cl, 0.01 M EDTA, 0.3% bovine-globulin, 0.005% Triton-X-100, 0.05% sodium azide, 0.0255 M NaH_2 PO_4 and 0.0245 M Na_2HPO_4, (pH 6.8) and kept frozen at −70°C . In total, 144 GCF samples from the Down syndrome group and 174 from the control group were collected. The concentration (picogrammes per millilitre) of IL-1β, IL-4, IL-6, IL-10, IL-12, IL-17, IFN-γ and TNF-α, respectively, was determined in GCF samples using the commercially available Bio-Plex Cytokine Assay (Bio-Rad Laboratories, CA, USA) in accordance with the manufacturer's instructions. Because of a lack of GCF material, IL-6 and IL-17 were analysed only in 21 out of 29 controls.

Statistical analysis

The subject was the statistical unit for all variables. The Student's independent *t* test (two-tailed) was used to compare the means and Chi-square exact test was used to compare the categorical variables of the groups. Pearson's correlation was used for calculating the correlation within the group. Fisher's Z transformation was used when testing the difference in correlation coefficients between subjects with Down syndrome and controls. To adjust for multiple testing, the Bonferroni analysis was performed. The Statistical Package for the Social Sciences (SPSS 13.0) and MedCalc version 10.2 (MedCalc Software, Mariakerke, Belgium) were used as statistical programmes.

Results

Cytokine concentration

The concentrations of the various cytokines and GCF in the Down syndrome group as well as in the control group are demonstrated in Table 2. The GCF volume (microlitres) was significantly higher in subjects with Down syndrome compared with controls ($P<0.001$). The cytokine concentrations (picogrammes per millilitre) of IL-1β ($P<0.001$), IL-4 ($P=0.002$), IL-6 ($P=0.005$), IL-10 ($P=0.001$), IL-12 ($P=0.003$), IFN-γ ($P=0.002$) and TNF-α ($P=0.002$) were also significantly higher in subjects with Down syndrome compared with controls. However, the concentration of IL-17 in GCF did not differ between the two groups ($P=0.253$; Table 2).

Table 2 Cytokine concentration (picogrammes per millilitre) in GCF (microlitres) in subjects with Down syndrome and controls

Variables cytokine	Down syndrome ($n=24$)		Controls ($n=29$)		*P* value*
	Mean	SD	Mean	SD	
GCF	0.3	0.1	0.2	0.1	<0.001
IL-1β	174.4	91.5	74.8	43.4	<0.001
IL-4	0.9	0.6	0.4	0.3	0.002
IL-6	2.9	2.9	0.9[a]	0.5	0.005
IL-10	0.3	0.1	0.2	0.1	0.001
IL-12	0.6	0.3	0.4	0.1	0.003
IL-17	5.7	5.5	4.3[a]	3.1	0.253
IFN-γ	33.5	18.7	17.9	11.9	0.002
TNF-α	13.5	9.0	6.2	4.0	0.002

Student's *t* test was used as the statistical method adjusted by Bonferroni

[a]The mean value is based on 21 subjects

*Significance level, $P<0.006$

Correlation analysis

The correlation coefficients between BOP% and volume of GCF, respectively, and the various cytokines are illustrated in Table 3. In subjects with Down syndrome, neither there were significant correlations between BOP% and the various cytokines (IL-1β, IL-4, IL-6, IL-10, IL-12, IL-17, IFN-γ and TNF-α) nor between GCF volume (microlitres) and the concentration of the cytokines. In the controls, however, the cytokine concentration (picogrammes per millilitre) of IL-1β (P=0.042), IL-4 (P<0.001), IL-10 (P=0.021), IFN-γ (P=0.016) and TNF-α (P=0.017), respectively, were positively correlated with GCF volume (microlitres; Table 3). Furthermore, the concentration of IL-1β, was correlated to gingival inflammation expressed as BOP% (P<0.05) in controls (Table 3)

We also studied the relationship between the Th1 cytokine, IFN-γ, and the Th2 cytokine, IL-4, in GCF in both subjects with Down syndrome and controls. The inclination of the regression line concerning the relationship between IL-4 and IFN-γ concentrations differed significantly in subjects with Down syndrome compared to controls (P=0.006; Fig. 1).

Discussion

The novel finding demonstrates higher levels of Th1-, Th2- and Th17-related cytokines in GCF with an altered proportion of the Th1 cytokine IFN-γ and Th2 cytokine IL-4 in subjects with Down syndrome compared to controls, matched regarding age and clinical degree of gingival inflammation (BOP%). The pathogenic mechanism(s) behind the enhanced risk for periodontal disease in subjects with Down syndrome is not fully explained, although factors associated with the genetic aberration itself, such as diminished humoral and cellular immunity, are suggested to play a significant role in periodontal disease development [26]. However, oral hygiene habits, dental calculus, periodontopathogens, poor mastication and tongue pressure might also be contributing factors [26, 27].

In previous studies, we have demonstrated enhanced level of the chemokine LTB_4 and the prostanoid PGE_2 in GCF of subjects with Down syndrome [15], whereas not for IL-1β [16]. We here report that the concentration of the following T-helper-related cytokines, IL-1β, IL-4, IL-6, IL-10, IL-12, IFN-γ and TNF-α in volume GCF were significantly higher in subjects with Down syndrome compared with controls. The enhanced level of the T-helper-related cytokines in GCF in Down syndrome subjects might be a result of the chronic inflammation in the periodontal tissue or a consequence of abnormalities in the innate and acquired immune system. Interestingly, Bloemers et al. [28] very recently reported that subjects with Down syndrome exhibit higher number of monocytes, referred as the nonclassical monocytes (CD 14^{dim} $CD16^{+}$), than healthy controls. These cells normally represent approximately 10% of the total population of monocytes [29] and have superior antigen presenting cell activity as well as produce significant amount of pro-inflammatory

Table 3 Correlations between BOP (%), volume GCF (microlitres) and the various cytokine concentrations (picogrammes per millilitre) in subjects with Down syndrome (*DS*) and controls

Cytokines		GCF		BOP%	
IL-1β	DS	−0.009		0.049	
	Control		0.381*		0.392*
IL-4	DS	0.171		0.214	
	Control		0.633**		−0.005
IL-6	DS	0.213		0.051	
	Control		0.187		0.080
IL-10	DS	−0.243		−0.005	
	Control		0.425*		0.309
IL-12	DS	0.181		−0.298	
	Control		0.104		0.042
IL-17	DS	−0.073		0.192	
	Control		−0.389		0.289
IFN-γ	DS	−0.140		0.071	
	Control		0.444*		0.235
TNF-α	DS	−0.120		0.105	
	Control		0.441*		0.054

Pearson's correlation was used as statistical method

*P<0.05

**P<0.001

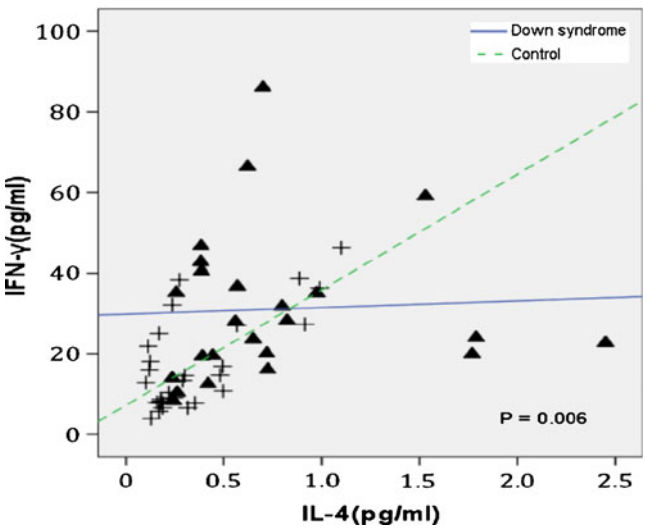

Fig. 1 The regression *line* between the concentration (picogrammes per millilitre) of IL-4 and IFN-γ in GCF (microlitres) in subjects with Down syndrome (*black up-pointing tiangle*; *line*) and controls (*plus sign*; *broken line*). Fisher's Z transformation was used when testing the statistical difference in the inclination of the regression *lines*

cytokines [30–33]. However, we did not have the possibility to identify the cytokine-producing cells in the periodontal tissue that contribute to the enhanced concentration of cytokines in GCF. Noticeably, the gingival inflammation determined clinically (BOP%), neither on individual level nor on GCF sample site level, differed between the two groups, which also was in agreement with the inclusion criteria of similar clinical degree of gingival inflammation in the two groups.

Our finding, demonstrating enhanced concentration of IL-1β, IL-4, IL-6, IL-10, IFN-γ and TNF-α in GCF, is not compatible with the situation in serum in which the cytokines IL-4, IL-10 and IFN-γ are enhanced whereas IL-6 and TNF-α are reported to be reduced in subjects with Down syndrome compared to healthy controls [34, 35].

In the controls we demonstrate a positive significant correlation between GCF volume (microlitres) and the concentration (picogrammes per millilitre) of the various cytokines, IL-1β, IL-4, IL-10, IFN-γ and TNF-α. In the Down syndrome group, the volume of GCF and concentrations of the various cytokines did not correlate significantly, indicating an altered host response regarding pro- and anti-inflammatory cytokines. In addition, there was a greater variation in cytokine concentrations in GCF in the Down syndrome group which also partly explains the lack of positive significant correlation between GCF volume and the various cytokine concentrations. Furthermore, one has to take into consideration the heterogenicity within subjects with Down syndrome, related to differences in genotypes, as well. Our findings further support the concept of an altered immune response in the periodontal tissue that in the long-term might predispose for early onset of periodontitis, in subjects with Down syndrome [3, 4].

We also studied the proportion of the Th1 and Th2 response in terms of the concentrations of IFN-γ and IL-4 in GCF. In the controls, there was a positive relationship between the concentration of Th1 cytokine, IFN-γ and the Th2 cytokine, IL-4; whereas in the Down syndrome group, such positive correlation was not demonstrated. The inclination of the regression line concerning the relationship between IL-4 and IFN-γ differed significantly between subjects with Down syndrome and controls indicating an altered Th1 and Th2 response in the Down syndrome group. This change of balance between pro- and anti-inflammatory cytokine reflects an altered host response in the Down syndrome group although we cannot state whether there is an enhanced or decreased inflammatory response. However, Cetiner et al. [35] demonstrated that increased levels of the anti-inflammatory cytokines (IL-4, IL-10) was related to high frequency of recurrent infection in subjects with Down syndrome.

The prostanoid, PGE_2, which we previously reported to be enhanced in GCF in subjects with Down syndrome [15, 16], has been reported to reduce the production of Th1-related cytokines like IFN-γ [36]. Whether the altered proportion of IFN-γ/IL-4 concentration in GCF in subjects with Down syndrome is directly or indirectly related to the arachidonic metabolite, PGE_2, is an important issue for further studies.

The enhanced concentration of cytokines in GCF and/or the altered proportion of IFN-γ/IL-4 in the Down syndrome group may negatively affect the homeostasis in the tissue as well. We previously reported enhanced level of MMP-9 in GCF and furthermore increased immunoreactivity of MMP-8 and MMP-9 in saliva has also been reported in the Down syndrome group compared to healthy controls [15, 37]. Although it has been demonstrated that IFN-γ suppresses TNF-α-induced MMP-9 production in monocyte-derived macrophages [38, 39], it is unclear whether an altered balance between pro- and anti-inflammatory cytokines also affects the MMP's in periodontal tissue in subjects with Down syndrome.

In conclusion, subjects with Down syndrome demonstrate higher concentration of Th1-, Th2- and Th17-related cytokines as well as an altered relationship between Th1 cytokine, IFN-γ, and Th2 cytokine, IL-4, in GCF compared to controls.

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Conflict of interest None.

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