

Plasma Nerve Growth Factor (NGF) and inflammatory cytokines (IL-6 and MCP-1) in young and adult subjects with Down Syndrome: An interesting pathway

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Abstract

OBJECTIVE: Down's syndrome (DS) is the most frequent chromosomal aberration in men and it is invariably associated with mental retardation.

MATERIAL AND METHODS: Plasma levels of nerve growth factor (NGF), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) from non demented DS subjects of three different age-cohorts (2-14 years; 20-50 yrs; > 60 yrs) and healthy controls were measured. No clinical and sub-clinical inflammation was apparent in DS patients.

RESULTS: Plasma levels of NGF were higher in children, adult and old DS subjects than in controls. However, a significant age-related decrease of NGF levels was present in DS subjects. Serum levels of IL-6 and MCP-1 were also increased in DS children and adults, but not in older DS patients.

CONCLUSIONS: High levels of circulating NGF might protect DS from clinical complications of atherosclerosis. However, the striking decrement of peripheral NGF levels with advancing age may predispose DS to clinical manifestation of dementia after adulthood.

Introduction

DS is the most frequent chromosomal abnormality in men [1], occurring in 0.8 out of 1000 live birth; the presence of an extra chromosome 21, or an its portion, causes several abnormalities. The syndrome is associated with several clinical alterations, a variable grade of mental retardation [2] and an accelerated ageing of different organs and tissues [3].

Altered immune responses are also frequently observed in DS subjects and have been considered responsible for the increased incidence of infections. A modest body of data regarding blood cytokines and their relations with cognitive decline in DS are on record. A recent investigation has shown that plasma levels of the macrophage inhibiting protein-1 (MIP-1) were higher in a small group of adult DS than in non DS mentally retarded controls and levels of IL-6 correlated with the degree of mental retardation only in DS subjects [4]. Moreover, IL-6 plasma levels also correlated with mental age [5].

NGF is a polypeptide that plays an important role in the growth, differentiation and survival of different cell types under various physiological and pathological conditions [6], and implicated in the regulation of immune and inflammatory response [7]. Recent reports, showing a decrement in circulating levels of NGF and another neurotrophin such as brain derived neurotrophic factor (BDNF) in acute coronary diseases, suggested a role of neurotrophic factor in the pathogenesis of human cardiovascular disease [8,9].

Prevalence of coronary artery diseases is low DS subjects and they rarely die because of atherosclerotic complications [10]. Moreover, a histopathological investigation showed no increase in atherosclerosis or absence of atherosclerosis changes in the syndrome [11]. It is of interest that plasma homocystein (tHcy) was increased and plasma folate decreased in DS subjects [12,13,14]. Moreover, increased tHcy is a well known risk factor for cardiovascular diseases [15]. Therefore, in spite of the presence of some classical biochemical risk factors for atherosclerosis, its clinical manifestation is low in DS. Reasons for this discrepancy remain unclear.

Peripheral NGF is implicated in the regulation of inflammatory responses [7] and maturation of hematopoietic cells [16]. IL-6 can modulate NGF synthesis in astrocytes from defined brain regions [17]. Neurons are able to produce IL-6 [18] and this cytokine may protect neuronal cell from insults [19]. It has been shown that impaired blood levels of the cytokine/NGF network might be implicated in the aging processes [20,21]. MCP-1, a member of CC chemokine family, plays a relevant role in inflammatory processes [22].

There is evidence indicating that levels of MCP-1 in plasma might be candidates as biomarkers for monitoring the inflammatory process in Alzheimer disease [23]. IL-6 and MCP-1 up-regulation has been demonstrated in neurodegenerative disease such as Alzheimer's disease (AD) [21]. It is of interest that DS have an high risk of

developing AD after the age of 50 year. Finally, increased blood levels of both IL-6 and MCP-1 cytokines were found in children with DS [24]. Recent findings showed that elevated levels of IL-6, soluble IL-6 receptor (sIL-6R), and soluble vascular cell adhesion molecule-1 (sVCAM-1) were increased in DS children [14] and suggested a possible remodelling of molecule network with regulatory function upon the vessel system in DS.

The aim of this study was to evaluate a possible role of circulating NGF in DS and its relationship with IL-6 and MCP-1 in DS subjects from different age cohorts. It is suggested that NGF might be a protective factor for atherosclerosis clinical manifestations in DS.

Material and methods

Three groups of DS subjects were studied: group 1 consisted of 23 children (age 2–14 years); group 2 of 14 adult (age 20–50 years) and group 3 of 13 elderly (>60 years) and a group of 20 healthy controls (age: 15–60 years). DS children and adults were living at home and elderly were living in institution. All DS were assessed by clinical examination and karyotype analysis, they showed a mild and variable degree of mental retardation, were free of other pathological conditions at the moment of the study and were in good health status. The project was approved by Ethics Committee of University of Milan and by Fondazione Antoniana of Bologna, Italy. Blood samples were collected from DS subjects. Plasma was obtained by centrifugation (1500 g for 15 min), transferred into coded plastic tubes, rapidly frozen and stored at -20°C until analysis.

NGF

NGF levels were measured by a highly sensitive two-site immunoenzymatic assay (PROMEGA, Madison, WI, USA), which recognizes human and murine NGF and does not cross-react with BDNF. Polystyrene 96-well microtube immunoplates (Nunc) were coated with affinity-purified, polyclonal goat anti-NGF antibody, diluted in 0.05 M carbonate buffer (pH 9.6). After overnight incubation at room temperature and 2 h incubation with a blocking buffer (0.05 M carbonate buffer (pH 9.5), 1% bovine serum albumine), the plates were washed three times with 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 0.5% gelatin and 0.1% Triton X-100. After extensive washing of the plates, the samples and the NGF standard solutions were diluted with sample buffer (0.1% Triton X-100, 100 mM Tris-HCl (pH 7.2), 400 mM NaCl, 4 mM ethylenediamine-tetraacetic acid, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM benzethonium chloride, 2 mM benzamidine, 40 U/ml aprotinin, 0.05% sodium azide, 2% bovine serum albumine and 0.5% gelatine); they were then distributed among the wells and left to stand overnight at room temperature. The plates were then washed three times and incubated with 4 mU/well anti- β -NGF-galactosidase for 2 h at 37°C ; after further washing, 100 μl of substrate solution (4 mg/ml of chlorophenol red, substrate buffer: 100 mM

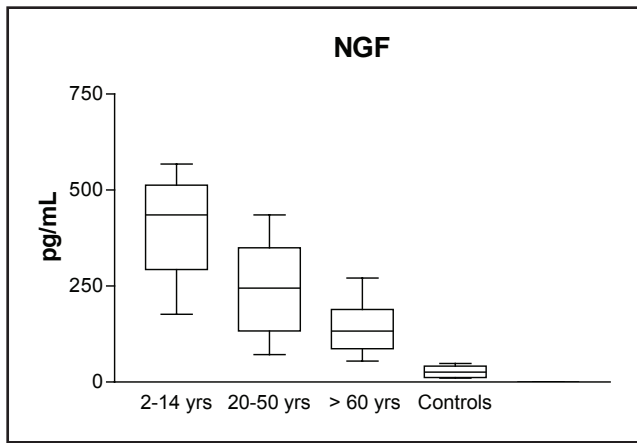


Figure 1. NGF plasma concentrations expressed as pg/mL. Data are presented as mean \pm Standard deviation (SD).

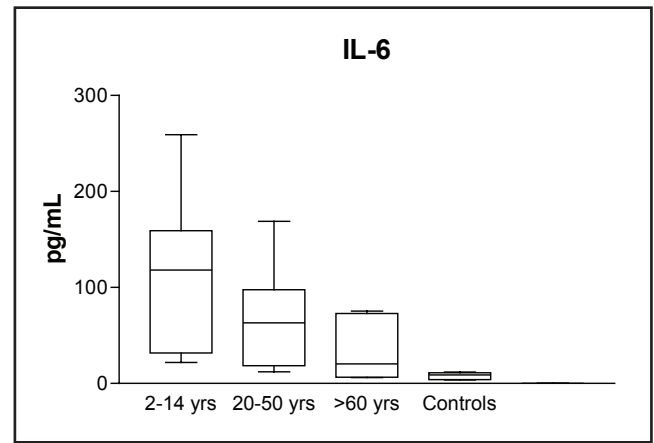


Figure 2. IL-6 plasma concentrations expressed as pg/mL. Data are presented as mean \pm Standard deviation (SD).

N-2-Hydroxyethylpiperazine- N²-2-ethanesulfonic acid, 150 mM NaCl, 2 mM MgCl₂, 0.1% sodium azide and 1% bovine serum albumine) was added to each well. After incubation for 2 h at 37° C, optical density was measured at 450 nm, using an ELISA reader (EL-600, BioTeck, Brussel, Belgium), and the values of standards and samples were corrected by taking non-specific binding into consideration. Under these conditions, the sensitivity was 3 pg/ml. The recovery of NGF in our assay ranged from 80 to 90%, and cross-reactivity with other molecules of the NGF family (i.e., NT-3 and NT-4/5) was less than 3%. NGF concentration was expressed as pg/ml for liquidsamples. All assays were performed in triplicate.

IL-6

The plasma concentration of IL-6 was measured using a commercial immunoenzymatic kit (Quantikine by R&D Systems, Minneapolis, MN, USA) following the instructions of manufacturer. The sensitivity of the assay was typically 0.70 pg/ml of IL-6 and no cross-reactivity or interference with other related interleukins was observed. Data were represented as pg/ml and all assay were performed in duplicate.

MCP-1(CCL2)

The plasma concentration of MCP-1 was measured using a commercial immunoenzymatic kit (Quantikine by R&D Systems, Minneapolis, MN, USA) following the instructions of manufacturer. The minimum detectable dose of sensitivity of MCP-1 was typically <5 pg/ml of IL-6 and no cross-reactivity with other related interleukins was observed. Data were represented as pg/ml and all assay were performed in duplicate.

The results are given as mean \pm standard deviation (SD). Comparison among groups were assessed by one way analysis of variance. Linear regression analysis between experimental variables was also performed. Significance values were taken as those with p values <0.05. Post-hoc comparisons within logical sets of

means were performed using Tukey-Kramer multiple comparisons test.

Results

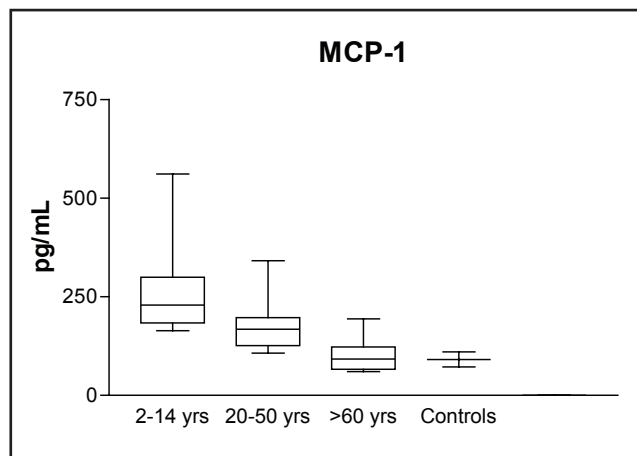
Serum levels of NGF are reported in Figure 1. Circulating levels of NGF were very high in DS subjects than in controls. NGF values of young and middle age subjects were significantly higher than controls (242.2 ± 116.4 SD pg/ml vs 29.4 ± 18.9 SD pg/ml, $p < 0.001$); differences were also found between elderly DS subjects and controls (139 ± 64.3 SD pg/ml vs 29.4 ± 18.9 SD pg/ml, $p < 0.05$).

However, a significant age related decrement of this neurotrophin was observed in DS. In fact, elderly subjects had significantly lower NGF values than they younger counterparts (139 ± 64.3 SD pg/ml vs 405.8 ± 125.4 SD pg/ml, $p < 0.001$). Middle age subjects had significantly lower NGF values than those from DS children (242.2 ± 116.4 SD pg/ml vs 405.8 ± 125.4 SD pg/ml, $p < 0.001$). Differences were also found between middle age and elderly subjects (242.2 ± 116.4 SD pg/ml vs 139 ± 64.3 SD pg/ml, $p < 0.05$).

Figure 2 shows plasma levels of IL-6. Plasma Concentrations of IL-6 in were significantly higher in young and middle age DS subjects than controls (111.2 ± 76 SD pg/ml vs 7.8 ± 4.6 SD pg/ml, $p < 0.001$ and 66 ± 50.3 SD pg/ml vs 7.8 ± 4.6 SD pg/ml, $p < 0.01$). No differences were observed between elderly DS subjects and controls were observed (33.1 ± 21.7 SD pg/ml vs 7.8 ± 4.6 SD pg/ml, $p > 0.05$). Elderly DS subjects had significantly lower IL-6 values than they younger counterparts (33.1 ± 21.7 SD pg/ml vs 111.2 ± 76 SD pg/ml, $p < 0.05$). No differences were found between middle age and elderly subjects (66 ± 50.3 SD pg/ml vs 33.1 ± 21.7 SD pg/ml, $p > 0.05$). On the other hand, DS children showed higher IL-6 values than adult with DS (66 ± 50.3 SD pg/ml vs 111.2 ± 76 SD pg/ml, $p < 0.05$).

Plasma levels of MCP-1 are shown in Figure 3 and were significantly higher in young and middle age DS subjects than controls (252.1 ± 91 SD pg/ml vs 91 ± 19

Figure 3. MCP-1 plasma concentrations expressed as pg/mL. Data are presented as mean \pm Standard deviation (SD).



SD pg/ml, $p < 0.001$ and 174.9 ± 62.3 SD pg/ml vs 91 ± 19 SD pg/ml, $p < 0.01$). No difference between elderly DS subjects and controls was observed (103.7 ± 42.1 SD pg/ml vs 91 ± 19 SD pg/ml, $p > 0.05$). Elderly DS subjects had significantly lower MCP-1 values than DS children (103.7 ± 42.1 SD pg/ml vs 252.1 ± 91 SD pg/ml, $p < 0.001$). Also DS adult subjects had significantly lower NGF values than DS children (174.9 ± 62.3 SD pg/ml vs 252.1 ± 91 SD pg/ml, $p < 0.01$). Our data show differences also between middle age and elderly subjects (174.9 ± 62.3 SD pg/ml vs 103.7 ± 42.1 SD pg/ml, $p < 0.05$).

Discussion

Our study showed that the plasma NGF of old subjects with Down syndrome decreased with age. NGF is the best known neurotrophin, which not only acts on central nervous system, but might also maintain a balance interplay between nervous, immune and endocrine system [7]. In fact the circulating levels of NGF increases in inflammatory responses, in various autoimmune diseases, in infections and in allergic disease [7]. Neurochemical and behavioural changes in the adult, which occur in most of the mental retardation, such as Down syndrome, are known to be critically influenced by brain plasticity, mediated by neurotrophins [25].

NGF is becoming an interesting target for neuropsychiatric research, and for our knowledge there are no data concerning NGF plasma levels in adults and elderly subjects with Down syndrome. Few studies have examined human NGF plasma concentration in different mental disorders and compared them to healthy controls; the data reported in these studies are scanty, and variation with age are sometimes contradictory.

An age related-decrement of NGF has previously been showed in healthy elderly without DS [26] and in cancer patients [27]. Data from the present investigation from the healthy of DS patients over 60 years shows a lower NGF plasma concentrations when compared to young patients. However, NGF circulating levels were higher than those from healthy controls. Our findings are at variance with those of another report which showed a decreased levels of peripheral NGF in children and teenagers with DS.

These Authors did not investigated cytokine blood levels in DS in their study [28]. On the other hand, we found a concomitant elevation of plasma IL-6 in DS, being this cytokine an activator of NGF release [17]. It is of interest that DS children and adults enrolled in our investigation were living at home and in good health status. Our findings on elevated NGF in DS paralleled those from a previous report showing elevated NGF and other neurotrophins blood levels in neonatal children with mental retardation [29]. Moreover, our data are also in line with several studies in which high NGF concentrations was shown to reflect an increased vulnerability to mental [30] or inflammatory disease [31].

IL-6 levels were increased in DS subjects, particularly in young subjects and these findings confirm previous observations showing elevated IL-6 and sIL-6R in children with DS assessed by different methods [5, 21]. Elevated IL-6 blood levels have been described in non DS-elderly and have been correlated with the age-associated frailty and cognitive impairment [20]. Increased circulating IL-6 [32] or sIL-6R [33] have also been reported in patients with AD and elevated plasma IL-6 predicted subsequent cognitive decline in subjects from the MacArthur Study of Successful Aging [34]. Recent findings showed that elevated levels of IL-6, soluble IL-6 receptor (sIL-6R), and soluble vascular cell adhesion molecule-1 (sVCAM-1) were increased in DS children [14] and suggested a possible remodelling of molecule network with regulatory function upon the vessel system in DS.

Here we showed that plasma IL-6 and MCP-1 levels were elevated in DS and these data further support the notion of vessel dysfunction in DS.

The plasma level increment of this cytochines could not be ascribed to a clinical inflammation, since DS subject were free of clinically apparent pathological conditions. A sub-clinical inflammation could also be unlikely, since neopterin plasma levels were normal in these DS subjects. Moreover, circulating CRP levels were within the normal range in these DS groups [14]. Therefore, it is likely that the plasma increment of these analytes could be ascribed to an endothelial activation without activation of immune responses. Previous investigations showing increased sICAM-3 and sVCAM-1 in DS plasma also

suggested the presence of mild activation and/or moderate dysfunction of endothelial cells in these subjects [35]. Elevated levels of IL-6 and intercellular adhesion molecules also have been associated with endothelial dysfunction in different pathological conditions, such as atherosclerosis and its complications [36]. However, DS is considered a human condition with low clinical atherosclerosis and low cardiovascular disease's risk during adulthood and ageing [37, 38].

An increment of molecules with functional relevance in endothelial cell activation should induce an elevated risk of atherosclerosis in DS. However, as already stated, DS has been considered an atheroma free model [11] and clinical investigations do not report elevated risk of cardiovascular disease in adult and elderly DS. Therefore, DS subjects represent a still unsolved biological/clinical paradox.

Recent studies have also reported the potential importance of neurotrophins in atherosclerosis and related disorders [8, 39]. A significant decrease in plasma NGF was also associated with the metabolic syndrome and atherosclerosis [9].

Conclusion

We suggest that in DS subjects elevated peripheral NGF may play a protective role against atherosclerosis and its clinical complications by regulating endothelial activation. However, a significant decrement of plasma NGF in elderly with DS might be related to the increased incidence of dementia in old subjects with the syndrome. In fact, recently, in an animal model for DS, middle-age trisomic mice (Ts65Dn), it has been demonstrated that decreased NGF levels in limbic areas correlate with progressive memory decline and cholinergic degeneration [40].

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