Increase of SCF plasma concentration during donepezil treatment of patients with early Alzheimer’s disease

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Abstract

Alzheimer’s disease (AD) can be treated with inhibitors of the enzyme acetylcholinesterase (AChE). There is evidence that AChE inhibitors promote neuroprotective effects and neurogenesis in the central nervous system (CNS). However, the mechanisms by which AChE inhibitors mediate these effects are still not well understood. One possible mechanism could be the up-regulation of haematopoietic growth factors (HGFs), also known to promote neuroprotective effects and to stimulate neurogenesis in the CNS. In the present study we investigated the impact of a 15-month treatment with the AChE inhibitor donepezil on blood levels of the HGFs stem cell factor (SCF), stromal cell-derived factor 1 (SDF-1), granulocyte colony-stimulating factor (G-CSF) and vascular endothelial growth factor (VEGF) in 19 patients with AD and 45 age-matched healthy controls. Before treatment with donepezil we found in AD patients significantly decreased SCF plasma concentrations (661.1 ± 40.0 pg/ml) compared to healthy controls (997.7 ± 33.7 pg/ml, p < 0.001) but no significant differences between both groups concerning blood levels of SDF-1, G-CSF and VEGF. After 15 months’ treatment SCF plasma levels increased significantly in the AD patients (764.5 ± 41.5 pg/ml, p = 0.016). In addition, we found a significant positive correlation between SCF plasma levels at baseline and changes of cognitive functions over the 15-month period (r = 0.521, p = 0.022). For the other HGFs we were unable to show a significant impact of donepezil treatment. Our findings indicate that donepezil treatment of AD patients is associated with an up-regulation of SCF plasma levels, which may contribute to neuroprotection and neurogenesis in the CNS.

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Key words: Donepezil, granulocyte colony-stimulating factor, stem cell factor, stromal cell-derived factor 1, vascular endothelial growth factor.

Introduction

Alzheimer’s disease (AD) is the most common cause of dementia in older people characterized by the accumulation of amyloid plaques and neurofibrillary tangles in the brain (Blasko & Grubeck-Loebenstein, 2003). A treatment with inhibitors of the enzyme acetylcholinesterase (AChE) is recommended by several guidelines. These drugs enhance the central concentrations of synaptic acetylcholine, thus improving interaction between neurons of the cholinergic system that are involved in memory function. Besides this symptomatic mechanism of action there is accumulating evidence that AChE inhibitors have neuroprotective effects and thereby a disease-modifying potential (Nordberg, 2006). In a previous study we were able to show that treatment with the AChE inhibitor donepezil is paralleled with an increase of brain-derived neurotrophic factor (BDNF) serum concentration in AD patients, reaching the levels of healthy controls. We concluded that up-regulation of BDNF might be part of a neuroprotective effect of AChE inhibitors (Leyhe et al. 2008).

Recent examinations have indicated increased neurogenesis in the hippocampus in two animal models of AD (Jin et al. 2004a; Lopez-Toledano & Shelanski, 2007) and in the active form of AD (Jin et al. 2004b).
In animal models it could be shown that donepezil promotes the survival of newborn neurons in the adult dentate gyrus and olfactory bulb (Kaneko et al. 2006; Kotani et al. 2006, 2008), brain regions that are involved early in the neurodegenerative process of AD.

Haematopoietic growth factors (HGFs) like stem cell factor (SCF), the chemokine CXCL12 (also known as stromal cell-derived factor 1, SDF-1), granulocyte colony-stimulating factor (G-CSF) and vascular endothelial growth factor (VEGF) are important for the mobilization, survival, proliferation, and differentiation of haematopoietic stem cells (HSCs) and other haematopoietic progenitor cells (Broudy, 1997; Gazitt, 2002; Greenberg & Jin, 2005; Lapidot et al. 2005; Zhou et al. 2007). However, recent studies have indicated that they might also be relevant for neuroprotection and neurogenesis in the central nervous system (CNS) (Cao et al. 2004; Dhandapani et al. 2005; Jin et al. 2002; Kawada et al. 2006; Komine-Kobayashi et al. 2006; Robin et al. 2006; Schneider et al. 2005; Shetty et al. 2005; Stumm & Höltt, 2007; Zhang & Fedoroff, 1999). Previous studies have shown decreased CXCL12 (SDF-1) plasma levels (Laske et al. 2008a), decreased plasma and cerebrospinal fluid levels of SCF (Laske et al. 2008b), decreased plasma levels of G-CSF (Laske et al. in press), and decreased VEGF serum levels in AD patients (Mateo et al. 2007).

In the present study we investigated the influence of a 15-month treatment with donepezil on blood levels of the HGFs: SCF, SDF-1, G-CSF and VEGF in AD patients hypothesizing that the AChE inhibitor might induce an increase of these factors with possible impact on neuroprotection and neurogenesis in the CNS.

Materials and methods

Subjects and clinical assessment

Nineteen AD patients and 45 healthy controls were investigated. All AD patients met the diagnostic criteria of probable AD according to DSM-IV (APA, 1994), ICD-10 (WHO, 1992) and the criteria of the National Institute of Neurologic and Communicative Disorders and Stroke (NINCDS) and the Alzheimer’s Disease and Related Disorders Association (ADRDRA; McKhann et al. 1984). The severity of dementia was assessed by Mini Mental State Examination (MMSE; Folstein et al. 1975) at baseline before treatment with donepezil and after 15 months’ treatment with 10 mg/d donepezil. The MMSE is a 30-point questionnaire that is used to test for cognitive impairment. It examines orientation, memory, attention, language use and comprehension, arithmetic, executive functions and constructional skills (Table 1). The investigator performing the MMSE after 15 months’ treatment was blinded for the MMSE scores at baseline. HGF measurements were performed without knowledge of the cognitive state. The AD patients and the control group underwent a physical, neurological, and psychiatric examination. In addition, an electroencephalography and a computed tomography or magnetic resonance imaging (MRI) were also performed to validate the diagnosis of AD. Routine laboratory tests included analysis of vitamin B12, folic acid and thyroid-stimulating hormone levels, to exclude other causes of dementia. The control subjects had a normal cognitive status according to clinical examination and MMSE score. Exclusion criteria for AD patients and control subjects were one of the following well-established cerebrovascular risk factors (hypertension, diabetes mellitus, chronic ischaemic heart disease, angina pectoris, myocardial infarction and heart failure), a current or a history of depression or psychosis, alcohol or substance abuse or use of psychoactive medications.

The ethical committee of the medical faculty at the University Hospital of Tübingen approved the study and written informed consent was obtained from each individual.

Sample collection

Blood was obtained in the morning (08:00–09:00 hours; in the fasting state) after 30 min rest into heparin plasma and serum tubes and then immediately placed in ice for 20 min. The plasma tubes were then centrifuged at 2500 g for 30 min at 4 °C, and the top third of the volumes of the resultant plasma supernatants were collected and frozen at –20 °C for evaluation. Plasma and serum tubes were also immediately immersed in melting ice. To minimize the source of platelets, plasma and serum was centrifuged within 30 min after collection and stored at –18 °C until further analysis.

In AD patients HGF concentrations were measured at baseline before treatment with donepezil, and after 15 months’ treatment with donepezil.

Measurement of SCF, CXCL12, G-CSF and VEGF concentrations in plasma/serum

Soluble SCF, CXCL12 and G-CSF plasma levels and VEGF serum levels were measured using ELISA kits (R&D Systems, Germany) according to the manufacturer’s instructions. Every ELISA kit contained a 96-well microplate pre-coated by the manufacturer with a monoclonal antibody (Ab) specific for the antigen. The ELISA kits for detection of SDF-1 (cat no. DSA00),
VEGF (cat no. DVE00) and SCF (cat no. DCK00) work with a monoclonal Ab from mouse as capture and with a polyclonal Ab from goat conjugated to horse-radish peroxidase (HRP) as detection Ab. The high sensitivity G-CSF ELISA (cat no. HSCS0) works with a monoclonal Ab from mouse as capture and with a polyclonal Ab from goat conjugated to alkaline phosphatase as detection Ab. All concentrations of reagents in these ready-to-use ELISAs are proprietary information of the manufacturer.

Recombinant soluble *E. coli* expressed VEGF\textsubscript{165}/SCF (KL-1 variant)/methionyl form of the 174 amino-acid residue isoform of human G-CSF/SDF-1\textsubscript{a} were reconstituted in 1 ml deionized water (for SDF-1\textsubscript{a}) or in an animal serum with preservatives named Calibrator diluent (1 ml for VEGF, 5 ml for SCF and G-CSF) and from this stock solution a dilution series was produced. SDF-1\textsubscript{a}, stock solution: 100 000 pg/ml; dilution series to produce the standard curve: 10 000–156 pg/ml. SCF and VEGF, stock solution: 2000 pg/ml; dilution series: 2000–31.2 pg/ml. G-CSF, stock solution: 80 pg/ml; dilution series: 80–1.25 pg/ml. The serum and plasma samples in these tests needed no dilution.

The assay procedure was as follows. First, 100 \(\mu l\) of a buffered protein base (assay diluent) was added to each well, followed by pipetting 100 \(\mu l\) of standard or undiluted sample (serum or plasma) in duplicates. Next, the plates were incubated for 2 h (3 h for G-CSF) at room temperature. After the incubation period, each well was aspirated and washed with 400 \(\mu l\) wash buffer per well, containing buffered surfactant, repeated 3–4 times (6 times for G-CSF). Subsequently, 200 \(\mu l\) of conjugate solution was added to each well, containing a recombinant lyophilized human polyclonal antibody conjugated to HRP (for G-CSF conjugated to alkaline phosphatase).

Again, the microplate was incubated for 2 h at room temperature. After the incubation period the wash step was repeated as described above. Then 200 \(\mu l\) (50 \(\mu l\) for G-SCF) freshly prepared substrate solution composed of stabilized hydrogen peroxide and tetramethylbenzidine as chromogen (for G-CSF: lyophilized NADPH with stabilizers) was added to each well and incubated for 20–30 min (1 h for G-CSF) at room temperature protected from light.

A blue colour developed in proportion to the amount of antigen in the sample/standard. The G-CSF

<table>
<thead>
<tr>
<th>Table 1. The items of the Mini Mental State Examination and their scoring (maximum points in parentheses)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Orientation (10)</strong></td>
</tr>
<tr>
<td>• The subject is asked: What is the year, season, date, day and month (1 point for each; maximum total 5 points)</td>
</tr>
<tr>
<td>• The subject is asked: Where are we: town, county, country, which hospital, surgery or house, and which floor (1 point for each; maximum total 5 points)</td>
</tr>
<tr>
<td><strong>Immediate recall (3)</strong></td>
</tr>
<tr>
<td>• Three objects are named and the subject is asked to repeat all three objects</td>
</tr>
<tr>
<td><strong>Attention and calculation (5)</strong></td>
</tr>
<tr>
<td>• The subject is asked to subtract 7 from 100, and then to subtract 7 from the answer until 65</td>
</tr>
<tr>
<td>• Alternatively the subject is asked to spell the word ‘world’ backwards</td>
</tr>
<tr>
<td><strong>Delayed recall (3)</strong></td>
</tr>
<tr>
<td>• The subject is asked to repeat the 3 objects of the immediate recall item</td>
</tr>
<tr>
<td><strong>Naming (2)</strong></td>
</tr>
<tr>
<td>• The subject is shown two common objects and asked to name them</td>
</tr>
<tr>
<td><strong>Repetition (1)</strong></td>
</tr>
<tr>
<td>• The subject is asked to repeat the following sentence exactly: ‘No ifs, ands, or buts’</td>
</tr>
<tr>
<td><strong>3-Stage Command (3)</strong></td>
</tr>
<tr>
<td>• The subject is given a piece of blank white paper and asked to follow a 3-stage command: ‘Take the paper in your right hand, fold it in half and put it on the floor’ (1 point for each part that is correctly followed)</td>
</tr>
<tr>
<td><strong>Reading (1)</strong></td>
</tr>
<tr>
<td>• The subject is asked to read and follow the command: ‘Close your eyes’</td>
</tr>
<tr>
<td><strong>Writing (1)</strong></td>
</tr>
<tr>
<td>• The subject is asked to write a complete sentence</td>
</tr>
<tr>
<td><strong>Copying (1)</strong></td>
</tr>
</tbody>
</table>
| • The subject is shown a drawing of 2 pentagons which intersect to form a quadrangle and asked to copy the design exactly as it is
kit is a highly sensitive kit and an additional step is needed because of the very low amount of antigen. Fifty microlitres of amplifier solution had to be added for 30 min after incubation with the substrate. This solution containing lyophilized amplifier enzymes and INT-Violet initiated the colour development.

Finally, the colour development was stopped by addition of 50 μl of 2 N sulphuric acid, which resulted in a change of colour from blue to yellow (for G-CSF it does not affect the former red colour). The optical density was determined within 30 min using a microplate reader (Tecan Sunrise, Switzerland) at a wavelength of 450 nm (correction at 540 nm) for SCF, SDF-1α, VEGF and 490 nm (correction at 620 nm) for G-CSF.

All samples and standards were measured in duplicate, and the means of the duplicates were used for statistical analyses. The intra- and inter-assay coefficients of variation of SCF in AD patients and healthy controls were <10%.

**Data analysis**

The data are presented as the mean ± standard error of mean (s.e.m.). For statistical analysis of gender differences between the AD patients and the healthy controls Fisher’s exact test was used. The Kolmogorov–Smirnov test was used to verify normal distribution of HGFs. All \( p \) values were between 0.176 and 0.996. Thus, all data have been proven to be normally distributed. Therefore we used \( t \) tests for further analyses. HGF concentrations of AD patients at baseline and after 15 months’ treatment with donepezil were compared by two-tailed \( t \) test for paired samples. The two-tailed \( t \) test for unpaired samples was used to compare HGF levels between the groups. After controlling for Gaussian distribution, correlations between variables were determined using Pearson’s test. Significance for the results was set at \( p < 0.05 \). All statistical analyses were carried out using the SPSS statistical analysis software package version 14.0 (SPSS Inc., Germany).

**Results**

AD patients and healthy controls were comparable regarding age and gender, showing no significant differences (Table 2).

Before treatment with donepezil we found in AD significantly decreased SCF plasma concentrations (661.1 ± 40.0 pg/ml) compared to healthy controls (997.7 ± 33.7 pg/ml, \( p < 0.001 \)). After 15 months’ treatment the SCF serum concentration increased significantly in the AD patients (764.5 ± 41.5 pg/ml, \( p = 0.016 \); Fig. 1, Table 3). The difference compared to healthy controls was still significant (\( p < 0.001 \), Fig. 1).

MMSE scores of AD patients after 15 months’ treatment with donepezil were significantly diminished

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**Table 2.** Demographic and clinical parameters of Alzheimer’s disease (AD) patients, before treatment with donepezil, and the control group

<table>
<thead>
<tr>
<th>Variables</th>
<th>AD patients ((n=19))</th>
<th>Control group ((n=45))</th>
<th>( t )</th>
<th>d.f.</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female, ( n )</td>
<td>4/15</td>
<td>19/26</td>
<td>–</td>
<td>1</td>
<td>0.155*</td>
</tr>
<tr>
<td>Age, yr (mean ± s.e.m.)</td>
<td>70.8 ± 2.0</td>
<td>68.0 ± 1.5</td>
<td>1.034</td>
<td>62</td>
<td>0.305 b</td>
</tr>
<tr>
<td>MMSE (mean ± s.e.m.)</td>
<td>23.5 ± 0.4</td>
<td>28.5 ± 0.2</td>
<td>– 12.040</td>
<td>62</td>
<td>&lt;0.0001 b</td>
</tr>
</tbody>
</table>

\( d.f. \), Degree of freedom; MMSE, Mini Mental State Examination; \( n \), number of subjects; s.e.m.; standard error of mean.

* Fisher’s exact test.

b Two-tailed \( t \) test.
Table 3. Mean haematopoietic growth factor concentrations of Alzheimer’s disease patients before treatment (AD-T0) and after 15 months’ treatment with donepezil (AD-T1)

<table>
<thead>
<tr>
<th>Variables</th>
<th>AD patients</th>
<th></th>
<th>AD patients</th>
<th></th>
<th>t</th>
<th>d.f.</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T0 (1)</td>
<td>T1 (2)</td>
<td>t</td>
<td>d.f.</td>
<td>p value</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n = 19)</td>
<td>(n = 19)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCF (mean ± S.E.M.) (pg/ml)</td>
<td>661.1 ± 40.0</td>
<td>764.5 ± 41.5</td>
<td>-2.656</td>
<td>18</td>
<td>0.016</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDF-1 (mean ± S.E.M.) (pg/ml)</td>
<td>2649.6 ± 119.9</td>
<td>2510.3 ± 127.3</td>
<td>1.668</td>
<td>18</td>
<td>0.113</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-CSF (mean ± S.E.M.) (pg/ml)</td>
<td>23.0 ± 2.9</td>
<td>23.4 ± 2.0</td>
<td>-0.108</td>
<td>18</td>
<td>0.915</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF (mean ± S.E.M.) (pg/ml)</td>
<td>336.6 ± 46.9</td>
<td>339.9 ± 54.7</td>
<td>-0.152</td>
<td>18</td>
<td>0.881</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

d.f., Degree of freedom; G-CSF, granulocyte colony-stimulating factor; SCF, stem cell factor; SDF-1, stromal cell-derived factor 1; S.E.M., standard error of mean; VEGF; vascular endothelial growth factor; n, number of subjects.

a Two-tailed t test for paired samples.

Discussion

To our knowledge this is the first study describing the impact of a treatment with an AChE inhibitor on HGF blood levels in patients with AD. After 15 months’ treatment with the AChE inhibitor donepezil we found a significant increase in SCF plasma levels, while no effect on the other investigated HGFs was seen. This result points towards a selective effect of donepezil on the induction of SCF. Increased SCF plasma levels can be due to increased cleavage of SCF from the cell surface by activation of protein kinase C (PKC) (Huang et al. 1992). Interestingly, AChE inhibitors have been demonstrated to activate PKC in vitro (Pakaski et al. 2001). This mechanism could explain how donepezil is able to specifically increase SCF plasma levels as demonstrated in the present study. The finding of increased SCF plasma levels under donepezil treatment might be of clinical relevance in AD, as higher SCF plasma levels at baseline were correlated with minor cognitive decline over the 15-month treatment period. This result indicates a potential protective effect of SCF plasma levels against cognitive decline in AD. As we did not find a significant correlation between SCF plasma levels and age in AD patients, age-related effects on SCF plasma levels could be excluded.

SCF, also known as steel factor, c-kit ligand or mast cell growth factor, is a HGF that binds to the receptor encoded by the proto-oncogene c-kit. SCF exists in two forms, membrane-bound and soluble. Soluble SCF is produced by the cleavage of membrane-bound SCF by metalloproteases. Both the soluble and the
membrane-bound form of SCF are biologically active (Anderson et al. 1990). SCF is a growth factor important for the mobilization, survival, proliferation, and differentiation of HSCs and other haematopoietic progenitor cells (Broudy, 1997). Alterations of circulating SCF levels and SCF gene mutations have been associated with haematological disorders of erythrocytes (e.g. sickle cell anaemia) and mast cells (e.g. mastocytosis) (Boissian et al. 2000; Croizat & Nagel, 1999).

While the important roles of SCF for haematopoiesis are broadly accepted, recent studies indicate that this chemokine has also direct effects on the CNS. SCF and its receptor c-kit are both highly expressed in the developing and adult CNS (Hirota et al. 1992; Manova et al. 1992; Matsui et al. 1990; Morii et al. 1992). Brain injury leads to an up-regulation of SCF and c-kit, implying an additional role in the cellular response to damage (Zhang & Fedoroff, 1999). In the CNS, SCF is a chemoattractant and a survival factor for neuronal stem/progenitor cells (NSPCs; Erlandsson et al. 2004), it exerts neuroprotective effects (Dhandapani et al. 2005), stimulates neurogenesis in vitro and in vivo (Jin et al. 2002) and modulates microglia (Zhang & Fedoroff, 1999). Sun et al. (2004) have demonstrated that SCF mRNA and protein are highly induced in neurons within the zone of injured brain, that the SCF receptor c-kit is expressed on NSPCs and that recombinant SCF induces potent NSPC migration through the activation of c-kit on NSPCs. Subcutaneous administration of SCF in the acute phase of focal brain ischaemia leads to increased NSPC proliferation, reduction in infarction size, and functional improvement (Kawada et al. 2006; Zhao et al. 2007a). Moreover, systemic administration of SCF increases bone marrow-derived neurons in the intact adult mouse brain (Corti et al. 2002). Previous studies have demonstrated deficits in spatial learning and memory and long-term potentiation in SCF mutant mice (Motro et al. 1996) and c-kit mutant rats (Katafuchi et al. 2000). These data suggest potentially important roles for SCF/c-kit in cognitive processes. Experimental data in rats have shown that SCF pass through the intact blood–brain barrier via receptor-mediated transport (Zhao et al. 2007b). Taken together, decreased SCF plasma levels may contribute to a disturbance of cognitive processes in AD patients and an increase evoked by donepezil treatment might exert neuroprotective effects and stimulate neurogenesis, as suggested by our study.

In a previous study we have shown that treatment with the AChE inhibitor donepezil is paralleled with an increase of BDNF serum concentration in AD patients (Leyhe et al. 2008). Interestingly, Zhang & Fedoroff (1998) have shown that recombinant SCF induces the expression of BDNF and inhibits inflammatory gene expression in microglia. Thus, the increase of BDNF during donepezil treatment could potentially be SCF mediated. Similarly, the induction of SCF may functionally limit microglial reactivity, a hypothesized factor in AD progression. Future studies should establish a temporal pattern of BDNF and SCF increase to determine whether the rise of SCF precedes increased BDNF expression.

The elevation of serum SCF was not paralleled by a cognitive stabilization or improvement in the AD patients in our study. MMSE score significantly decreased during donepezil treatment. Generally there is a decline in cognition to below baseline levels after ~1 yr treatment with AChE inhibitors, but the level of cognition remains above that predicted for those not receiving pharmacological treatment (Winblad & Jelic, 2004). Thus, it appeared to be unethical to put AD patients on placebo for 15 months. We cannot say whether a placebo group would have shown more cognitive decline and no up-regulation of SCF serum levels. In addition, the small sample size of the study should be mentioned. The results of the present investigation should be confirmed in larger studies. Moreover, it should be examined whether the rise of SCF could also be induced by other AChE inhibitors.

In summary, the results of the present study confirm data of a prior investigation showing a decrease of SCF plasma levels in AD patients. Treatment with the AChE inhibitor donepezil is paralleled with an increase of SCF plasma concentration in AD. This up-regulation of SCF might be part of a neuroprotective effect of AChE inhibitors. The molecular mechanism of this potentially disease-modifying mechanism of action of donepezil should be clarified.

Acknowledgements

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Statement of Interest

None.

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