Chapter 8

Inflammation and Stroke

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ABSTRACT

Background Experimental evidence indicates that IL-10 deficiency is associated with the development of cardiovascular and cerebrovascular disease. We analyzed the relation between low Interleukin-10 (IL-10) production levels, a history of stroke and incident fatal stroke.

Methods All 85-year-old inhabitants of Leiden (n=599) were visited at their place of residence (response rate 87%). Production levels of the anti-inflammatory cytokine IL-10 were assessed in a whole blood assay whereby lipopolysaccharide was used as a stimulus. Plasma concentrations of C-reactive protein (CRP) were also used as a marker of inflammation. A history of stroke was obtained at baseline (prevalence 10%). The number of fatal strokes were prospectively obtained for a median follow-up of 2.6 years (incidence 1.82 per 100 person-years at risk).

Results Subjects with a history of stroke had significant lower median IL-10 production levels at baseline than subjects without stroke (558 pg/ml versus 764 pg/ml, p<0.05). They also had significant higher median CRP concentrations (6 mg/l versus 3 mg/l, p<0.05). The odds ratio for a history of stroke increased to 2.30 (95 % CI 1.12-4.72) over strata representing decreasing production levels of IL-10. The relative risk for incident fatal stroke was 2.94 (95 % CI 1.01-8.53) when we compared both subjects with low or intermediate baseline IL-10 production levels to those with high production levels of IL-10.

Conclusion Our data support the hypothesis that subjects with low IL-10 production levels have an increased risk of stroke.
Introduction
Accumulating evidence suggests that inflammation plays an important role in the development of cardiovascular and cerebrovascular disease\textsuperscript{1-3}. Markers of inflammation, such as C-reactive protein (CRP)\textsuperscript{1,2}, and pro-inflammatory cytokines are associated with stroke\textsuperscript{4}.

Interleukin-10 (IL-10) is a centrally operating anti-inflammatory cytokine, which plays a crucial role in the regulation of the innate immune system. It has strong de-activating properties on the inflammatory host response, and potently inhibits the production of pro-inflammatory cytokines\textsuperscript{5}. Animal models investigating the protective role of IL-10 in atherosclerosis show that IL-10 deficient mice have a high susceptibility to atherosclerosis\textsuperscript{6}. Moreover, IL-10 deficient mice have an increased stroke lesion size after ligation of the mid-cerebral artery\textsuperscript{7}, whereas rats treated with IL-10 have a decreased stroke lesion size\textsuperscript{8}.

Here, we tested the hypothesis that a pro-inflammatory cytokine response predisposes to stroke. We therefore analyzed the association between low IL-10 production levels and a history of stroke. We also determined the association between low IL-10 production levels measured at baseline and incident fatal stroke.

Methods
Between 1st September 1997 and 1st September first 1999, 705 inhabitants of Leiden reached the age of 85 years and were eligible to participate in the Leiden 85-plus Study. There were no selection criteria on health or demographic characteristics. The Medical Ethical Committee of the Leiden University Medical Center approved the study. Fourteen inhabitants died before they could be enrolled. The response rate was 87%, a total of 599 subjects (397 women, 202 men) participated. There were no significant differences for various demographic characteristics between the 599 respondents and the source population.

The WHO definition of stroke “rapidly developing clinical signs of focal (at times global) disturbance of cerebral functioning lasting > 24 hours” was used to identify subjects with a history of stroke. All subject’s general practitioners or the individual subject’s treating (nursing home) physician were interviewed to obtain a complete medical history, including a history of stroke. The advantage of using general practitioners to obtain a history of stroke was that subjects with a history of stroke, who were not hospitalized, were also included in the study. All subjects were followed up for mortality until 1st September 2001. The primary and secondary causes of death were obtained from subjects’ general practitioners or treating physicians. Fatal stroke was classified according to the ICD-10 codes I60-I69.

IL-10 production in lipopolysaccharide stimulated whole blood samples varies between individuals. This interindividual variation has a strong genetic basis. Family studies of first-degree relatives and analysis of twins indicate that as much of 75% of the differences in quantitative IL-10 production in humans derive from heritable factors\textsuperscript{9,10}. The innate IL-10 production was assessed with an \textit{ex vivo} whole-blood
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assay. The full methods by which whole-blood samples were simulated with 10 ng/ml of lipopolysaccharide have been described elsewhere. Unstimulated baseline samples were obtained to serve as a control for contamination. Subjects with detectable Tumor Necrosis Factor-α concentrations (TNF-α) under unstimulated conditions (TNF-α > 100 pg/ml) were therefore excluded from further analysis. Plasma levels of CRP were measured using a fully automated Hitachi 911.

Subjects were classified as having diabetes when they met at least one of the following criteria: (1) history of diabetes, obtained from the subject’s general practitioner, or treating physician, (2) use of sulphonylureas, biguanides or insulin, obtained from the subject’s pharmacist, or (3) non fasting glucose concentrations of 11.1 mmol/l or higher. Subjects were classified as having hypertension when they met at least one of the following criteria: (1) history of hypertension, (2) use of β-blockers, ACE-inhibitors, thiazide diuretics or calcium antagonists, obtained from the subject’s pharmacist, or (3) a diastolic blood pressure of 95 mm Hg or higher or a systolic blood pressure of 180 mm Hg or higher. Subjects were classified as having cardiovascular disease, when they met at least one of the following criteria: (1) history of myocardial infarction, angina pectoris, arterial surgery, or intermittent claudication, (2) signs of myocardial infarction or myocardial ischaemia, recorded on the electrocardiogram, which was obtained in all subject’s. Finally, use of non steroidal anti-inflammatory drugs, including aspirin, was obtained from the subject’s pharmacist.

Data analysis

Data are presented as medians with corresponding 95% confidence intervals for the median, representing the range of values, which includes the “true” median. The non-parametric Mann-Whitney test was used, because IL-10 production levels and CRP concentrations were not normally distributed and skewed to the right. The production levels of IL-10 were grouped in three equal strata representing decreasing IL-10 production levels. This was done separately for women and men, since women had a lower IL-10 production than men. CRP concentrations were grouped using a similar approach. Multivariate odds ratios and 95% confidence intervals were obtained by logistic regression analysis, to determine the risk of a history of stroke depending on IL-10 production levels and plasma CRP concentrations at baseline (age 85). The p-values for trend over strata of IL-10 production and stroke and strata of CRP concentrations and stroke were determined using the log-likelihood statistic with one degree of freedom. Mortality risks were determined using multivariate Cox regression. To prevent that low IL-10 production levels were markers for imminent death, we excluded in an additional analysis those subjects, who died during the first half-year of follow-up.
Results

Data on the history of stroke were incomplete in two out of the 599 subjects. IL-10 production levels upon stimulation with endotoxin (lipopolysaccharide) in whole blood could not be obtained in 46 subjects. Since seven subjects died before a blood sample could be drawn, 30 subjects refused to give a blood sample and, nine subjects had detectable TNF-α concentrations (TNF-α $>$ 100 pg/ml) underunstimulated conditions and were therefore excluded from all analyses \textsuperscript{10,11}. Complete data on stroke and IL-10 production levels were therefore available for 551 subjects.

Cross-sectional study

Table 1 shows clinical and inflammatory characteristics of the subjects at baseline. Subjects with a history of stroke had lower median IL-10 production levels and higher median CRP concentrations than subjects without stroke (IL-10 production levels 558 pg/ml versus 764 pg/ml, $p=0.047$ and CRP 6 mg/l versus 3 mg/l, $p=0.004$). We found a dose-response relationship between IL-10 production levels and subjects without a history of stroke, subjects with one stroke ($n=45$) and 10 subjects with two or more strokes (IL-10 production levels 764 pg/ml, 597 pg/ml and 542 pg/ml respectively, $p$ for trend $=0.06$). In an additional analysis, we excluded subjects who used non-steroidal anti-inflammatory drugs ($n=153$). The IL-10 production levels and plasma CRP concentrations in subjects with a history of stroke compared to those without stroke remained similar (IL-10 production levels 545 pg/ml versus 756 pg/ml, $p=0.12$ and CRP 8 mg/l versus 3 mg/l, $p=0.003$).

| Table 1 Clinical and inflammatory characteristics in relation to a history of stroke |
|-----------------------------------------|-----------------------------------------|------------------|
| Stroke                                  | Absent ($n=496$)                        | Present ($n=55$) |
|                                         | p value                                 |                  |
| Clinical characteristics                |                                         |                  |
| Age (years)                             | 85                                      | 85               | 0.83            |
| Women                                   | 332 (67%)                               | 36 (65%)         | 0.41            |
| Type 2 diabetes                         | 78 (16%)                                | 11 (20%)         | 0.33            |
| Hypertension                            | 277 (56%)                               | 38 (69%)         | 0.06            |
| Cardiovascular disease                  | 285 (57%)                               | 36 (65%)         | 0.25            |
| Use of NSAID*                           | 125 (25%)                               | 28 (51%)         | <0.001          |
| Inflammatory characteristics †         |                                         |                  |
| Interleukin-10 (pg/ml)                  | 764 (727-803)                           | 558 (465-817)    | 0.047           |
| C-reactive protein (mg/l)               | 3 (3-4)                                 | 6 (3-9)          | 0.004           |

*NSAID, including use of aspirin. † Data are presented as medians and corresponding 95% confidence intervals.
The odds ratio for a history of stroke, adjusted for type 2 diabetes, hypertension, use of non-steroidal anti-inflammatory drugs, and cardiovascular disease increased to 2.30 (95 % CI 1.12-4.72) over strata representing decreasing production levels of IL-10 (figure 1, p for trend =0.018). The adjusted odds ratio for a history of stroke increased to 2.11 (95 % CI 1.00-4.40) over strata representing increasing CRP concentrations (p for trend =0.031). Each 500-pg/ml increase of IL-10 production corresponded to a 26% lower risk of having a history of stroke, odds ratio 0.74 (95 % CI 0.52–1.00). The results remained similar after adjustment for gender, type 2 diabetes, hypertension, use of non-steroidal anti-inflammatory drugs, and cardiovascular disease, odds ratio 0.70 (95 % CI 0.52–1.00).

Figure 1 Risk of stroke in relation to Interleukin-10 production and C-reactive protein
The odds ratios represented here are adjusted for diabetes, hypertension, use of non-steroidal anti-inflammatory drugs and cardiovascular disease. Bars represent 95% confidence intervals. Production level of IL-10 and plasma CRP concentrations are presented as medians.
Follow-up study

In total, 147 subjects died during a median follow-up of 2.6 years (incidence 1.82 per 100 person years at risk; 95 % CI 1.12-2.53). Twenty-six of them suffered a fatal stroke. Eight out of the 26 subjects with fatal stroke had a history of stroke at baseline. Only 4 out of the 183 subjects (2.1%) with high IL-10 production levels suffered a fatal stroke, whereas 12 subjects out of the 185 subjects (6.5%) with intermediate IL-10 production levels and 10 out of the 183 (5.5%) with low IL-10 production levels suffered a fatal stroke. Figure 2 shows the cumulative mortality for stroke, of the 551 participating subjects, over strata of IL-10 production and strata of CRP. The highest cumulative mortality for stroke was present for those with low or intermediate IL-10 production levels (p=0.06, Cox regression), and those with high or intermediate CRP (p=0.095, Cox regression).

The crude relative risk for incident fatal stroke was 2.94 (95 % CI 1.01-8.53) when we compared both subjects with low or intermediate production levels of IL-10 measured at baseline to those with high IL-10 production levels. Each 500-pg/ml increase of IL-10 production corresponded to a 36% decrease in mortality due to stroke, risk ratio 0.64 (95 % CI 0.39–1.00). The results remained similar after adjustment for gender, type 2 diabetes, hypertension, use of non-steroidal anti-inflammatory drugs, and cardiovascular disease, risk ratio 0.67 (95 % CI 0.41–1.00). The median IL-10 production level, measured at baseline, was lower in those with a fatal stroke (n=26) compared to those without a fatal stroke (n=525), 764 pg/ml vs. 715 pg/ml, p=0.07. The median CRP concentration, measured at baseline, was higher in those with a fatal stroke compared to those without a fatal stroke, 5mg/l vs. 3mg/l, p=0.14. To prevent that low IL-10 production levels were markers for intercurrent fatal disease we excluded those subjects who died during the first half-year of follow-up (n=11), leaving 540 subjects in the analysis. Adjustments were made for diabetes, hypertension, use of non-steroidal anti-inflammatory drugs, history of stroke and presence of cardiovascular disease at baseline, using Cox regression. After the age of 85.5 years the adjusted relative risk for incident fatal stroke (n=25) was 3.63 (95 % CI 1.08-12.21) when we compared both subjects with low or intermediate production levels of IL-10 measured at baseline to those with high IL-10 production levels.
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Figure 2  Cumulative mortality for stroke
Discussion
This analysis of the Leiden 85-plus Study shows that low IL-10 production levels are associated with both a history of stroke, in the cross-sectional study, and increased mortality due to stroke, as obtained in the prospective follow-up study. These associations persisted after adjustment for known risk factors for stroke. In line with earlier clinical studies we also showed that high CRP was associated with stroke\(^1\).\(^2\) Our findings extend data from animal models, which showed that IL-10 deficiency predisposes to atherosclerosis [6] and increases stroke lesion size\(^3\).\(^8\).

Since both our cross-sectional and longitudinal data showed an association between low IL-10 production levels and an increased risk of stroke, it is tempting to speculate that the association between low IL-10 production levels and stroke is causal. Furthermore we have previously shown associations between innate IL-10 production and multiple sclerosis\(^10\) and used a family design in which the cytokine response of the patient was estimated in first-degree relatives\(^9\)-\(^11\) . Also, the genetic basis of IL-10 production favors a causal interpretation of the association. Finally, other findings suggest that the cytokine response in whole blood induces the same effects in the brain across the blood-brain barrier\(^13\).

In our study both high CRP and low IL-10 production are associated with stroke. We feel that CRP and IL-10 at least partly represent the effect of an inflammatory response on stroke. Studies on cerebral ischaemia emphasize the relevance of an inflammatory response on lesion size, in which IL-10 is a key regulator\(^5\).\(^7\).\(^8\) . IL-10 is a powerful suppressor of the immune response, produced by T cells, B cells, monocytes, macrophages and microglia \(^5\).\(^14\) . It inhibits pro-inflammatory cytokines such as TNF-\(\alpha\) and IL-6\(^5\) . It could also inhibit CRP, since it has been suggested that IL-6 partly regulates CRP production\(^15\) . Moreover, IL-10 limits the size of ischaemic brain damage, occurring after occlusion of cerebral arteries\(^8\) . IL-10 could therefore represent a potential therapeutic agent for inflammatory diseases such as atherosclerosis and stroke.

In summary, low IL-10 production levels and high plasma CRP concentrations are associated with an increased risk of stroke, obtained at baseline and during follow-up. These findings support the hypothesis that a pro-inflammatory response predisposes to stroke.
References