The role of neutrophils and interleukin-8 in acute ischemic stroke

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ABSTRACT

الهدف: لكشف دور لانترليوكين (IL–8) والذي هو جاذب ومنشط كيميائي قوي للكريات المتعادلة في التلف الإفتقاري وتوسع الآفة عن طريق قيادة تراكم الكريات المتعادلة في المناطق الإفتقارية.

الطريقة: شملت الدراسة 76 مريضاً تم إدخالهم مستشفى ومركز حيدرباسا نومون للتدريب والبحث باسطنبول بتركيا في الفترة ما بين سبتمبر 2001م ويونيو 2002م مع التشخيص الأولي بجلطة افتقارية حادة ومجموعة التحكم المكونة من 28 شخصاً مع توافق في متوسط العمر. تم الحصول على مستويات مصل (IL–8) خلال 24 ساعة من التعرض للجلطة وتم تقييمها بواسطة طريقة (ELISA) . تم تقسيم المرضى الى أربع مجموعات وفقاً لتوسع موضع الآفات الإفتقارية. تم تقييم التوقع بعاقبة المرض بواسطة نقاط رانكين المعدلة (Rankin Scale) .

النتائج: بالمقارنة بين مجموعة المرضى ومجموعة التحكم، كان هنالك فرقاً إحصائياً ملحوظاً في مستوى (IL–8) ($p<0.001$) ومستويات الكريات المتعادلة ($p=0.000$) . كانت مستويات مصل (IL–8) متحدة مع توسع الآفة ($p<0.01$) . على الرغم من كون مستويات مصل (IL–8) أعلى بشكل ملحوظ في المجموعة المستقلة ($p>0.05$) . لم يكن هنالك أيضاً فرقاً ملحوظاً وفقاً للعمر ونوع الجنس وأسباب المرض بين مستويات (IL–8) ومستويات الكريات المتعادلة.

خاتمة: ارتفاع مستويات مصل (IL–8) متحداً مع التوقع بعاقبة المرض. يهدف تطوير الأدوية الوقائية العصبية الجديدة الى وقاية التهاب الكريات المتعادلة المحرض بواسطة (IL–8) ويعد ذلك كحالة حرجة في علاج الجلطة والوقاية من ازدياد الحالة السريرية سوءاً.

Objectives: To investigate the crucial role of interleukin 8 (IL-8) as an inflammatory marker in infarct evolution, and course of the disease.

Methods: The study included 76 patients that were admitted to Haydarpasa Numune Training and Research Hospital, Istanbul, Turkey between September 2001 and June 2002 with an initial diagnosis of acute ischemic stroke, and 28 control subjects with a corresponding mean age. The serum IL-8 levels obtained within 24 hours of the stroke were assessed by the enzyme-linked immunoabsorbent assay method. The patients were divided into 4 groups according to the extent, and localization of the ischemic lesions. Prognosis was evaluated by modified Rankin Scale.

Results: In comparison between patients and control groups, there was a statistically significant difference in ($p<0.001$) IL-8, and neutrophil (net) levels ($p=0.000$). The serum IL-8 levels were associated with the extent of the lesion ($p<0.01$). Though the serum IL-8 levels were significantly higher in the dependent group ($p<0.05$), there was no significant difference between net levels, and prognosis ($p>0.05$). There was also no significant difference according to age, gender, and etiology between IL-8 and net levels.

Conclusion: The high serum IL-8 levels are associated with prognosis. The development of new neuroprotective treatments aimed to prevent neutrophil-mediated-inflammation induced by IL-8 is critical in the treatment of stroke, and prevention of clinical worsening.

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Although pathophysiological mechanisms involved in acute ischemic stroke are not clearly understood, attention has been focused in the last decade on the significant role of the neuroinflammatory response, and peripheral blood components in the development of cerebral ischemia, and of the improvement in the clinical course as a result of inhibiting this mechanism.[1-3] The chemokines, which are chemotactic cytokines, cause cellular chemotaxis by regulating the relation between several inflammatory cell groups and the damaged tissue, and they take part in the lesion formation.[4] Endothelial cells contribute to the accumulation of neutrophils, and the synthesis of interleukin 8 (IL-8), which is a potent neutrophil chemoattractant, and

activator.[5] By leading to neutrophil accumulation, IL-8 gives rise both to mechanical obstruction in the collateral microcirculation in the capillaries by means of rheological impact, and to the secretion of toxic oxygen radicals, and enzymes from neutrophils such as protease, gelatinase, and collagenase, which causes damage in the tissues.[6-8] Hence, IL-8 and neutrophils take part in the augmentation of ischemic damage and extent of lesions, and the elevation of IL-8 is correlated with CNS injury.[9,10] Increased local expression of IL-8 is not only limited to the CNS, but also establishes a concentration gradient over the brain blood barrier, and it is systemically detectable in body fluids such as cerebrospinal fluid (CSF), plasma, and bronchoalveolar lavage samples.[11-14] It has also been shown that intrathecal synthesis of IL-8 is increased in patients with ischemic stroke.[11,12] The leukocytes accumulated within the damaged tissue in the brain after acute ischemia have a significant contribution to the inflammation. Though the primary role of the neutrophils, the earliest leukocyte subgroup participating in infections, is to defend the organism against several infectious agents, they are also as effective as potent mediators in non-infectious conditions such as ischemia, malignancy, and inflammatory demyelination syndromes like multiple sclerosis.[15-17] The attachment of neutrophils on the endothelium by adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), and P-selectin, and the migration of neutrophils by chemotactic factors in acutely developed inflammation after ischemia, has a critical role in infarct evolution that is also correlated with the concentration gradient of chemotactic factors.[9,18-20] In the present study, we determined the serum levels of IL-8 expressed as a response to acute ischemia in the brain, and examined the association of IL-8 and peripheric neutrophil count with the extent of the ischemic lesion, localization, and neurological outcome. Our aim was to investigate the crucial role of IL-8 as an inflammatory marker in infarct evolution, and course of the disease.

Methods. This prospective study included 76 patients, who were admitted to the Department of First Neurology, Haydarpasa Numune Training and Research Hospital, Istanbul, Turkey between September 2001 and June 2002. The patients with a first-ever acute ischemic stroke within the first 24 hours from onset were included in the study. The patients with a temporary ischemic attack or intracerebral hemorrhage, a history of previous stroke, a history of infection within the last 2 weeks, an acquired infection after the admission, a head trauma within the last one month, an autoimmune and immunosuppressive disease, or immunosuppressive drug use, newly acquired cardiac disease, rheumatic, hepatic or renal disease, and brain tumor or systemic cancer disease that may affect the serum levels of IL-8 were excluded. Each patient in the study group underwent a detailed anamnesis, physical and neurological examination, routine biochemical and hematological tests, urinary test, electrocardiography, chest radiography, echocardiography, bilateral carotid-vertebral artery Doppler ultrasonography assessments as well as cranial CT and/or MRI. According to the results of cranial CT or MRI at admission, and at day 3, dimensions of the infarct were assessed in all cases, and defined as follows: large infarct (LI) if the widest diameter is ≥3 cm, small infarct (SI) if the widest diameter is <3 cm,[21] and as cortical or subcortical according to their localization in the imaging. The cases were divided into 4 groups: cortical large infarct (CLI), cortical small infarct (CSI), subcortical large infarct (SLI), and subcortical small infarct (SSI). For routine hematological and biochemical tests, peripheral blood samples were obtained from an antecubital vein within the first 24 hours of the stroke. Suspensions of serum were centrifuged within 30 minutes at 1500g for 10 minutes, and immediately frozen and stored at -80°C until analysis. The serum IL-8 values were measured using enzyme-linked immunoabsorbent assay (ELISA) method according to the manufacturer's instructions (Immunotech, Marseille, France). The IL-8 determinations were performed blinded to clinical and radiological data. The etiology of ischemic stroke was described as large vessel disease (LVD), cardioembolic (CE), small vessel disease (SVD), other causes (OC), and undetermined according to the classification of TOAST (Trial of Org 10172 in Acute Stroke Treatment).[22] The neurological outcome was determined by the neurological examination of the patients, which was assessed by the modified Rankin Scale (mRS) at 2 months after the stroke. According to this scale, a mRS point of 0, 1, 2 was described as independent (good outcome), and of 3 or more as dependent (bad outcome).[23] Twenty-eight healthy subjects were included in the study as a control group, who had no ischemic stroke, and temporary ischemic attack, complying with age, and exclusion criteria. The objective of this study was described, and informed consent was obtained from all patients, and local ethical committee permission regarding this study had been obtained from the hospital's ethical committee.

Statistical evaluation. For data assessment, SPSS for Windows, version 10 was used, and for descriptive statistical values, arithmetical mean, standard deviation, minimum and maximum values, and percentage values were used. In analytical evaluations, Kruskal Wallis test was used for comparing the median values of more than 2 groups while Mann Whitney U test was employed for comparing the median values of 2 groups. The Wilcoxon

sign test was used to determine the prognosis. The relation between the unstable variables was evaluated with Pearson's correlation test. A p value of less than 0.05 was considered statistically significant.

Results. Seventy-six patients with a mean age of 64.9±11.8, and first-ever ischemic stroke were included in the study. Forty of the patients were female (52.6%), and 36 were male (47.4%). Twenty-eight healthy subjects were selected as the control group with a mean age of 63.3 ± 8.3. The patients were divided into 4 groups according to the cranial CT/MR results, and there were 18 patients in the CSI group, 20 patients in the CLI group, 20 patients in the SSI group, and 18 patients in the SLI group. The risk factors of the patients are shown in **Table 1**, and the blood parameters are shown in **Table 2**. Hypertension, cardiac disease, diabetes mellitus, and high levels of triglyceride (Tg) and low density lipoprotein-cholesterol (LDL-chol) were compared, and a significant difference was observed between the patient, and control groups. White blood cells (WBC), and monocyte (Mon) were significantly higher than the control group (p=0.000). There was also a statistically significant difference in IL-8 (p<0.0001), and the net levels (p<0.001) **Table 2**. According to the extent of the lesion, the patients were grouped with large infarct (CLI + SLI), and with small infarct (CSI + SSI). In the large infarct group, the median serum IL-8 level was 33.5±21.2, and the median net level was 8980.1±3223.9. In the small infarct group the median serum IL-8 level was 21.1±10.3 and the median net level was 8321.3±2643.8. The serum IL-8 levels were found significantly higher in the large infarct group than the small infarct group, and they were associated with the extent of the lesion (p=0.006). However, no association was found between the net levels, and the extent of the lesion (p>0.05). The patients were grouped as under age 55 (n=18) and over age 55 (n=58). They were evaluated based on LVD, CE, SVD, OC and unknown according to the etiology of the stroke. According to localization (subcortical-cortical), gender, age, and etiology there were no statistical difference between serum IL-8, and net levels (p>0.05) **Table 3**. For prognosis, patients were assessed by mRS at 2 months upon admission. The patients with a mRS score of 0-2 (n=13) were described independent, while the ones with a score of 3-6 (n=57) as dependent. When the 2 groups were compared, the serum IL-8 values were significantly higher in the dependent group (p<0.05), but no association was found between net levels and prognosis (p>0.05). Twenty-

Table 1 - Risk factors of the patients.

Risk factors	n (%)	*P*-value
Hypertension	50 (65.8)	0.017*
Cardiac disease	48 (63.1)	0.000*
Diabetes mellitus	26 (31.6)	0.016*
Smoking	14 (18.4)	0.234
Hyperlipidemia	12 (15.8)	0.585
Increased hematocrit level	6 (0.08)	0.325
Alcohol abuse	5 (0.07)	0.367
Obstructive apnea syndrome	2 (0.03)	0.503

*significant values

Table 2 - Comparison of blood parameters, neutrophil, and IL-8 levels between the control and patient groups.

Blood parameter	Cortical small infarct (N=18)	Cortical large infarct (N=20)	Subcortical small infarct (N=20)	Subcortical large infarct (N=18)	Control (N=28)	*P*-value
Fib	149.8 ± 63.1	129.7 ± 50.8	145 ± 59.8	144.6 ± 91.9	98.9 ± 10.6	0.018*
Chol	190.3 ± 46.8	193.4 ± 54.1	220 ± 56.1	208.4 ± 59.5	182.8 ± 38.6	0.352
Tg	126.6 ± 44.7	149.8 ± 163.8	194.6 ± 113	134.8 ± 53.6	116.1 ± 43.6	0.013*
HDL-chol	48.3 ± 16.1	44.8 ± 9.9	39.8 ± 11.3	42.6 ± 13.9	45.1 ± 8.1	0.267
LDL-chol	129.6 ± 41.2	121.4 ± 44.7	159 ± 51.6	134.6 ± 50.5	102.5 ± 15.8	0.003*
WBC	10456.2 ± 2641.4	12310.5 ± 3636.8	9921.1 ± 2385.9	10287.5 ± 3104.6	7165.9 ± 1710.6	0.000*
Hb	12.9 ± 2.4	12.8 ± 17	13.2 ± 1.9	14.5 ± 6.4	13.5 ± 0.9	0.706
Plt	253812.5 ± 73022.5	244684.2 ± 864041	25289.7 ± 70542.2	246937.5 ± 68842.3	260995.5 ± 69186	0.617
Mon	859,6 ± 478,6	767.9 ± 342.3	594.7 ± 256.5	509.4 ± 228.2	427.3 ± 158.7	0.000*
Net	8352.1 ± 2501.9	9585.6 ± 3515.1	8078.2 ± 2692.7	8496.2 ± 2851.5	5084.5 ± 1269.2	0.000*
IL-8	20.9 ± 10.5	30.1 ± 18.2	22.1 ± 9.9	29.2 ± 15.8	14.4 ± 4.6	0.000*

Fib - fibrinogen, Chol - cholesterol, Tg - triglyceride, HDL-chol - high-density lipoprotein cholesterol, LDL-chol - low-density lipoprotein cholesterol, WBC - white blood cell, Hb - hemoglobin, PLT - platelet, Mon - monocyte, Net – neutrophil, IL-8 - interleukin-8, *significant values

Table 3 - Comparison of median neutrophil and IL-8 levels according to gender, age, etiology, and prognosis.

Variable	n	Neutrophil (median)	*P*-value	IL-8 (median)	*P*-value
Gender					
Female	40	8171.1 ± 2461.6	0.359	24.2 ± 12.1	0.910
Male	36	9144.1 ± 3315.1		30.3 ± 22.5	
Age					
<55 years	18	9450.3 ± 3490.9	0.712	25.1 ± 11.9	0.252
>55 years	58	8276.9 ± 2605.2		27.1 ± 17.2	
Etiology					
Large vessel disease	19	8399.9 ± 2426.9	0.569	20.9 ± 5.3	0.278
Cardioemboli	48	8560.3 ±3117.6		29.9 ± 21.3	
Prognosis					
Independent	17	9492.8 ±2935.9	0.210	19.2 ± 10.8	0.046*
Dependent	59	8439.6 ± 2913.1		29.3 ± 18.6	

*significant value, IL-8 - interleukin-8

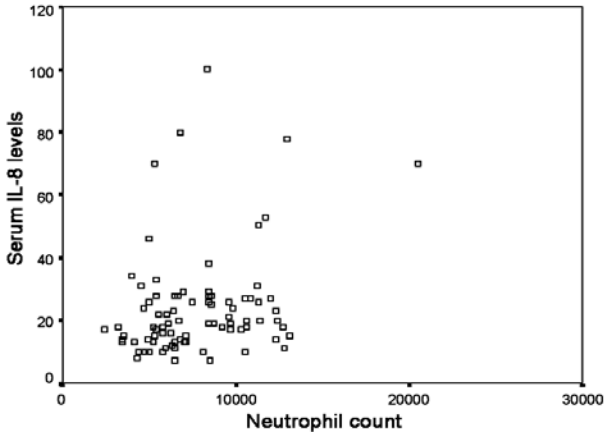

Figure 1 - Correlation between serum IL-8 and neutrophil levels.

three of the dependent patients had progression in the neurological examination, and 9 patients had died. Thirty-five patients from the large infarct group and 23 from small infarct group had poor prognosis, and while there was a relation between the extent of the lesion and prognosis (p<0.01), no difference was found between the localization and prognosis (p>0.05) **Table 3.** The correlation between IL-8 levels and all other values was assessed, and a positive correlation was found between IL-8 and mRS assessment at day 60 (r=0.29, p=0.02) as well as a positive correlation (r=0.28, p=0.01) between IL-8 and net values (**Figure 1**).

Discussion. The chemokines, proinflammatory chemotaxic cytokines with a low molecular weight, are divided into 4 groups based on the positions of the sistein residuals they include. The alpha (CXC), and beta (CC) are the major groups. The first 2 sistein of the alpha subgroup are combined with the amino acid residuals, and are mostly effective on the neutrophils.[24] The IL-8 belongs to the CXC group, and the functions of the neutrophils are regulated by high affinitive IL-8 receptors, CXCR1, and CXCR2.[25] The IL-8 is secreted mostly by macrophages, and by neutrophils, monocytes, endothelial cells, T-lymphocytes, fibroblasts, and astrocytes.[26,27] They are not particularly ready within the cell, they are mostly synthesized when they are induced by factors like hypoxy, lipopolysaccharides, mytogens, and cytokines such as IL-1β and tumor necrosis factor (TNF)-α.[28] Macrophage/microglia induce leukocyte accumulation, and activation by secreting IL-1β and TNF-α in the area of brain damage whereas astrocytes induce IL-8 expression.[9,29] In neutrophil mediated acute inflammation, the IL-8 is vital as a neutrophil chemotaxic, and activating factor.[19] By stimulating the binding of neutrophils to the receptors, it causes a conformational change in the integrin molecules, and facilitates adhesion and transmigration.[17] While stimulating the secretion of neutrophil granules and their respiratory burst, it provides prolonged impact by delaying their apoptosis.[30,31] It has been clearly shown that local expression of IL-8 in the ischemic brain establishes a concentration gradient over the blood-brain barrier, and as a result of movement of migrating cells toward this gradient the neutrophils rapidly penetrate into the brain parenchyma, and cause

local inflammation.[32] The synthesis in the damaged tissue starts before determination of the IL-8 in the plasma.[28] In the present study, there was a significant relation between the serum IL-8 values, and extent of lesion in the cranial CT/MR imaginations performed at day 3. The serum IL-8 levels of the large infarct group were significantly higher compared to the small infarct, and control groups (p<0.001). In our study, as there is a significant correlation between the response of IL-8 assessed within the first 24 hours of stroke and the extent of the brain lesion, determinations of IL-8 levels may allow the indication of the extent of the lesion when CT/MRI fail to estimate the extent of the lesion within hours of onset. High levels of serum IL-8 may be suggestive of large ischemic areas, while low levels may be suggestive of small ischemic areas. In previous studies with pro-inflammatory cytokines like IL-6, the plasma cytokine levels of the patients with cortical infarcts were found higher than with the subcortical infarcts[33] whereas in our study, no relation was found between the values of IL-8, which is a pro-inflammatory chemotaxic cytokine, and the subcortical-cortical lesions (p>0.05).

The association of IL-8 with net values in the acute phase of ischemic stroke was assessed by experimental studies. The studies performed in rats with permanent MCA occlusion have shown that neutrophils in the capillary lumen appeared within the 30 minutes of onset, and they reached their peak at 12 hours in the capillary lumen whereas in the parenchyma they appeared within the first 12 hours of onset and reached to their peak at 24 hours, and then they started to decrease.[34,35] It has been found that reperfusion occurring after a temporary focal ischemia in rabbits induced neutrophil infiltration and aggregation, resulting in severe brain edema and infarct, and it induced IL-8 expression in the vascular wall.[36] It has been also shown that in rats with systemic administration of monoclonal antibodies against IL-8 during the early cerebral perfusion damage, the net accumulation and brain edema decreased, the blood flow in the damaged brain area increased, and there was a reduction of up to 50-60% in the extent of the infarct.[37,38] It has been reported that IL-8 was expressed locally in the brain, and it was an important mediator of cerebral perfusion, and a significant target in the prevention of the damage.[4,36] The number of studies examining the plasma IL-8, and net levels in the patients with acute ischemic stroke is very rare. The histological evidence of leukocyte accumulation in the lesion of human ischemia, contrary to the experimental ischemia has been restricted to a few studies on necropsy. Therefore, clinical studies of acute ischemic stroke were mainly carried out with peripheral leukocytes.

In their study series, Kostulas et al[39] found high plasma and CSF IL-8 levels in patients with acute ischemic stroke as well as a positive correlation between the IL-8 and mRNA secreting mononuclear cells. Higher IL-8 levels in the CSF show that IL-8 is expressed in the damaged tissue of the brain, and it is parallel to the rise in the plasma. In a study by Grau,[40] the comparison of patients with acute ischemic stroke with the control group showed no relation between the increased plasma levels within the first 24 hours of onset and the number of monocytes, and it was suggested that increased plasma IL-8 levels caused accumulation of neutrophils in the ischemic area, taking part in the tissue damage. In our study, net levels were significantly higher in all patient groups compared to the control group. While a positive correlation was observed (r=0.28, p=0.01) between the IL-8 and net values, there was no relation between both IL-8 and net levels with age, gender, localization, and etiology (p>0.05). However, according to the mRS assessed at 2 months, the serum IL-8 levels of dependent patients were significantly higher than the independent patients (p<0.05). During the assessment of association between large lesion infarcts with remarkably increased IL-8 levels and prognosis, no relation was found between net levels, and lesion size or clinical course.

In conclusion, the fact that the serum levels of IL-8 are correlated with the extent of the lesion in the early phase of the stroke shows that IL-8 is expressed from the ischemic lesion in the brain, and is consistent with the rise in the plasma. The limitation of our study was the lack of observation of IL-8 in CSF, however, we think a reliable correlation would have been determined between serum and CSF IL-8 levels if we could evaluate IL-8 in CSF. Based on our findings, we can suggest that the localization of the lesion has no impact on the secretion of IL-8. We also suggest that there is a correlation between IL-8 and neutrophils, and the neutrophils play a role in the pathology of stroke, however, the absence of any correlation between the neutrophil levels and the extent of lesion in our patients may be associated with the fact that the impact of neutrophil is independent from its amount in the peripheral circulation. The development of new neuroprotective treatments aiming to prevent neutrophil-mediated-inflammation induced by IL-8 is critical in the treatment of stroke, and prevention of clinical worsening.

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