Effect of Non-Steroidal Anti-Inflammatory Drugs on Natural Killer Cell Activity in Patients with Dementia

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Abstract

Background: Alteration of innate and acquired immunity can play a role in the mechanism involved in the development of dementia. Epidemiologic studies indicate that the use of non-steroidal anti-inflammatory drugs can delay the onset or slow progression of Alzheimer disease.

Objectives: To determine whether the use of NSAIDs is associated with natural killer activity alteration in AD and multi-infarct vascular dementia patients, as compared with non-demented elderly and healthy young people.

Methods: In this prospective open study four groups of subjects (AD, VD, non-demented elderly, and healthy young people) were treated with an NSAID drug (rofecoxib 12.5 mg/day or ibuprofen 400 mg twice daily) for 7 days. Natural killer cell cytotoxicity was measured after flow cytometry analysis before and after treatment.

Results: Of the 49 subjects studied, 15 had a diagnosis of AD (3 men, 12 women; mean age 83.5 $\pm$ 8.1 years), 15 had a diagnosis of multi-infarct VD (7 men, 8 women; mean age 75.5 $\pm$ 8.4), 13 were non-demented elderly (1 man, 12 women; mean age 80.2 $\pm$ 7.2), and 6 were healthy young volunteers (3 men, 3 women; mean age 36.8 $\pm$ 4.4). While all examined subjects showed decreased NK cell cytotoxicity after treatment, this decrease was most prominent and statistically significant in elderly patients suffering from vascular dementia – from an average of 30.5 $\pm$ 11.8% before treatment to 22.5 $\pm$ 16% after treatment (P = 0.04). The decrease in NK cell cytotoxicity was only moderate and not statistically significant in all other elderly and young subjects. Young healthy volunteers exhibited a significantly higher total NK cytotoxicity before and after treatment compared to all age groups (P < 0.001).

Conclusion: These findings suggest that NSAIDs decrease NK activity in vascular dementia patients. Our findings also suggest that natural killer activity alteration cannot explain the ability of anti-inflammatory drugs to delay the onset or slow the progression of AD.

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Inflammatory processes play a role in the development and progression of Alzheimer's disease [1]. The clinical symptoms of Alzheimer's disease are determined to a great extent by the inflammatory response in the brain. It was found that the formation of the amyloid-microglia complex is a relatively early pathogenic event that precedes the process of inflammatory destruction of neurons [2].

The pathophysiologic relevance of inflammation in AD is related to several lines of evidence: activated microglia cells in AD brains [1]; high Interleukin-1 and other cytokines in brain regions with AD pathology [3]; IL-1, IL-6, tumor necrosis factor and complement factors in the vicinity of amyloid peptide [4]; and complement fixation and lysis of neurites in the AD brain [5].

Epidemiologic studies indicating that the use of anti-inflammatory drugs can prevent or retard AD have stimulated the idea that these agents have a neuroprotective effect with regard to the development of AD [6]. Of more interest is the ability of anti-inflammatory drugs to improve symptoms or delay progression in patients with AD. Preliminary limited data suggest that treatment with anti-inflammatory drugs might provide some improvement [7], but most targeted studies of anti-inflammatory therapy in AD had negative results [8]. Natural killer cells are cytotoxic cells that also express cytokine receptors, secrete immunoregulatory cytokines, and play a critical role in the innate immune response. Experimental data such as increased cytokine-mediated cytotoxic NK cell activity that were recently demonstrated in patients with AD suggest the involvement of immune cellular components in the development of AD [9].

Vascular dementia is a controversial diagnosis; nonetheless, diagnostic criteria require a temporal profile of illness compatible with vascular etiology. Evidence has accumulated of a correlation between the presence and extent of cerebral and systemic atherosclerosis, chronic ischemia and cognitive impairment in vascular dementia patients, especially the multi-infarct type. Knowledge on the involvement of mediators of innate and acquired immunity in various stages of atherosclerosis is fairly new [10]. Immunocompetent cells infiltrate atherosclerotic plaques of all stages. Plaque-infiltrating T cells elicit antibody responses, which have been proposed as markers of disease activity. Cytokines secreted by activated T cells may control macrophage activation, modulate smooth-muscle proliferation, nitric oxide production, apoptosis, and induce endothelial activation [10]. In recent years atherosclerosis has often been considered an unusual form of chronic inflammation occurring within the artery wall, however only a few studies examined the role of NK cells in human atherosclerosis. Although macrophages predominate in atherosclerotic lesions, NK cells in smaller numbers are present as well [11]. Atherosclerosis in elderly people, measured by the ankle-brachial arterial index, was found to be associated with decreased NK cytotoxicity, but only on a per cell basis [12]. NK cells were also

NSAIDs = non-steroidal anti-inflammatory drugs
AD = Alzheimer disease
VD = vascular dementia
NK = natural killer
IL = interleukin

found to have the capacity to directly contribute to acute coronary syndromes [13].

The non-steroidal anti-inflammatory drugs block prostaglandin E2 biosynthesis via inhibition of cyclooxygenase activity. However, the effects of NSAIDs on immune responses are not fully understood. To our knowledge, the effects of NSAIDs on natural killer function in elderly and demented people have not been studied. We conducted the present study to determine whether the use of NSAIDs was associated with NK alteration in patients with Alzheimer disease and patients with multi-infarct vascular dementia compared to healthy elderly and healthy young people.

Patients and Methods

Participants

The present study was carried out in four groups: a) 15 patients with probable AD (mean age 83.5 $\pm$ 8.1 years, 12 women and 3 men); b) 15 patients with vascular dementia of the multi-infarct type (mean age 75.5 $\pm$ 8.4 years, 8 women and 7 men); c) 13 non-demented subjects (mean age 80.2 $\pm$ 7.2 years, 12 women and one man); and d) 6 young healthy volunteers (mean age 36.8 $\pm$ 4.4 years, 3 women and 3 men). Participants were selected from geriatric departments of the Fliman Hospital, a 210 bed public geriatric facility located in Haifa, Israel and affiliated with the Technion Faculty of Medicine, and from the Hemdat Avot nursing home in Haifa, a long-term care facility for patients who require assistance with activities of daily living or skilled nursing care.

Clinical criteria for the diagnosis of AD and VD were according to the Diagnostic and Statistical Manual of Mental Disorders (fourth edition) [14]. Assessment and diagnosis, according to the DSM-4, were performed before the study in most cases by a specialist in the field, and in all other cases by the authors. All our AD patients were defined as probable AD according to criteria of the NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and Stroke – Alzheimer's Disease and Related Disorders Association): Dementia established by clinical examination and documented by mental status questionnaire; Dementia confirmed by neuropsychological testing; Deficits in two or more of the following areas of cognition: memory, language, perceptual skills, attention, praxis, orientation, problem-solving and functional abilities; progressive worsening of memory and other cognitive functions; no disturbance of consciousness; onset between ages 40 and 90; and absence of systemic or other brain diseases capable of producing a dementia syndrome. The controls were non-demented (Mini-Mental State Examination score above 26) elderly patients hospitalized for orthopedic rehabilitation (hip fracture or knee replacement, at least 3 weeks after the operation).

Patients were eligible if they did not have co-morbid conditions that increased the risk of adverse events associated with NSAID treatment; i.e., hypersensitivity to aspirin or NSAIDs, active peptic ulcer disease within 5 years, renal insufficiency (serum creatinine level >1.5 mg/dl), clinically significant liver disease, poorly controlled hypertension, congestive heart failure, or bleeding disorder. Individuals were excluded if they had co-morbid conditions that might respond to NSAIDs (e.g., inflammatory arthritis). Individuals were also excluded if within the prior 2 months they had regularly used anti-inflammatory medications (aspirin at a daily dose $\leqslant$ 325 mg was allowed). Inclusion criteria included age older than 50 years and diagnosis of AD or VD according to the DSM-4. Patients who met the above entrance criteria and who were treated with an NSAID drug for a musculoskeletal painful condition without inflammatory signs were enrolled to the study. Patients were randomly assigned to one of two treatment regimens – rofecoxib 12.5 mg administered once daily or ibuprofen 400 mg administered twice daily, for 7 days each. Immediately before and after the 7 day trial a sample of 15 ml heparinized blood was taken.

We obtained approval for the study from our local institutional committee and from the Ministry of Health Helsinki Committee. Written informed consent was obtained from participants and/or legally authorized representatives.

Isolation of peripheral blood mononuclear cells

PBMC were isolated from heparinized whole blood by density gradient centrifugation over Hypaque 1077 (Pharmacia, Sweden) [15]. The PBMC were suspended in medium composed of RPMI 1640, 10% fetal calf serum, 1% glutamine, 1% antibiotics (Biological Industries, Israel).

Flow cytometry analysis

Fresh PBMC were washed twice in Dulbeco phosphate-buffered saline, and $1.0x10^5$ cells were resuspended in 100 µl PBS and incubated for 30 minutes at 4°C with fluorescein-isothiocyanate conjugated CD3, CD4, CD8, CD16, CD56, CD94 (Becton Dickinson, Oxnard, CA, USA). For determination of background staining, cells were incubated with the relevant mouse isotype antibodies as negative controls. Labeled cells were washed twice with Dulbeco PBS, fixed with paraformaldehyde and analyzed by a flow cytometer (Becton Dickinson). Lymphocytes were distinguished from monocytes on the basis of their forward versus right-angle light scatters. A lymphocyte gate was used. The subsequent computer analyses were carried out using PC lysis software from Becton Dickinson.

NK cell activity assays

NK cell cytotoxicity was measured using K562 in a 51 Cr release assay as previously described [16,17]. Briefly, fresh PBMC were added in different concentrations to target cells: Effector/Target (E/T) cell ratios of 50/1, 25/1, 12.5/1 were used. Triplicates of each E/T ratio were incubated for 4 hours at 37°C, 5% CO_2 incubator in short-term assays. The plates were centrifuged for 10 min, 50 µl supernatant was transferred to new tubes and radioactivity was determined. The spontaneous release was measured by incubating target cells with medium alone. Maximal release was measured by incubation of target cells with 5% Triton X-100.

The percentage of 51 Cr release (NK cell activity) was calculated by the formula

$$\% \text{ cytotoxicity} = \frac{\text{cpm} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

PBMC = peripheral blood mononuclear cells
PBS = phosphate-buffered saline

Statistical analysis

Mean values are presented together with the standard deviation (SD). The ANOVA test was used throughout the study to determine any significant differences between the mean values measured for each group. Groups were also compared by a two-tailed *t*-test for independent samples (before treatment versus after treatment; control versus treated). A *P* value less than 0.05 was assumed to indicate statistical significance.

Table 1. Patients' characteristics

Characteristics	Probable AD	Vascular dementia	Non-demented elderly	Healthy young
N	15	15	13	6
Mean age (yrs ± SD)	83.5 ± 8.1	75.5 ± 8.4	80.2 ± 7.2	36.8 ± 4.4
Range	65–93	61–91	67–92	32–44
Male	6	7	4	3
Female	9	8	9	3

Results

Patient characteristics

There were no statistically significant differences with respect to most of the patients' characteristics between the three groups of elderly patients [Table 1].

Cytotoxicity of NK cells before and after treatment

Comparing the alteration of percent cytotoxicity between groups, vascular dementia patients exhibited a significant decline in cytotoxicity of NK cells – from an average of 30.5 ± 11.8% before treatment to 22.5 ± 16% after treatment ($P = 0.04$) [Table 2]. A small decline in NK cytotoxicity was also observed in AD patients, non-demented subjects and healthy young volunteers, but this decline did not reach statistical significance. Young healthy volunteers exhibited a significantly higher total NK cytotoxicity before and after treatment as compared to all age groups ($P < 0.001$). Comparison between the mean values of cytotoxicity measured for each group of elderly subjects did not exhibit any significant difference [Table 2]. No significant differences in NK cytotoxicity were observed between groups of subjects treated with a selective Cox-2 inhibitor NSAID (celecoxib) and those treated with a non-selective NSAID (ibuprofen) [Table 3].

Table 2. Percent cytotoxicity of NK cells before and after treatment

	Before treatment (% cytotoxicity)	After treatment (% cytotoxicity)	P value*
Probable AD	25.5 ± 11.4	20.92 ± 13.5	0.279
Vascular dementia	30.5 ± 11.8	22.5 ± 16	0.04
Non-demented elderly	19.5 ± 12.7	16.6 ± 11.4	0.365
Young healthy	56.8 ± 17.2	46.3 ± 15.6	0.295
P value**	0.176	0.526	

* Comparison using two-tailed *t*-test

** Comparison of groups of elderly subjects using ANOVA

Table 3. Percent cytotoxicity of NK cells before and after treatment with ibuprofen (non-selective NSAID) and celecoxib (selective Cox-2 inhibitor NSAID)

	Before treatment (% cytotoxicity)	After treatment (% cytotoxicity)	*P* value
Ibuprofen			
Probable AD (n=11)	26.0 ± 15.7	24.8 ± 13.6	0.816
Vascular dementia (n=11)	33.7 ± 17.1	24.3 ± 18	0.07
Non-demented (n=6)	17.3 ± 8.2	17.8 ± 12.4	0.934
Celecoxib			
Probable AD (n=4)	24.1 ± 11.8	10.25 ± 4.8	0.057
Vascular dementia (n=5)	21.8 ± 16.5	15 ± 9.1	0.178
Non-demented (n=7)	21.4 ± 16	15.6 ± 11.3	0.96

Flow cytometry analysis

No statistically significant variations were observed in the purity of T cell fractions after treatment of T lymphocyte cells with antibodies to specific cytokines (CD3, CD4, CD8, CD16 and CD56, CD94 for NK cells) in all groups before and after treatment with an NSAID.

Discussion

The major finding in the present study was the statistically significant decrease in NK cell cytotoxicity in elderly patients with vascular dementia after treatment with an NSAID (7 day oral treatment with a Cox-2 selective or non-Cox-2 selective drug). A decrease in NK cell cytotoxicity was found in all other elderly and young subjects examined, but this decrease was only moderate and not statistically significant.

The most anti-inflammatory effect of NSAIDs is mediated through the inhibition of cyclo-oxygenase 2 (Cox-2) and reduction of PG levels. It has been postulated that the effect of NSAIDs on NK cell activity is via negative modulators of NK cytotoxicity that includes PGE2 [18]. The inhibitory action of PGE2 on NK cells is mediated via elevation of intracellular cAMP [19]. Increased cAMP is thought to suppress NK cytotoxicity by interfering with specific steps along the lytic pathways. This sequence of events can be countered by NSAIDs, which block PGE2 synthesis [20].

Of interest is our finding that vascular dementia patients exhibited a decline in NK activity after treatment with an NSAID. Atherosclerosis is the most prevalent root cause of multi-infarct vascular dementia. It is well known that the innate and the adaptive immune system are deeply involved in the development of atherosclerosis, with immunocompetent cells participating in the formation of atherosclerotic plaque. T cells and macrophages enter the intima in response to the expression of adhesion molecules (VCAM1 and others) [10]. The accumulation of macrophages and their internalization of cholesterol constitute the streak (a macrophage foam cell lesion). Advanced human plaque contains a mixture of smooth-muscle cells, macrophages and T cells (CD4>CD8 2:1). T cells can be and probably are activated [10]. Our finding that young healthy people have a higher total NK cytotoxicity is compatible with most studies, which report a decreased cytotoxicity per NK cell in the elderly and an age-associated compensatory increase percentage of NK cells among

PG = prostaglandin

VCAM1 = vascular cell adhesion molecule 1

lymphocytes [21]. Well-preserved NK activity has been associated with successful aging. The mild difference we observed between the groups of elderly subjects in NK activity before treatment was not statistically significant.

Our finding that NSAIDs suppress NK cell activity in patients with vascular dementia (multi-infarct type) may offer another insight into the inter-relation between NSAIDs, NK cells, and atherosclerosis. The question whether NK cells participate in atherosclerosis is further complicated by the fact that low NK activity is associated with higher morbidity and mortality in the elderly. In this context our finding suggests that NSAIDs might have a negative effect in these patients.

Regarding inflammation in Alzheimer's disease, our study does not support the hypothesis that natural killer lymphocytes are involved in AD. Clinical studies suggesting that anti-inflammatory drugs may delay the onset or slow the progression of AD cannot be explained by alteration in natural killer activity. Another specific element of the inflammatory process must be the key, and other studies may still be needed to target the right point in the inflammatory pathway. The argument that the lack of NK activity alteration in AD in our study may be related to the short duration of therapy, insufficient for NK cytotoxicity to change, seems unlikely pharmacokinetically but must be considered because in most epidemiologic studies that examined the effect of anti-inflammatory drugs on AD the treatment period was longer. Our results are compatible with the hypothesis that although an inflammatory mechanism plays a chronic but important part in the damage to the brain in AD, it does not appear to involve lymphocytes [22]. The nature of AD inflammation has a highly interactive mechanism involving endogenous cell-mediated responses, particularly complement, cytokine, and acute-phase responses [23]. Our results are in line with those of most studies examining the humoral, antibody-mediated responses in AD.

Our findings are further complicated by recent publications on the convergence of risk factors for AD and stroke that have led some to speculate that the underlying pathogenic mechanisms are similar – i.e., both diseases begin with vascular insufficiency [24]. Evidence has accumulated that vascular abnormalities have a role in the development of both types of dementia. Patients with stroke are at increased risk for both vascular dementia and AD [25].

In conclusion, these findings suggest that alteration in NK activity might be connected to the presence of severe cerebral atherosclerosis and that atherosclerosis in VD is associated with decreased NK activity. Our findings also suggest that alteration in natural killer activity cannot explain the ability of anti-inflammatory drugs to delay the onset or slow the progression of AD. Clinical studies examining the effect of NSAIDs on the onset or progression of atherosclerosis can help answer these questions.

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