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Serum CD44 Levels Preceding the Diagnosis of Non-Hodgkin's Lymphoma

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Serum CD44 (s-CD44) concentrations were measured in sera taken from 49 individuals who were diagnosed with non-Hodgkin's lymphoma 0.9 to 7.2 years after taking the blood sample, and from 49 controls matched for age. The serum samples had been collected in conjunction of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) study, which evaluated the influence of vitamin supplementation on cancer prevention. S-CD44 was measured using chemiluminescence enzyme immunoassay. S-CD44 concentrations of the cases were significantly elevated before the diagnosis of lymphoma when compared to the serum levels found in the controls (median, 447 ng/mL; range, 108–780 ng/mL vs. median, 364 ng/mL; range, 53–660 ng/mL; p=0.012). Individuals who were later diagnosed with high grade lymphoma according to the Kiel classification (n=21) had significantly higher values than the controls 0.9–4.0 years before the diagnosis, but such a difference could not be detected if serum samples had been taken more than 4 years before the diagnosis. The s-CD44 levels were not significantly elevated among individuals who were later diagnosed with low grade malignant non-Hodgkin's lymphoma (n=25) as compared to their controls. The prediagnostic s-CD44 levels in cases and controls overlapped markedly, and a value higher than the highest value found among the controls (660 ng/mL) was found only in 5 (10%) samples taken from individuals who were later diagnosed with lymphoma. We conclude that serum CD44 may be elevated a few years preceding the diagnosis of non-Hodgkin's lymphoma, but the levels overlap markedly with those found in individuals without lymphoma.

Keywords: adhesion molecules, diagnosis, non-Hodgkin's lymphoma, screening

INTRODUCTION

CD44 is a multifunctional adhesion molecule expressed on several hematopoietic and nonhematopoietic cells. Most hematopoietic cells express the standard, 90 kDa form, but larger forms up to 250 kDa have been described (1,2), and are thought to arise from alternative splicing and/or post-translational modifications of a single gene.(3,4) CD44 is involved in many cell-cell and cell-matrix interac-

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tions, and has been shown to be involved in lymphocyte adhesion to the vascular endothelium in the high endothelial venules and the extracellular matrix, in T-cell activation and adherence, and in lymphohematopoiesis.(5–9) Moreover, CD44 has been described to function as a signaling molecule (10), and some forms have been shown to bind growth factors.(11) Both *in vitro* and *in vivo* data suggest that CD44 may have an important role in dissemination of non-Hodgkin's lymphoma and other malignancies.(12–16)

Elevated serum concentrations of CD44 (s-CD44) have been measured at the time of the diagnosis in patients with non-Hodgkin's lymphoma, and they have been found to decrease in parallel with the treatment response and to increase at the time of disease progression.(17) In one study, patients with higher than the median s-CD44 level at diagnosis had poorer survival than those with a lower level, and high s-CD44 levels were associated with a high International Prognostic Index.(18) In patients with gastric or colon cancer, s-CD44 measured at the time of the diagnosis correlate with the tumor burden, and the serum concentrations have been found to decrease after resection of the tumor.(19,20) Elevated s-CD44 levels have also been measured in other malignant diseases, such as in cervical cancer.(21) However, there are still few data on the functions and clinical importance of circulating CD44 in malignant diseases. S-CD44 has been shown, at least in part, to have its origin in cancer cells.(22,23) Although s-CD44 lacks the cytoplasmic tail, it seems to be biologically active.(23)

Although elevated s-CD44 levels have been measured in patients with non-Hodgkin's lymphoma at the time of the diagnosis, there are currently no data on s-CD44 levels in individuals who have been later diagnosed with lymphoma. The rate of increase in s-CD44 before the diagnosis might be of interest, because little is known about the length of the pre-clinical period in different subtypes of lymphoma. Moreover, there are currently no data available on whether s-CD44 can be used in early diagnosis or screening of primary or recurrent lymphoma. In the present study we analyzed the CD44 levels from serum samples taken from individuals who were diagnosed with non-Hodgkin's lymphoma a few years after sampling and their age-matched controls.

MATERIAL AND METHODS

Patients and Controls

The serum samples for the study were obtained from of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) study, which was a controlled trial designed to test the hypothesis that supplementation with alpha-tocopherol or beta-carotene reduces the incidence of cancer in smokers. The trial included 29,133 male smokers, aged 50 to 69 years, who were randomly assigned to one of four regimens: alpha-tocopherol 50 mg per day, beta-carotene 20 mg per day, both alpha-tocopherol and beta-carotene, or to placebo. The details and results of the trial have been published elsewhere.(24)

We identified the patients who participated in the ATBC study and were later diagnosed with lymphoma from the study follow-up data and the files of the Finnish Cancer Registry. The lymphoma diagnoses were confirmed by reviewing of the medical records and the histopathological specimens. Among the participants of the trial 57 histologically confirmed cases of non-Hodgkin's lymphoma were diagnosed during the follow-up period from 1985 to 1993. Forty-nine of these 57 cases were included in the present study. One case was excluded because of concurrent myeloma and seven patients because of lacking serum samples from the time period preceding the diagnosis of lymphoma. The median age of the 49 men at ATBC study entry was 58 years (range, from 50 to 70), and at the time of the diagnosis of lymphoma 60 years (range, from 54 to 76).

For each patient with lymphoma a control was chosen from the ATBC study participants who were at risk at the time of the lymphoma diagnosis of the case and who had no malignancy diagnosed by April 1994. The control subject was matched for the type of vitamin supplementation and age. The median age of the 49 controls at entry to the ATBC study was 58 years (range, from 50 to 70).

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TABLE I Grade of malignancy defined by the Kiel classification and stage according to the Ann Arbor classification in 49 non-Hodgkin's lymphomas studied

	Low grade malignancy	*High grade malignancy*	*No grading available*	*All*
Stage I-II	10	10	2	22
Stage III-IV	14	11	1	26
Staging not available	1			1
All	25	21	3	49

The staging of lymphomas was done according to the Ann Arbor classification based on staging examinations carried out in several hospitals.(25) (Table I). Ten of the 49 patients had stage I, 12 stage II, two stage III, and 24 stage IV disease. In one case lymphoma could not be staged because no staging examinations had been performed. The histological slides were centrally reviewed by one pathologist with a special interest in lymphoma (K.F.). Lymphomas were classified according to the REAL classification(26) (Table II), and malignancy grading was done according to the updated Kiel classification.(27) The classification was mainly based on morphological examinaton, but in 18 cases immunohistochemical studies had also been performed. Twenty-five of the lymphomas were considered to be of low grade and 21 of high grade of malignancy, and in 3 cases the grade of malignancy could not be determined because of inadequate quality of the histological samples available (Table II).

Blood Samples

A blood sample was drawn at entry to the ATBC study, and the sera were stored frozen at −70°C until assay. The serum samples had been taken 0.9 to 7.2 years before the diagnosis of lymphoma.

Chemiluminescence Enzyme Immunoassay

S-CD44 was analyzed with a chemiluminescence enzyme immunoassay (EIA) with a method that has been described in detail elsewhere.(18) Briefly, streptavidin-coated 96-well EIA-plates (White Combiplate Streptavidin Coated, Labsystems, Helsinki, Finland) were incubated with biotinylated Hermes-3 for 60 minutes in RT. The biotinylation was performed using a standard procedure with biotin-NHS (Calbiochem, Calbiochem-Novobiochem Corporation, La Jolla, CA). Nonspecific binding sites were blocked by incubation with PBS containing 1% dried non-fat milk powder and 1% gelatin for 45 minutes. Diluted serum samples were added and incubated for 60 minutes. The detecting MoAb, 1F1, was then added and incubated for 60 minutes. Peroxide-conjugated monoclonal rat anti-mouse IgG2b (Zymed Laboratories, Inc., San Francisco, CA) was used as the second-stage antibody. Between incubations, the wells were washed extensively with PBS containing 0.3% Tween-20. Immunodetection was performed using CBM chemiluminescence ELISA Reagents (Boehringer Mannheim GmbH, Mannheim, Germany). The degree of luminescence was quantified using a luminometer (Luminoscan, Labsystems). CD44-depleted serum was used as a negative control, where soluble CD44 had been removed by incubation with Hermes-3 coupled to CNBr-activated Sepharose 4B beads (Pharmacia, Uppsala, Sweden). Serum with a high concentration of CD44 and tonsil lysates with a known concentration of soluble CD44 were used as positive controls.

TABLE II Histological subtype by the REAL classification and grade of malignancy defined by the Kiel classification among 49 non-Hodgkin's lymphomas

REAL classification	*Number of patients (%)*	*Low malignancy*	*High malignancy*
		n	*n*
B-cell lymphomas:			
Small lymphocytic lymphoma	6 (12)	6	–
Mantle cell lymphoma	6 (12)	6	–

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REAL classification	*Number of patients (%)*	*Low malignancy*	*High malignancy*
		n	*n*
Follicular lymphoma	7 (14)	6	1
Marginal zone lymphoma	6 (12)	6	–
Diffuse large cell lymphoma	18 (37)	–	18
T-cell lymphomas:			
Mycosis fungoides	1 (2)	1	–
Peripheral T-cell lymphoma, unspecified	1 (2)	–	1
Large anaplastic cell (Ki-1+) lymphoma	1 (2)	–	1
Unclassified non-Hodgkin's lymphoma	3 (6)		

Statistical Analysis

S-CD44 distributions in different groups were compared with the paired Mann-Whitney U-test. All P-values are 2-tailed.

RESULTS

Serum CD44 Levels

S-CD44 concentrations in the 49 prediagnostic samples of the individuals who were later diagnosed with non-Hodgkin's lymphoma were significantly higher than those of their controls (median, 447 ng/mL; range 108–780 ng/mL vs. median, 364 ng/mL; range, 53–660 ng/mL; p=0.012). However, a marked overlap in serum s-CD44 values between the cases and the controls was found. Values lower than the median value in the control group (364 ng/mL) were measured in 15 (31%) lymphomas and a value higher than the highest value found among the controls (660 ng/mL) was found only in 5 (10%) samples taken from individuals who were later diagnosed with lymphoma.

Stage

Individuals who were later diagnosed with stage I or II lymphoma had significantly higher prediagnostic s-CD44 levels than their controls (P=0.009, Table III). No significant difference in s-CD44 between the cases who were later diagnosed with stage III or IV lymphoma and their controls was found (P=0.36).

The Prediagnostic Time Period

We compared the s-CD44 values between the cases and the controls in samples taken 0.9 and 3.9 years preceding the diagnosis of lymphoma, and in samples taken 4.0 to 7.2 years before the diagnosis. The cut-off value of four years was chosen, because this cutoff divided the series into two roughly equally large subgroups. The samples taken 0.9–3.9 years before the diagnosis turned out to have significantly higher s-CD44 levels as compared to the controls, while no such difference was found when the samples taken more than four years before the diagnosis were compared (Table IV). When the samples taken from individuals who were diagnosed with high grade malignant lymphoma and those of their controls were compared taking the time of sampling into account, it turned out that the cases had significantly higher s-CD44 levels 0.9–3.9 years before the diagnosis as compared to the controls (P=0.023), but such a difference could not be demonstrated in samples taken earlier than four years preceding the diagnosis. No difference in s-CD44 levels was found between the cases who were later diagnosed with low grade malignant lymphoma and their controls.

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TABLE III S-CD44 by stage of non-Hodgkin's lymphoma among 48 cases and their age-matched controls. One patient could not be staged

Stage	*Number of case-control pairs*	*S-CD44 (ng/mL)*		
		Cases	*Controls*	
		Median (Range)	*Median (Range)*	*p*
I and II	22	468 (154–780)	358 (53–660)	0.009
III and IV	26	417 (108–676)	353 (152–578)	0.36

TABLE IV S-CD44 in two different prediagnostic periods of the 49 cases with non-Hodgkin's lymphoma and their age-matched controls

	Number of case/control pairs		*S-CD-44 (ng/mL)*		
			Cases	*Controls*	
			Median (Range)	*Median (Range)*	*p*
All	49		447(108–780)	364(53–660)	0.012
≥0-<4 yrs before diagnosis		29	451(108–780)	367(53–660)	0.020
≥4 yrs before diagnosis		20	403(182–720)	329(192–578)	0.31
High grade of malignancy	21		447(218–780)	339(53–660)	0.050
≥0-<4 yrs before diagnosis		15	451(343–780)	364(53–660)	0.023
≥4 yrs before diagnosis		6	330(218–511)	321(192–446)	0.84
Low grade of malignancy	25		447(108–720)	367(139–595)	0.20
≥0-<4 yrs before diagnosis		13	450(108–720)	373(139–595)	0.46
≥4 yrs before diagnosis		12	425(182–554)	314(205–578)	0.37

Histological Subtypes

The small number of prediagnostic samples available per a single histological subtype of non-Hodgkin's lymphoma limited analysis according to the histological subtype. However, the 18 cases in the largest single subtype, diffuse large B-cell lymphoma (Table I) tended to have higher values (median, 439 ng/mL; range, from 218 to 780 ng/mL) than their controls (median, 347 ng/mL; range, 53 to 660 ng/mL, P=0.14). The levels of s-CD44 tended to be higher also among cases with follicular B-cell lymphoma (median, 466 ng/mL: range, 154 to 735 ng/mL) and among marginal zone B-cell lymphoma (median, 472ng/mL; range, 403 to 720ng/mL) than in their controls (median, 336 ng/mL; range, 139 to 506 ng/mL; P=0.16 and median, 328 ng/mL; range, 209 to 595 ng/mL; P=0.09, respectively).

Grade of Malignancy

Individuals who were later diagnosed with high grade malignant lymphoma by the Kiel classification had significantly higher s-CD44 levels than their matched controls (P=0.050, Table IV). On the other hand, the prediagnostic s-CD44 levels were not significantly higher than those of the controls in cases who were later diagnosed with low grade lymphoma (P=0.20).

DISCUSSION

It has been shown that many untreated patients with non-Hodgkin's lymphoma have elevated s-CD44 levels at diagnosis and that these levels decrease if a response to treatment is achieved(17), but there have been no data available on the s-CD44 levels in lymphoma patients preceding the diagnosis. In the present study prediagnostic s-CD44 concentrations were

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found to be significantly higher among patients with high grade non-Hodgkin's lymphoma when compared to controls matched for age. It needs to be pointed out that the range of s-CD44 levels measured in the controls was wide, and with few exceptions all s-CD44 values measured in individuals who were later diagnosed with lymphoma were lower than the highest values found among the normal controls. It has been reported that healthy smokers have higher s-CD44 levels than non-smokers.(28) In the present series all cases and controls were smokers at the time of blood sampling, which may have influenced the s-CD44 levels, and it is not known whether the differences in the s-CD44 serum levels between the cases and the controls are greater among non-smoking subjects.

Based on *in vivo* and *in vitro* studies the origin of elevated s-CD44 in patients with non-Hodgkin's lymphoma lies in lymphoma deposits.(23) Up to 90 % of non-Hodgkin's lymphomas express at least some cell surface CD44 in immunohistochemical studies.(13) Strong expression of CD44 in lymphoma tissue has been found to be associated with a low histological grade of malignancy, but, paradoxically, with a relatively poor prognosis possibly due to enhanced lymphoma dissemination, whereas the lack of CD44 has been found to be associated with a high cellular proliferation rate but with a low Ann Arbor stage.(13) Endogenous protease activity is a likely cause for shedding of CD44, because protease inhibitors have been shown to decrease the amount of shed CD44 from the cell surface.(29) While located on the cell surface, CD44 may bind growth factors and reduce their binding to their cognate receptors, which, in turn, might result in a slow or absent cell proliferation. According to this hypothesis shedding of s-CD44 allows enhanced access of growth factors to their receptors, which provides a signal for cell proliferation.

In individuals who were later diagnosed with high grade non-Hodgkin's lymphoma the median s-CD44 level was higher than in the controls when the samples had been taken within 4 years before the diagnosis, suggesting that in some lymphomas shedding of CD44 is a relatively early phenomenon in the malignant process. Loss of adhesion molecules that control lymphoma dissemination at an early stage of tumor development would explain why bulky lymphoma deposits may contain billions of lymphoma cells without any clinical sign of dissemination. Although the loss of normal adhesion molecules may be an important step in the malignant progression of some lymphomas, our finding of very low s-CD44 levels only a few months before the diagnosis in some cases suggests that marked shedding of s-CD44 does not always take place, although little is known about the fate and half-life of shed CD44 in the circulation.

We found somewhat higher s-CD44 levels among patients with stage I or II lymphoma when compared to patients with stage III or IV lymphoma, although the difference was not significant ($p=0.13$). S-CD44 functions as an adhesion molecule in lymphocyte extravasation in high endothelial venules, and lymphomas that contain cells that have not shed their s-CD44 from their outer surface are more likely to be able to disseminate than those that lack s-CD44. Ann Arbor stage reflects the extent of lymphoma dissemination, and the finding that stage I and II lymphomas have higher prediagnostic levels of s-CD44 than stage III or IV lymphomas suggests that some stage I or II lymphomas have a reduced tendency to disseminate because they, for an unknown reason, shed more easily their cell surface CD44.

Non-Hodgkin's lymphoma is a very heterogenous disease consisting of subtypes that may have a greatly different clinical course. Serum samples taken before the diagnosis of lymphoma are very difficult to find and, due to the limited number of serum samples available, prediagnostic serum s-CD44 levels in single histological types of the disease could not be examined in depth. However, patients with B-cell diffuse large cell lymphoma, marginal zone lymphoma, or follicular lymphoma appeared to have elevated serum s-CD44 levels a few years before the diagnosis when compared to the controls.

In conclusion, s-CD44 may often be elevated a few years before the diagnosis of non-Hodgkin's lymphoma, especially among patients later diagnosed with high grade malignant non-Hodgkin's lymphoma. However, the serum levels are only slightly higher than those found in the controls, and they are only sel-

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dom higher than the highest values measured in individuals who will not develop lymphoma suggesting strongly that s-CD44 determinations cannot be used in screening of lymphoma. Further study is needed to find out whether longitudinal measurements of serum s-CD44 are of value in the diagnosis of lymphoma relapse in patients who have been successfully treated for non-Hodgkin's lymphoma.

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