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Substance P – a potent risk factor in childhood lymphoblastic leukaemia

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The study focused on determining the expression of substance P (SP) in neoplastic bone marrow cells in childhood acute lymphoblastic leukaemia (ALL) in terms of its mRNA and the level of protein production. An attempt has also been made to demonstrate a correlation of SP with leukaemia risk factors and treatment failure. The study group comprised 120 children treated for ALL. Expression of SP was examined by *in situ* hybridisation with a 5′-biotinylated probe and by immunocytochemistry with specific anti-human SP antibody. Out of 80 patients with common ALL, the expression of SP was demonstrated in 33 cases (41.2%). In the group of 24 children with pre-B ALL, the presence of SP was noted in six cases (25.0%). Of 16 patients with T-cell leukaemia, SP expression was demonstrated in 13 cases (81.2%). The percentage of immunopositive cells in the SP-positive cases ranged from 79.8 to 97.3. Treatment failure in the children with ALL was closely related to the expression of SP observed at the beginning of treatment. The results showed a connection between the presence of SP-positive blasts and leukaemia relapse. This may indicate that SP expression, involved in the proliferation of the tumour cells, may represent a novel risk factor in ALL.
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Introduction

Substance P (SP), a neuromediator, is an oligopeptide consisting of 11 amino acids (H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2), with a C-terminal fragment typical for tachykinins.[1] SP and neurokinin A (NK-A) are products of the common preprotachykinin I (PPT-I) gene and are capable of modulating the functions of the immune and haematopoietic systems.[2] SP stimulates proliferative activity of cells with surface receptors for SP (NK-1R).[3] According to some authors, peptidergic nerve terminals in the closest proximity to immunocompetent cells in the organs most exposed to exogenous antigens represent a morphological exponent of functional interrelations between the nervous and immune systems.[4,5] It is of particular significance that neoplastic lymphoblasts in acute lymphoblastic leukaemia (ALL) in children carry a three- to four-fold excess of NK-1R as compared to normal lymphocytes.[6]

ALL constitutes up to 30% of all neoplastic diseases in children.[7] According to the Polish Paediatric Leukaemia/Lymphoma Study Group, the most important independent risk factors that critically affect selection of treatment include a patient's age below 12 months of life and blast hyperleucocytosis >50 G/l.[7] A blast level <50 G/l and a patient's age exceeding 12 months represent unequivocal criteria for assigning a child to a low-risk (LR) group.[7] A baseline leucocytosis >50 G/l, a patient's age of less than 12 months or the presence of at least one risk factor (poor response to 7-day prednisone treatment: a decrease in peripheral blood blasts by less than 50%, a delayed start of remission and translocations) allocate a child to a high-risk (HR) group.[8–10] The treatment protocol for the HR group patients (New York II scheme) [11] is quite different from that of the LR group (ALL-BFM-90 programme).[12]

Despite extensive recognition of the molecular bases of bone marrow proliferative diseases, the mechanisms responsible for disease progression need further investigation. Both the complex bone marrow microenvironment and the presence of peptidergic nerve endings may facilitate proliferation of neoplastically transformed haematopoietic cells, although no convincing proof for this hypothesis has, so far, been produced. The choice of SP as a substance, which may be involved in the pathogenesis of leukaemia in children relies on its established role in normal haematopoiesis,[3] its broad distribution and bioavailability[3,4] and the probability of its presence in leukaemic cells, as indicated by the results of our earlier immunocytochemical studies on SP in bone marrow hypoplasia.[13]

The present study is aimed at determining the expression of SP at the level of mRNA and its protein product in neoplastically transformed bone marrow cells in childhood ALL. The results of these tests, performed prior to the initiation of treatment, demonstrated that SP expression in the neoplastic cells correlated with known leukaemia risk factors and with treatment failure.

Materials and methods

Bone marrow samples taken from children treated in the Department of Paediatric Haematology and Oncology at the University of Medical Sciences in Poznan between 1998 and 2002 were used for the study. The research protocol was approved by the Ethical Commission of the University. One hundred and fourty children refered to our Department were included in the investigation. Bone marrow biopsies were performed on them all and evidence of neoplasia was found in 120. The remaining 20 children, all of whom presented with only one enlarged lymph node, served as the control group. Here, histopathological examination of the enlarged node indicated an inflammatory response only. Subsequent observation of the children for around 12 months in the Outpatient Clinic for Hyperplastic Diseases, Institute of Paediatrics, detected no clinical traits of a neoplastic disease.

Of the patients, 80 children were diagnosed with common ALL, 24 with pre-B ALL and in the remaining 16 children T-cell leukaemia was diagnosed. The relevant data are presented in Table 1.

The samples of bone marrow were taken from the posterior superior iliac spine. Bone marrow smears were fixed in 96% ethanol (30 min at room temperature) within 24 h of sampling and kept at -20°C until immunophenotyping and *in situ* hybridisation was performed.

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Table 1 Clinical pretreatment characteristics, selected laboratory data and results of SP determination in children with ALL

Study group	Age	Number of patients	ALL phenotype	Risk group	% SP-positive cases	SP % blast		P-value (McNemar's analysis)
						ICC	ISH	
1	4–17	69	Common	LR	43.5	78.4	82.3	0.8125
2	6–16	11	Common	HR	27.3	87.9	89.9	0.9399
3	3–14	18	pre-B	LR	22.2	84.6	88.9	0.8201
4	4–17	6	pre-B	HR	33.3	85.3	89.6	0.7624
5	6–13	5	T cell	LR	60.0	88.1	92.1	0.8231
6	5–16	11	T cell	HR	90.9	93.6	94.7	1.0

Common=common acute lymphoblastic leukaemia antigen (CALLA, CD10) immunophenotype; pre-B=CD10, CD19, CD20, CD22 immunophenotype; T cell=CD2, CD3 immunophenotype; LR=low-risk patients; HR=high-risk group; SP % blast=the percentage of SP-positive blast cells to the total number of nucleated cells in SP-positive children; ICC=immunocytochemistry; ISH=*in situ* hybridisation; (P-value) statistically significant if <0.05.

In order to demonstrate the presence of SP in bone marrow cells, an indirect immunocytochemical procedure was performed with rabbit antibodies against human SP (Serotec, PEP A40) and the StreptABComplex/HRP method modified by the application of biotinylated tyramine (Dako Catalysed Signal Amplification System, Peroxidase, K 1500).[14] Heat-induced antigen demasking pretreatment was also carried out (Target Retrieval Solution, Dako S 1699).[15] The endogenous activity of peroxidase was blocked by 10 min preincubation in 10% hydrogen peroxide. The smears were then incubated with anti-SP antibodies diluted 1:500–1:2000 for 12 h at +4°C. Incubation with a second antibody (biotinylated goat anti-rabbit, Dako E 0432, diluted 1:300) was performed at room temperature for 60 min followed by incubation with diaminobenzidine (DAB, Dako S 3000).

Immunocytochemical staining for HLA-DR, CD2, CD3, CD10, CD19, CD20, CD22, CD79a antigens was used in blast immunophenotyping.[16]

For detection of mRNA encoding the amino-acid sequence of human SP, a 5′-biotinylated probe of the following nucleotide sequence: 5′GAG TTT GGA TTC TGA TGA CCT CCC AAG CCG GCA 3′, was used.[17] The probe was synthesised by the DNA-Gdańsk company and was complementary to the nucleotide sequence of human SP (GenBank; www.ncbi.nlm.nih.gov). The smears were incubated with the probe (concentration: 200 ng/1 ml) for 18 h at +37°C in a hybridisation chamber. This was followed by incubation with a streptavidin–biotin complex (Dako, K 1500, diluted 1:100) at room temperature for 60 min and incubation with DAB for 5 min. Both techniques complied with the principles of positive and negative controls.[18] Both analyses were performed blind on coded samples. Results of the immunocytochemical reactions and of the *in situ* hybridisation were examined under a light microscope (Eclipse 600, Nikon), at 1:200–1:400 magnification. Based on the results of the haematological staining, which were analysed through the use of Microimage (Olympus) morphometric software, the content of reaction-positive cells was determined by comparing the number of cells with a positive reaction for SP or mRNA SP with the total number of blasts. A percentage of SP-positive cells less than 5% was deemed to reflect technical errors and was classified as a negative result.

The clinical diagnosis of ALL remission was confirmed by blood morphology, microscopical analysis of subsequent bone marrow samples (May-Grunwald-Giemsa staining, haematological remission) and a second immunophenotyping of bone marrow nucleated cells (immunological remission).[19]

Statistical analysis

Since the studied group was relatively small, the statistical analysis was based on McNemar's analysis and Fisher's exact test. The first was used to verify the difference in percentage of SP-positive cells noted by the immunocytochemical and *in situ* hybridisation techniques. Comparison of frequencies of leukaemia treatment failure and of risk factors between the SP-positive and SP-negative patients was performed using Fisher's exact test. Significance was defined at $P=0.05$.

Results

Expression of SP (in both the immunocytochemical and with the hybridisation technique) was confirmed in 33 patients with common ALL (41.2%). and in six patients with pre-B ALL (25.0%). Out of 16 patients with T-cell leukaemia, SP was expressed on the blast cells of 13 patients (81.2%). The percentage of immunopositive blasts in the three groups ranged from 78.4 to 94.7% (mean of 84.5% blast cells for *in situ* hybridisation and 81.8% with the immunocytochemical technique). McNemar's analysis revealed no significant difference between the results obtained by the immunocytochemical and *in situ* hybridisation techniques (*P*-values ranged from 0.7624 to 1.00). The detailed information is presented in Table 1.

SP was absent from the cytoplasm of the normal haematopoietic cells of the 20 children in the control group.

During the observation period (13–48 months), 12 cases of early leukaemia relapse were noted. These relapses developed at least 18 months after the start of treatment but before the 6th month after completion of the treatment. There were also five cases of disease progression with a poor response to a cytoreducing 7-day treatment with prednisone; a reduction in the peripheral blood blast level by less than 50% accompanied by a delayed remission, after the 28th day following introduction of the full steroid dose. These disease progressions (ie failed inductions) resulted, in all of the cases, from drug resistance to glucocorticoid treatment. The relapses and progressive cases consisted exclusively of those children with SP-positive blasts before the start of treatment.

As demonstrated by Fisher's exact test (Table 2), the risk of leukaemia treatment failure (progression or relapse), when all the patients were considered (regardless of leukemia phenotype and type of failure), was found to be significant in the HR group ($P=0.018$).

Table 2 Correlation between treatment failure (disease progression or disease relapse) and risk factors in HR children with ALL (Fisher's exact test)

ALL type	*Correlation of HR group patients to*		
	Disease progression (P-value)	*Early disease relapse (P-value)*	*Σ of treatment failure (progression + relapse) (P value)*
1. Common	0.13	0.53	0.18
2. Pre-B	0.75	0.59	0.71
3. T cell	0.7	0.63	0.63
Σ	0.08	0.12	**0.018**

Common=common acute lymphoblastic leukaemia antigen (CALLA, CD10) immunophenotype; pre-B=CD10, CD19, CD20, CD22 immunophenotype; T cell=CD2, CD3 immunophenotype; LR=low-risk patients; HR=high-risk group; (*P*-value) statistically significant if < 0.05.

Table 3 Correlation between treatment failure (disease progression and disease relapse) and SP expression in blast cells of children with All (Fisher's exact test)

Study group	*Risk group*	*Correlation of SP-positive blast pattern to*		
		Disease progression (P-value)	*Disease relapse (P-value)*	*Σ of treatment failure (progression+relapse) (P-value)*
1.	LR	—	**0.0317**	**0.0317**
2.	HR	0.27	0.27	0.054
Σ (1+2)		0.41	0.099	**0.037**
3.	LR	1.0	**0.039**	**0.049**
4.	HR	—	0.067	0.33
Σ (3+4)		0.25	0.099	**0.014**
5.	LR	1.0	1.0	0.3
6.	HR	1.0	1.0	0.545
Σ (5+6)		0.51	0.39	0.15
Σ (1+2+3+4+5+6)		**0.01**	**0.00**	**0.00**

LR=low-risk patients; HR=high-risk group; (*P*-value) statistically significant if <0.05; '–'=absence of the trait.

On the other hand, as shown in Table 3, the risk of relapse was significantly greater in the LR group with SP-positive blasts and additional common and pre-B ALL phenotypes (study groups 1 and 3) than in all the HR patients and T-cell LR patients who were SP-positive (study groups 2, 4, 5 and 6). It should be emphasised that the distribution of disease relapse in study groups 1 and 3 depended on the assignment to a given risk group and, hence, on baseline leucocytosis. Analysis of disease relapse in all the patients (study groups 1–6) revealed a significantly higher risk for SP-positive children ($P=0.00$).

The distribution of leukaemia progression in every study group was independent of the SP-positive blast level (*P*-value ranged from 0.25 to 1.0), even if analysis of all the patients, without their categorisation into leukaemia types, demonstrated a significantly more frequent progression in SP-positive children ($P=0.01$).

Statistical analysis of those patients in whom no treatment failure was noted during the observation period revealed that the probability of permanent remission of leukaemia was significantly higher in those LR patients with SP-negative blasts who were initially assigned to the common ALL group ($P=0.0317$) and pre-B ALL group ($P=0.049$).

Discussion

The most important determinant of a favourable prognosis in cases of ALL is an appropriately selected and fully implemented treatment regimen. However, in determining the best form of therapy some of the current indices are not conclusive. Using standard criteria, namely age and initial leucocytosis and no response to steroid therapy on days +7 and +28, we allocated 28 children to the HR group and 92 to the LR group. Over the 48-month period of the investigation there were nine treatment failures (31%) in the HR and eight (8.7%) in the LR group ($P=0.018$, Table 2). However, a more detailed analysis, with proper attention given to the type of unfavourable episode (disease progression or relapse of the disease) and to the type of leukaemia (common, pre-B or *T*-cell leukaemia), failed to disclose a significantly higher risk of treatment failure in children of the HR group (Table 2). It would therefore be very useful to introduce a new prognostic index, which would accurately reflect prognostic differences between individual types of ALL.

We were led to the choice of SP as a potential prognostic factor in ALLs by the results of our earlier observations regarding the risk of neoplastic transformation in patients affected by bone marrow hypoplasia.[13] In patients affected by this condition, immunocytochemical detection of SP in normal bone marrow cells demonstrated that, within 6–18 months after establishing the diagnosis, SP-positive patients developed neoplastic transformation which led to acute leukaemia. We therefore assumed that the presence of immunocytochemically SP-positive blasts in ALL patients might be of prognostic significance.

In order to make the findings as reliable as possible, we examined SP expression at the level of mRNA (*in situ* hybridisation) and at the level of the synthesised peptide (immunocytochemistry). The results are presented in Table 1.

The absence of correlation between the presence of SP-positive blasts and an unfavourable course in patients with T-cell leukaemia requires some discussion. The observation may be explained by the significantly higher proportion of SP-positive patients in that group (81%) as compared to the remaining patients (38%) (McNemar's analysis: $P=0.00$). As reported in the literature,[20] SP may also be present in normal, mature T lymphocytes in persons manifesting no lymphoproliferative processes in the bone marrow. This might indicate that, in patients with T-cell leukaemia, proliferation of blast cells leading to relapse of the disease takes place along SP-independent pathways.

The available literature on the subject repeatedly draws the reader's attention to the fact that SP, outside the nervous system, may be produced and released by monocytes, T lymphocytes, eosinophils and dendritic cells.[20,21] The results of our study suggest that SP mRNA and SP peptide are also expressed in some blast cells of common, pre-B and T origin showing a low level of differentiation.

The results obtained so far have shown that, in normal haematopoiesis, the nerve-ending released SP represents an important factor capable of stimulating this process.[22] It enables the expression of genes for other cytokines, as well as their synthesis and release by cells of the bone marrow stroma. Based on the available data, SP is regarded as the only recognised NK-1 receptor agonist and, apart from IL-1, SP represents the most important agent capable of stimulating bone marrow stromal

cells to synthesise cytokines and indicating the presence of adhesion molecules (LEC-CAM, ELAM-1, ICAM-1, CD43, CD45RA, CD164) on their surface.[23]

According to the number of disease relapses observed in our study groups and its correlation to the SP-positive blast pattern in bone marrow blasts, we suggest that SP may not only be an important factor involved in the proliferation of leukaemic cells, but may also be regarded as a novel risk factor in children with common and pre-B leukemia who are currently classified as low risk. On the other hand, the lack of any statistical significance in correlation between disease relapse and the SP-positive blast pattern in HR patients with common ALL, indicates that the more aggressive New York II treatment programme may provide a longer lasting remission in these children.

Conclusions

The possible connection between the SP-positive blast pattern and leukaemia relapse may prove that SP is involved in proliferation of the tumour cells. It may also justify routine determination of SP as a potent risk factor before the treatment regimen of ALL is chosen and initiated.

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