The predicting role of substance P in the neoplastic transformation of the hypoplastic bone marrow

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Aims: To estimate the expression of substance P in the haematopoietic cells of hypoplastic bone marrow and define its relationship with the course of bone marrow hypoplasia.

Methods: Bone marrow specimens were obtained from 42 children with bone marrow hypoplasia who were hospitalised in the Department of Pediatric Oncology, Hematology and Transplantology, Poznan University of Medical Sciences, Poznan, Poland, between 1996 and 2003. Substance P and Ki-67 expression were evaluated using immunocytochemical and hybridisation techniques. The expression of substance P (as evidenced by both immunocytochemical and hybridisation techniques) was confirmed in the cytoplasm of B lymphocytes in 8 of 11 children who developed acute leukaemia in 45 (SD 12) days. The percentage of substance P-positive cells ranged from 67.6 to 95.8 (mean of 81.5% cells with immunocytochemistry and 84.3% with in situ hybridisation). The risk of development of leukaemia secondary to bone marrow hypoplasia was found to be significant (p<0.001) in those children who expressed substance P in normal-looking lymphocytes at the initial bone marrow evaluation.

Conclusions: The presence of substance P in B lymphocytes of hypoplastic bone marrow may predict its neoplastic transformation. A marked correlation between substance P-positive bone marrow pattern and the expansion of tumour cells may prove the potential value of this oligopeptide in the pathogenesis of leukaemia.

Bone marrow hypoplasia seems to be a morphological equivalent of clinically diagnosed haematopoietic insufficiency. It is defined by the following laboratory values: haemoglobin <8.5 g/dl, mean corpuscular volume <88 fl, a white cell count <2 g/l with a neutrophil count <1 g/l, a platelet count <50 g/l and reticulocytes <0.1%. Bone marrow aspirate contains hypocellularity. Transient bone marrow hypoplasia is usually caused by infections (both viral and bacterial) or by different chemical (including iatrogenic) or physical factors.

A considerable number of cases of bone marrow hypoplasia may be subjected to spontaneous remission and may not even be recognised. The first hospitalisation of a child with bone marrow hypoplasia is usually after two or more ineffective courses of antibiotic treatment (for some undiagnosed chronic infection), followed by symptoms of anaemia and thrombocytopenia. In such cases, bone marrow hypoplasia can be diagnosed as myelodysplastic syndrome, severe aplastic anaemia or neoplasia. The correct diagnosis usually takes several weeks, during which time the affected children are not treated but usually receive continuous monitoring, with laboratory evaluation of peripheral blood, bone marrow, chest radiographs and so on being carried out at least once a month.

The acute leukaemia of both lymphoblastic and non-lymphoblastic types, which develops secondary to bone marrow hypoplasia, usually has a bad prognosis. This may, at least in part, result from the late introduction of a chemotherapy regimen. In cases of neoplastic transformation, the type of chemotherapy used will depend on a precise recognition of the neoplasm concerned. Such treatment, however, can probably be introduced much faster if, in the mean time, the expression of certain markers of early neoplastic transformation can be detected sooner. The difficulty at present is that the full list of possible markers is not universally accepted. On the other hand, some authors emphasised that several of the cytokines which regulate physiological haematopoiesis may themselves be possible risk factors of neoplastic transformation in bone marrow hypoplasia.

One of these cytokines is substance P, a neuromediator and 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 of tachykinins. It is widely distributed in the central and peripheral nervous systems, being responsible for contraction of skeletal and smooth muscle, salivation, micturition, vasodilatation and sensory perception. Substance P and neurokinin A are products of the common preprotachykinin I gene and are also capable of modulating the functions of the immune and haematopoietic systems.

Substance P stimulates proliferative activity of cells with surface receptors for substance P. It is generally accepted that substance P shows a preference for neurokinin-1 receptor (NK-1R) and has less affinity for NK-2R and NK-3R. The expression of NK-1R has been reported in T and B lymphocytes, macrophages, haematopoietic progenitor (CD34) cells, endothelial cells and CD10 lymphoblasts. It is of particular significance that neoplastic lymphoblasts in acute lymphoblastic leukaemia (ALL) in children carry a threefold to fourfold excess of NK-1R as compared with normal B and T lymphocytes.

According to some authors, peptidergic nerve terminals in closest proximity to immunocompetent cells in the organs most exposed to exogenous antigens represent a...
marginal increase of functional inter-relations between the nervous and immune systems.

However, substance P present in the bone marrow can be also of non-neural origin. It is synthesised in the macrophages, eosinophils and neoplastically transformed lymphoblasts. It has been shown that substance P induces interleukin 1 (IL1) and stem cell factor in bone marrow stromal cells, with levels of IL1b (a major isoform of this cytokine) being twice those of IL1a. Both cytokines also perform a potent autoregulatory role in NK-1R expression in bone marrow stromal cells.7

Therefore, our study aimed to estimate the expression of substance P in haematopoietic cells of hypoplastic bone marrow and define its relationship with the future course of bone marrow hypoplasia.

PATIENTS AND METHODS

Patients

Patients were children with bone marrow hypoplasia, hospitalised in the Department of Pediatric Oncology, Hematology and Transplantology (Poznan University of Medical Sciences, Poznan, Poland) between 1 January 1996 and 31 December 2003. In all, 485 children were referred to our department during that time. Bone marrow biopsies were performed on all of them and evidence of bone marrow hypoplasia was found in 42. Twenty children, all of whom presented with only one enlarged lymph node, served as the control group. Histopathological examination of the enlarged node indicated an inflammatory response only. Subsequent observation of these children in the outpatient clinic for hyperplastic diseases for around 12 months detected no clinical traits of a neoplastic disease. This research protocol was approved by the ethical commission of the university.

To participate in the investigation, children had to meet the following eligibility criteria: age 4–18 years, laboratory values: haemoglobin <8.5 g/dl, mean corpuscular volume <88 fl, white cell count <2 g/l with a neutrophil count <1 g/l, platelet count <50 g/l and reticulocytes <0.1%, hypocellularity of bone marrow aspirate, with no neoplastic cells evaluated by microscopy and flow cytometry test; normal chest radiograph; no enlarged lymph nodes, spleen or liver; and sterile blood cultures (obtained at least three times) at the time of the first hospitalisation. All the children underwent reverse transcriptase-polymerase chain reaction evaluation for hepatitis C virus, Epstein–Barr virus and cytomegalovirus. Table 1 presents the relevant data.

Bone marrow smear preparation

The samples of bone marrow were taken from the posterior superior iliac spine. Bone marrow smears prepared on Super Frost were fixed in 96% ethanol (30 min at room temperature) within 24 h of sampling and kept at −80°C until

immunophenotyping and in situ hybridisation (ISH) were carried out.

Antibodies and immunocytochemistry

To show the presence of substance P in bone marrow cells, an indirect immunocytochemical procedure was carried out using rabbit polyclonal antibodies against human substance P (Serotec, PEP A40, Oxford, UK) and the streptavidin–biotin complex method with the use of horseradish peroxidase modified by application of biotinylated tyramine (Dako Catalysed Signal Amplification System, Peroxidase, K 1500, Dako, Copenhagen, Denmark).14 Heat-induced antigen demasking pretreatment was also carried out (Target retrieval solution, Dako S 1699).14 The endogenous activity of peroxidase was blocked by 10-min pre-incubation in 10% hydrogen peroxide. The smears were then incubated with anti-substance P antibodies diluted 1:2000 for 12 h at 4°C. Incubation with a secondary antibody (biotinylated goat anti-rabbit, Dako E 0432, diluted 1:300) was carried out at room temperature for 60 min followed by incubation with diaminobenzidine (Dako S 3000).

Ki-67 antigen detection included an immunocytochemical procedure with mouse monoclonal antibodies directed against human Ki-67 (Dako, M 7187). The smears were incubated with anti-Ki-67 antibodies, diluted 1:100 for 12 h at 4°C. Incubation with the secondary antibody (biotinylated goat anti-mouse, Dako E 0433, diluted 1:300) was carried out at room temperature for 60 min.

After incubation with antibodies against human leucocyte antigen-DR, CD3, CD10, CD13, CD14, CD15, CD20, CD23, CD34, CD41, CD61 and glycophorin A were used in white cell (including blasts) immunophenotyping.18

In situ hybridisation

For detection of mRNA encoding the amino acid sequence of human substance P, a 5’-biotinylated probe of the nucleotide sequence 5’-TCT GGG TTC GGA GTC GTC AAG AAA CCT AAT TAC 3’, was used.17-19 The probe was synthesised by the DNA-Gdansk (Gdansk, Poland) and was complementary to the nucleotide sequence of human substance P ( locus = 7q21–q22, gene = “TAC1”, product = “substance P”, bp = 68–100; http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val = M68907). The smears were incubated with the probe (concentration: 200 ng/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 diaminobenzidine for 5 min.

Controls and microscopical analysis

Immunocytochemistry (ICC) and hybridocytochemistry complied with the principles of positive and negative controls.20 Both analyses were carried out blind on coded samples.

<table>
<thead>
<tr>
<th>Study group</th>
<th>No of patients</th>
<th>Age (year)</th>
<th>RT-PCR viral infection markers (n)</th>
<th>12-month observation (n)</th>
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<td>HCV</td>
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<td>17</td>
<td>10 (6.2)</td>
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<td>II</td>
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<tr>
<td>III</td>
<td>11</td>
<td>10 (1.8)</td>
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<td>6</td>
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<tr>
<td>IV</td>
<td>4</td>
<td>14 (2.7)</td>
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Age was expressed as the mean (SD).

ALL, acute lymphoblastic leukaemia; ANLL, acute non-lymphoblastic leukaemia; CMV, cytomegalovirus; EBV, Epstein–Barr virus; HCV, hepatitis C virus; RT-PCR, reverse transcriptase-polymerase chain reaction. —, absence of the trait in all patients from a given group.
control incubations, primary antibodies or oligoprobes were replaced by rabbit or mouse serum (positive control) or phosphate-buffered saline (negative control). The results of the immunocytochemical reactions and of the ISH were examined under a light microscope (Eclipse 600, Nikon), at magnifications of 1:200–1:400. On the basis of 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 substance P, Ki-67 or mRNA substance P with the total number of blasts. A percentage of substance P-positive cells <5% was deemed to reflect technical errors and was classified as a negative result.

**Statistical analysis**

As the group studied was relatively small, statistical analysis was based on McNemar analysis and Fisher’s exact test. The first was used to verify the difference in percentage of substance P-positive cells noted by the immunocytochemical and in ISH techniques. The relationship between substance P-positive cases and an unfavourable course of bone marrow hypoplasia was determined using the Fisher’s exact test. Significance was defined at p<0.05.

**RESULTS**

**12-month monitoring**

Every child from the study group underwent a 12-month period of observation. During that time, laboratory values of peripheral blood were evaluated at least once a week; bone marrow, depending on the clinical course, was aspirated every 30–60 days; and chest radiographs and abdomen ultrasonography were carried out every 3 months. Reverse transcriptase-polymerase chain reaction markers of viral infection (see Patients and methods) were estimated only once. Of the patients, 17 children underwent a spontaneous remission (subgroup I, median time of observation 93 (standard deviation (SD) 23) days) and 10 children developed severe aplastic anaemia (subgroup II, median time 212 (162) days). Eleven children were diagnosed with acute leukaemia (subgroup III): eight had ALL with a median time of 44 (12) days and three had acute non-lymphoblastic leukaemia (ANLL) with a median time of 48 (13) days. In the remaining 4 children (subgroup IV), bone marrow hypoplasia did not develop during the 12-month period of observation, and these children were excluded from further analysis (table 1).

**Outcome**

The 17 children who underwent spontaneous remission are still alive. The median time of observation is 37 (10.2) months. These patients are regularly evaluated for clinical traits of aplastic anaemia or neoplastic disease.

Of the 10 patients who developed severe aplastic anaemia (haemoglobin <7.0 g/dl, mean corpuscular volume <85 fl, white cell count <1.0 g/l with a neutrophil count <0.5 g/l, platelet count <20 g/l), 6 underwent allogenic bone marrow transplantation and they are alive at the first remission. Three children in this group were given thymoglobulin (they are also alive at the first remission). The one remaining child died 50 days after first hospitalisation due to a cytomegalovirus infection. The median time of observation of the children of subgroup II who survived is 32 (12.5) months.

The children of subgroup III had the worst outcome. Six of the eight patients with ALL died, with no clinical and haematological signs of remission (median time of observation after the development of ALL was 45 (12) days). In the remaining two children ALL remission was diagnosed, but they later relapsed (median time of observation 284 (68) days) and underwent allogenic bone marrow transplantation. They are still alive in the second remission, but the period of observation is not long (12 (4.5) months). Of the three patients, two with ANLL died, with no clinical and haematological signs of remission (median time of observation after ANLL was recognised as 34 (16) days). The remaining child with ANLL is in remission and is still alive (time of observation 24.5 months).

Overall, 9 of the 42 patients (21.4%) investigated in this study died between 1 January 1996 and 31 December 2003, 27 (64.3%) are alive at the first remission and 2 (4.8%) at the second remission. Four patients (9.5%) were excluded from the analysis. The relevant data are presented in fig 1.

**White cell immunophenotyping**

In all the children of the study group the population of nucleated cells in the hypoplastic bone marrow was mainly composed of lymphocytes (mean of 96.4%). The percentage of B lymphocytes (CD20) was >95% (mean of 95.1%), whereas T lymphocytes (CD3) constituted <4% of all lymphocytes. Other individual nucleated cells were first represented by neutrophils (fig 2A). After neoplastic transformation B lymphocytes constituted <5% of all nucleated cells (fig 2B), which consisted mostly CD10-positive blasts in cases of ALL, or CD13/CD14-positive cells in cases of ANLL (data not shown).

**ICC and ISH**

Before and at the time of neoplastic transformation the expression of substance P (in both the immunocytochemical and hybridisation techniques) was confirmed exclusively in the cytoplasm of B lymphocytes in 8 of the 11 children assigned to subgroup III (fig 2,C,E). The number of substance P-positive cells ranged from 67.6% to 95.8% (mean of 81.5% cells for ICC and 84.3% with ISH). This positive expression was also found in the subsequent bone marrow examination and the range of positive cells did not differ markedly from that in the initial findings. During the 12-month observation period, seven substance P-positive patients developed ALL and one developed ANLL. The mean percentages of substance P-positive cells at this stage of the diseases were 82.7 (ICC, fig 2D) and 88.9 (ISH, fig 2F), respectively. No differences were observed among the densities from substance P staining in individual patients.

The expression of Ki-67 antigen in the bone marrow haematopoietic cells was not evaluated at the first hospitalisation. However, it was detected at the time of (fig 2G), and after (fig 2H), neoplastic transformation, when the percentage of Ki-67-positive cells (blasts) ranged from 39.6% to 49.8% (mean of 42.6% blast cells) of all nucleated cells.

Substance P was absent from the cytoplasm and Ki-67 from the nucleus of the normal haematopoietic cells of the 20 children in the control group (data not shown). Table 2 presents the information in detail.

**Statistical analysis**

McNemar analysis showed no significant difference between the results obtained by the ICC and ISH techniques (p values ranged from 0.25 to 1.00).

When all the patients were considered (regardless of leukaemia type), the risk of developing leukaemia secondary to bone marrow hypoplasia was found to be significantly greater (p<0.001, Fisher’s exact test) in those children who showed expression of substance P in normal-looking lymphocytes at the initial bone marrow evaluation—that is, before neoplastic transformation. However, in subgroup III the risk of death resulting from progression of the leukaemia was not significantly greater in the children who were substance P-positive at the initial bone marrow examination.
(ALL-related death: p = 0.248; ANLL-related death: p = 1.0; death regardless of leukaemia type: p = 0.153). On the other hand, statistical analysis of those patients in whom no treatment failure was noted during the observation period showed that the probability of remission of bone marrow hypoplasia was significantly greater in those children who were initially substance P-negative (p < 0.001).

No marked relationship was found between initially detected viral infection and the outcome of bone marrow hypoplasia.

**DISCUSSION**

The search for causes and aids to the diagnosis of bone marrow hypoplasia includes taking a thorough history, a detailed physical examination and several laboratory tests, including microscopic analysis of bone marrow, sampled by needle biopsy or during surgery, determination of infectious diseases and, occasionally, genetic studies of patients and their families. The application of ICC techniques in the diagnosis of bone marrow hypoplasia has been tested already. However, attempts to use this technique have been restricted to the differentiation of myelodysplastic syndromes from various grades of bone marrow aplasia. Among other antigens, Ki-67 antigen and proliferating cell nuclear antigen are useful in such a procedure. In patients with bone marrow aplasia the markers could not be seen in nucleated cells, whereas in patients with myelodysplastic syndromes the percentage of immunopositive cells ranged from 20% to 60%. These studies, however, have not attempted to determine the future trend of such bone marrow hypoplastic lesions.

Our selection of substance P as an indicator of the potential evolving trend of bone marrow hypoplasia was prompted by detection of its role and the role of other neuropeptides in the physiological control of haematopoesis. Owing to the peptidergic nerve endings in bone marrow, substance P...
easily comes into contact with haematopoietic and stromal cells of the bone marrow. Previous investigations have proved that substance P is released in bone marrow by macrophages, eosinophils and vascular endothelial cells. Most bone marrow cells—that is, haematopoietic cells, the cells forming bone marrow stroma, and T and B lymphocytes present in bone marrow—are equipped with the substance P-specific NK-1R receptor. Substance P, tested in short-term human bone marrow cultures in methylcellulose, is capable of supporting haematopoiesis in vitro. This indicates that at a concentration of $10^{-8}$ to $10^{-11}$ mol/l it can substitute for IL3 and for granulocyte–monocyte colony stimulating factor, the presence of which is indispensable for growth of the colonies. However, substance P cannot

Figure 2  (A) Immunohistochemical expression of CD20 in the hypoplastic bone marrow of a 7-year-old child assigned to subgroup III. Individual cells present at the time of neoplastic transformation are mostly B lymphocytes. (B) A bone marrow specimen of a 7-year-old child who developed acute lymphoblastic leukaemia. Mature CD20-positive B lymphocytes form a minority of all blasts. (C) Substance P (SP) immunoreactivity in normal-looking B lymphocytes before neoplastic transformation of a 5-year-old child. Its expression is present in the cytoplasm of most nucleated cells. (D) The same 5-year-old child (as in C), who developed acute non-lymphoblastic leukaemia. Weak substance P expression in all blasts. (E) A hypoplastic bone marrow of a 9-year-old child assigned to subgroup III. Expression of substance P mRNA in individual groups of nucleated cells. (F) Expression of SP mRNA in the same 9-year-old child (as in E) after neoplastic transformation (common acute lymphoblastic leukaemia). SP transcript immunoreactivity in all nucleated cells. (G) Expression of Ki-67 antigen at the moment of neoplastic transformation. Strong nuclear immunocytochemical reaction in about half of the normal-looking B-lymphocytes (7-year-old child assigned to subgroup III). (H) Immunocytochemical expression of Ki-67 in the bone marrow specimen of a 7-year-old child who developed acute leukaemia. Ki-67-positive cells constitute one third of all nucleated cells. Scale bar = 50 μm. ICC, immunocytochemistry; ISH, in situ hybridisation.
Substance P was also found to act,

Substance P can also induce synthesis of IL1 and

that substance P acts by inducing synthesis of the cyto-

cytokines. Substance P can also induce synthesis of IL1 and

Some investigators have suggested that substance P

released from nerve endings not only augments IL1 synthesis

but also increases the density of IL1 receptors. In turn, IL1,

released by the sublayer cells, augments the density of NK-1R

receptors on the surface of haematopoietic and sublayer cells.

We have previously reported that substance P is also

secreted by blast cells in ALL. A possible connection

between the substance P-positive blast pattern and leukaemia

relapse may prove that substance P is associated with the

proliferation of the tumour cells.

Our studies of the bone marrow of healthy people (who

formed the control group) showed that the immunocyto-

chemical expression of peripherally located substance P

(most probably coupled to NK-1R) on nucleated cells of the

bone marrow was \( \leq 5\% \). These results prompted us to carry

out analogous determinations in the material sampled from

patients with clinical symptoms of bone marrow hypoplasia.

In some of them the proportion of substance P-positive cells

among all nucleated cells was 67.6–95.8\% (mean of 81.5\% cells

for ICC and 84.3\% with ISH), with an absence of neoplastic cells. Subsequent observation of these patients showed that they developed a proliferative disease of the bone marrow. Throughout the entire time preceding the neoplastic transformation, an increased number of substance P-positive cells was noted even if the proportion of Ki-67-positive cells was similar to control values. Neoplastic transformation was manifested by the presence of poorly differentiated cells with a positive reaction for Ki-67 (39.6–49.8\%) in bone marrow. In line with the above, we suggest that the presence of substance P in normal-looking bone marrow lymphocytes before neoplastic transformation could not predict the death of the patient, which is certainly related to the small number of patients in subgroup III and the different chemotherapy protocols used in the treatment of ALL and ANNL.

Bone marrow hypoplasia, followed by neoplastic transformation, often remains asymptomatic. Only a few patients receive specialist care before the symptoms of bone marrow proliferative disease become evident. In these few patients, the diagnosis of bone marrow hypoplasia leads to subsequent check-ups, during the course of which immunocytochemical analysis of cellular inducers of differentiation on the surface of bone marrow cells may provide a simple screening test pointing to the potential trend of evolution of the lesion.

CONCLUSIONS

1. Substance P expressed in the B lymphocytes of hypo-

plastic bone marrow may predict its leukaemic transforma-

tion.

2. The significant correlation between a substance P-

positive bone marrow pattern and expansion of tumour

cells in the bone marrow may prove the potential role of

this oligopeptide in the pathogenesis of leukaemia.

3. The results reported here, although promising, require

confirmation by the investigation of a larger group of

patients.

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Competing interests: None.

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Predicting role of substance P


