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ORIGINAL PAPER

Bone marrow hyaluronan and reticulin in patients with malignant disorders

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Abstract Myelofibrosis is commonly seen in patients with chronic myeloproliferative diseases and sometimes in myelodysplastic syndrome, acute leukaemia and lymphoproliferative diseases. The fibrotic process is evaluated by grading the amount of collagen deposited in the bone marrow interstitium. The established method to evaluate bone marrow fibrosis is staining for reticulin to visualise the collagen fibres. However, the extra cellular matrix does not only contain collagens but also other components, e.g. glycosaminoglycans of which hyaluronan is the most abundant. Hyaluronan is important for structural and cellular functions. Earlier studies have shown that there is a positive correlation between hyaluronan and reticulin staining in healthy volunteers and in patients with de novo acute myeloid leukaemia. In this study bone marrow biopsies from 43 patients with a malignant disease involving the bone marrow were compared with 18 patients with a malignant disease not involving the bone marrow. The intensity of hyaluronan grading was significantly higher in the patients with disease involving the bone marrow compared to the healthy controls but not compared to the patients without disease involving the bone marrow. The staining intensity of reticulin in the bone marrow was significantly higher in the patients with disease involving the bone marrow, compared to those without disease involving the bone marrow and to the controls. In all patients and the controls there was a correlation between hyaluronan and reticulin.

Keywords Hyaluronan · Reticulin · Fibrosis · Bone marrow · Tumour · Malignant disorder

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Introduction

Development of bone marrow fibrosis is a severe complication in haematological malignancies. In patients with chronic myeloproliferative disease (CMPD) fibrosis is also a common finding and an important purpose of treatment is to reverse fibrosis [1–3]. Myelofibrosis also occurs in myelodysplastic syndrome (MDS), especially in chronic myelomonocytic leukaemia (CMML). In MDS myelofibrosis is a poor prognostic sign and can precede transformation to acute leukaemia [4, 5]. In lymphoproliferative diseases, e.g. hairy-cell leukaemia (HCL), fibrosis is regularly seen in the bone marrow and can be pronounced [6–9]. Myelofibrosis is occasionally seen in acute myeloid leukaemia (AML) especially in acute megakaryoblastic leukaemia but there is a lack of information about the frequency of myelofibrosis in other subtypes of leukaemia. Whether fibrosis in AML is a poor prognostic factor and of importance for outcome has been discussed [10–12].

Bone marrow fibrosis is evaluated by the amount of collagen deposited in the bone marrow interstitium and staining for reticulin is the common way of visualising myelofibrosis [13, 14]. This extracellular space does not

only contain collagen fibres but also many other components, e.g. glycosaminoglycans (GAGs). One such GAG is hyaluronan (HA), which is ubiquitously distributed in connective tissues. HA is a high molecular weight polymer, important for both structure and stability of the interstitium. HA is normally produced at the inner surface of the plasma membrane by hyaluronan synthases (HAS) 1, 2 and 3, and metabolised within hours of synthesis. HA can act as a barrier and filter, and has a significant osmotic capacity and important cell biological properties influencing for example cell maturation and cell proliferation [15–18]. Also low molecular weight forms of HA exist, which participate in many biological functions including neovascularisation. Moreover, the molecule has been designated a significant impact on tumour initiation and progression via stroma and cancer cell interactions [19, 20]. HA is obviously an important component of bone marrow matrix not only from a structural point of view but also regarding cellular functions.

We have previously described the distribution of HA and reticulin in bone marrow biopsies from healthy volunteers, patients with de novo AML and CMPD patients [21–23]. In these studies HA was found in a pattern that was concordant with reticulin staining.

The aim of this study was to further explore the occurrence of HA and reticulin in the bone marrow in patients with various malignant diseases, with and without bone marrow engagement and investigate to which extent bone marrow involvement is correlated to an increase of both HA and reticulin.

Materials and methods

Patients

Bone marrow trephine biopsies from the posterior iliac crest were collected from 61 patients with a malignant diagnosis, 39 males (M) and 22 females (F), median age 57 years (range 26–85 years). These patients were divided in two groups; 43 patients median age 59 years (range 26–83 years) with engagement of the disease in the bone marrow and 18 patients median age 55 years (range 37–81 years) without engagement of the disease in the bone marrow. The 43 patients with bone marrow involvement were as follows: one patient with CML, 14 MDS (two of these were RAEBT/refractory anaemia with excess blasts in transformation, one RAEB/refractory anaemia with excess blasts and one CMML), 7 AML, 3 acute lymphoblastic leukaemia (ALL), 4 hairy-cell leukaemia (HCL), 3 chronic lymphatic leukaemia (CLL), 1 B-cell lymphoma not otherwise specified (NOS), 1 T-cell lymphoma NOS, 6 myeloma and 3 Mb Waldenström had bone marrow involvement (Table 1). The 18 patients with non bone marrow involvement were as follows: one patient with bladder cancer, one prostate cancer, 7 B-cell lymphoma NOS, 1 T-cell lymphoma NOS, 2 mantle cell

Table 1 HA and reticulin grading in patients *with* malignant disease in the bone marrow

Pat	Sex	Age	Diagnosis	Reticulin	HA
1	M	43	AML	2+	3
2	F	49	AML M2	1+	1
3	M	44	AML M2	1+	2
4	F	73	AML M3	N	3
5	M	47	AML M4-M5	2+	3
6	M	56	MDS-AML M2	3+	4
7	M	70	MDS-AML	2+	3
8	M	36	ALL	3+	4
9	F	60	ALL	2+	2
10	F	37	ALL	3+	3
11	F	83	MDS I	3+	4
12	F	50	MDS I	1+	2
13	M	43	MDS I	2+	3
14	M	43	MDS I	2+	2
15	F	62	MDS I	2+	3
16	M	76	MDS I	2+	3
17	F	75	MDS I	3+	1
18	M	73	MDS II	N	1
19	M	79	MDS III	1+	2
20	M	70	MDS III	2+	3
21	F	75	MDS RAEB	1+	2
22	F	26	MDS RAEBT	3+	1
23	M	75	MDS RAEBT	3+	4
24	F	76	(MDS) CMML	1+	2
25	F	80	CML	3+	3
26	M	43	HCL	2+	2
27	M	54	HCL	2+	2
28	M	56	HCL	4+	4
29	M	43	HCL	3+	1
30	M	65	CLL	1+	2
31	F	47	CLL	3+	3
32	F	58	CLL	2+	2
33	M	48	Myeloma	3+	3
34	M	59	Myeloma	3+	2
35	F	75	Myeloma	2+	2
36	F	53	Myeloma	1+	3
37	M	79	Myeloma	3+	4
38	M	45	Myeloma	1+	3
39	F	72	Mb Waldenström	2+	2
40	M	67	Mb Waldenström	3+	3
41	M	66	Mb Waldenström	2+	3
42	M	65	B-cell lymphoma	3+	2
43	M	45	T NHL	2+	3

Age and gender distribution

Table 2 HA and reticulin in patients *without* malignant disease in the bone marrow

Pat	Sex	Age	Diagnosis	Reticulin	HA
1	M	71	Mb Hodgkin	2+	2
2	M	37	Mb Hodgkin	2+	3
3	M	39	B-cell lymphoma	N	1
4	M	44	B-cell lymphoma	1+	2
5	M	49	B-cell lymphoma	2+	2
6	M	61	B-cell lymphoma	1+	2
7	F	65	B-cell lymphoma	1+	3
8	F	54	B-cell lymphoma	2+	3
9	M	52	B-cell lymphoma	1+	2
10	M	70	T-cell lymphoma	2+	3
11	F	54	Mantle cell lymphoma	2+	3
12	M	62	Mantle cell lymphoma	1+	2
13	M	69	DLBCL	N	1
14	F	75	DLBCL	1+	2
15	M	56	Plasmacytoma	1+	2
16	F	81	Plasmacytoma	1+	2
17	M	49	Bladder ca	1+	2
18	M	54	Ca prostate et met	1+	1

Age and gender distribution

lymphoma, 2 diffuse large B-cell lymphoma (DLBCL), 2 Mb Hodgkin, and 2 plasmacytoma had no bone marrow involvement (Table 2).

The myeloid neoplasms were classified according to FAB [24] and the lymphoid according to REAL [25].

All biopsies came from the University Hospital of Tromsö, Norway.

Controls

Bone marrow biopsies from 30 healthy volunteers, 10 males and 20 females, median age 29.5 years (range 18–60 years), served as controls [21].

Bone marrow biopsies

Serial sections of the bone marrow biopsies were mounted on glass slides for haematoxylin–eosin staining for evaluation of morphology, silver impregnation for visualisation of reticulin fibre content according to Laidlaw [26] and histochemical localisation of HA with Hyaluronan-Binding Protein (HABP). The isolation and biotin labelling of HABP was performed in the same manner as described earlier [21, 27]. The HABP was kindly donated from Corgenix Inc. (Westminster, Co, USA).

The bone marrow reticulin was quantified using the following scoring system described by Bauermeister [13]; 0, no reticulin fibres demonstrated; N, occasional fine individual fibres only; 1+, occasional fine individual fibres plus foci of fine fibre network; 2+, fine fibre network throughout most of the section with no coarse fibres demonstrated; 3+, diffuse fibre network and scattered thick, coarse fibres but no true collagen; and 4+, diffuse, often coarse, fibre network with areas of collagenisation. According to Bauermeister the upper normal limit for bone marrow biopsies is 2+.

The content of HA in bone marrow was graded according to intensity in a four-graded scale: 1 sparse; 2 weak; 3 moderate; and 4 intense. This grading scale was used for the control group. Sparse and weak were considered normal [21]. Figures 1 and 2 show bone marrow specimens stained for HA and reticulin.

All samples were coded and examined by one microscopist M.H.

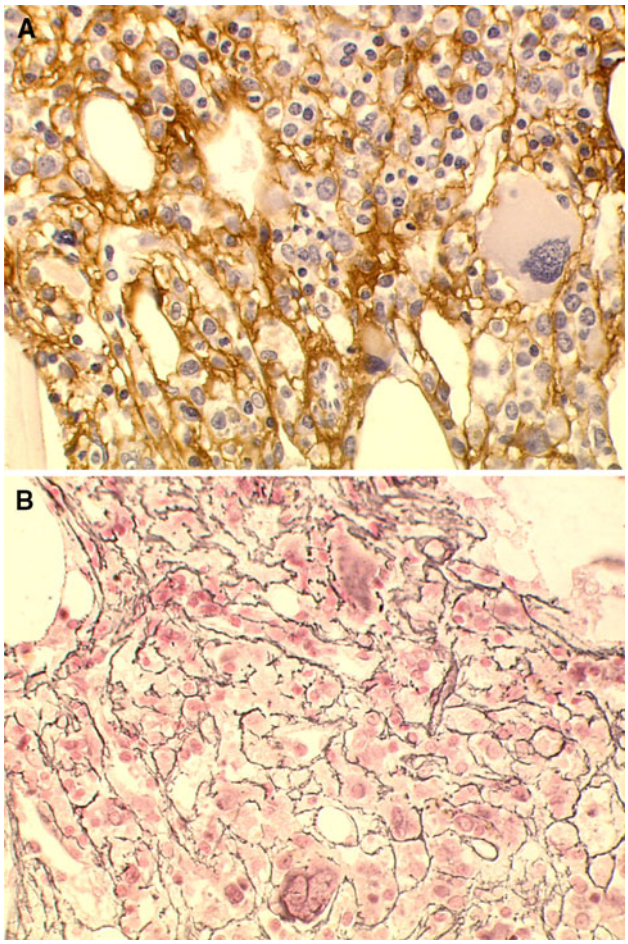

Fig. 1 **a** Bone marrow sample from a male, 75-year-old, with MDS RAEBT. The HA staining is intense, grade 4. HA is stained brown. Magnification 360×. **b** The reticulin staining is diffuse with scattered thick coarse fibres, grade 3+. Magnification 360×

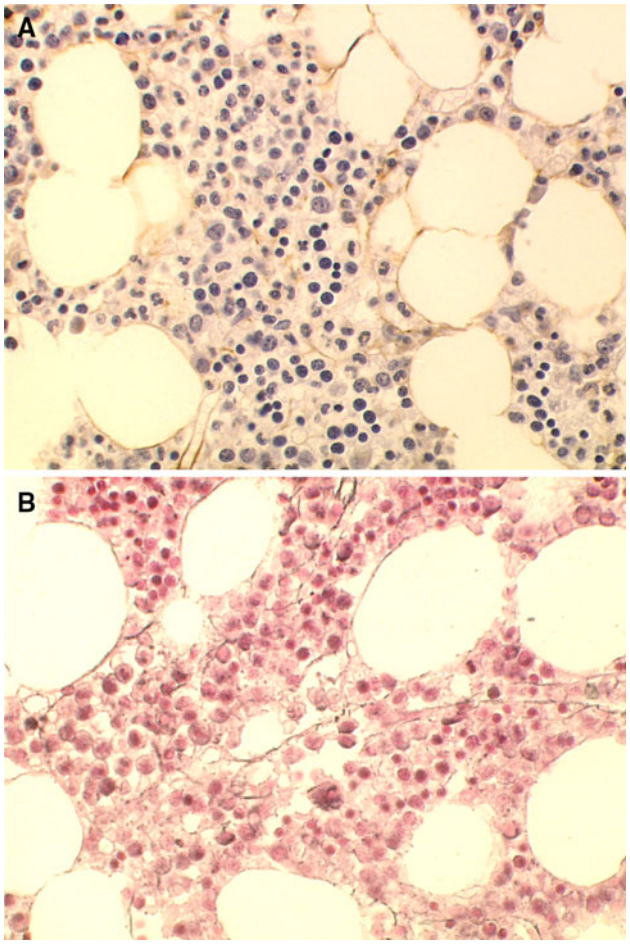

Fig. 2 **a** Bone marrow sample from a male, 39-year-old, with B-cell lymphoma NOS, not involving in the bone marrow. The HA staining is sparse, grade 1. Magnification 360×. **b** The reticulin staining shows occasional fine individual fibres, grade N. Magnification 360×

Statistics

The HA and reticulin grading were compared between the controls and the patients with and without involvement of a malignant disease in bone marrow, using Kruskal–Wallis and Mann–Whitney U test. The relation between HA and reticulin grading was analysed using Pearson's chi-square test. Differences were considered significant when the P value was below 0.05.

Results

The HA grading was significantly higher in the 43 patients with malignant disease involving the bone marrow compared to the healthy controls ($P < 0.001$). However, a trend but not a complete significant correlation was seen between the patients with malignant disease involving the bone marrow compared to the 18 patients without bone marrow involvement ($P = 0.059$).

Table 3 HA and reticulin grading in patients *with* and *without* malignant disease involving the bone marrow, given as a percentage

HA and reticulin score	Controls %	Without bone marrow involvement %	With bone marrow involvement %
HA 1–2	96.7	72.2	46.5
HA 3–4	3.3	27.8	53.5
Reticulin N–2+	100	100	62.8
Reticulin 3+ to 4+	0	0	37.2

HA score 1–2 and reticulin score N–2+ are considered normal

The reticulin grading was significantly higher in the patients with a malignant disease involving the bone marrow, compared to the patients without ($P = 0.001$) and to the healthy controls ($P < 0.001$).

In the patients with a malignant disease not involving the bone marrow, the HA grading was significantly higher compared to the healthy controls ($P < 0.001$). This was not seen for reticulin grading ($P = 0.54$) (Table 3).

In all patients as well as in the healthy controls, a significant correlation was seen between HA and reticulin grading ($P < 0.001$), showing that HA and reticulin are not independent variables.

Discussion

The aim of this study was to analyse the content of HA and reticulin in biopsies from patients with and without a malignant disease involving the bone marrow. The majority of the patients with disorders involving the bone marrow had a malignant haematological diagnosis. Both HA and reticulin staining intensities were significantly increased in the patients with the disease involved in the marrow, compared to the controls. In all patients the HA and reticulin staining intensities correlated significantly, as was reported in earlier studies in patients with de novo AML and myeloproliferative disorders [22, 23]. However, HA staining intensity in involved bone marrows was not significantly higher compared to non-involved marrows. In the patients without bone marrow involvement, the HA grading was significantly higher compared to healthy controls. This was not the case with reticulin grading which did not differ from the controls.

These findings indicate that malignant diseases involving the bone marrow activate cells to produce components essential for a fibrotic process. The cells most prone to be activated in this process are fibroblasts. Haematopoietic cells, including leukaemic cells, have been reported to synthesize HA [28].

Also in the patients without bone marrow involvement, the HA grading was significantly higher compared to the healthy controls. This was not the case with the reticulin grading, which did not differ from the controls.

The trend of increased HA staining in the bone marrow without any sign of disease involvement is an interesting observation and there is no obvious explanation. One hypothesis is that the malignant disease has a systemic influence, also involving the bone marrow, in spite of no demonstrated malignant cells in the marrow. Both cytokines and growth factors are activated in malignancies and can stimulate HA synthesis. An alternative explanation is that malignant cells might be present in other parts of the bone marrow, not sampled by the biopsy but influencing the adjacent marrow spaces. There is no doubt that the local milieu is an important prerequisite for cell function [29, 30].

In this study the patients with malignant diseases, mostly haematological, demonstrated an increase of HA in bone marrow involved of disease and to a less extent in non-involved marrows. Increased reticulin staining was seen only in involved marrows. It has earlier been discussed if HA could be an early sign of impending fibrosis, which can be supported by these findings [21–23]. However, in recent review articles, the impact of HA in initiation and progression of tumour spread is proposed which add another important aspect to the increased HA content in both the involved and non-involved bone marrow. These findings may signal not only impending fibrosis but also an active malignant process. Further studies, on selected groups of patients with detailed diagnosis and treatment, should focus not only on quantification of HA but also on identification of type of HA-synthase, activation of receptors and if possible, chain length.

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