Lymphocytes from patients with early stage of B-cell chronic lymphocytic leukaemia and long survival synthesize decorin

Salvatore Campo a,*, Giuseppe M. Campo a, Angela Avenoso a, Angela D’Ascola a, Caterina Musolino b, Luana Calabrò b, Giacomo Bellomo b, Eugenia Quartarone b, Alberto Calatroni a

a Department of Biochemical, Physiological and Nutritional Sciences, University of Messina, Messina, Italy
b Department of Internal Medicine and Medical Therapy, Faculty of Medicine, University of Messina, Messina, Italy

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Abstract

mRNA/cDNA gene expression of both small leucine-rich proteoglycans decorin and biglycan was evaluated by PCR real time in lymphocytes collected from patients with chronic lymphocytic leukaemia (CLL) at different stages of disease and from healthy controls. Lymphocytes obtained from healthy controls showed no or very low levels of mRNA expression of both decorin and biglycan. Biglycan expression was very low in CLL patients, values being close to those of controls. On the contrary, decorin mRNA was clearly expressed in patients with early B-cell CLL, while a low expression was found in advanced clinical stages. Furthermore, a significant higher decorin expression was found in patients with non-progressive CLL type in comparison with patients with aggressive type of the disease. Decorin expression resulted especially high in the low-progressive low-risk patients. The synthesis of decorin was also assessed by Western blot analysis. The peculiar occurrence of decorin in the non-aggressive type of CLL is consistent with its suggested anti-oncogenic role. Intracellular Bcl-2 level does not correlate with decorin mRNA transcription, suggesting that a Bcl-2 independent anti-cancer mechanism may occur. The measurement of galactosamine-containing proteoglycans concentration in plasma confirmed decorin expression results, with significant differences between CLL patients and controls. Significant changes were also seen between groups of patients of Rai stage 0 with recent diagnosis (less than 5 years, from analysis), (low amount of decorin) and less recent diagnosis (more than 5 years), (high amount of decorin).

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1. Introduction

Chronic lymphocytic leukaemia (CLL), a monoclonal proliferation of mature B-lymphocytes, is a heterogeneous disease with highly variable clinical courses. The clinical staging systems, Rai and Binet, are unable to prospectively identify patients at higher risk of disease progression. Therefore, continuous efforts have been made to identify additional events which may help to discriminate between the rapidly evolving patients from those with a stable disease for decades. CLL is generally described as a disease of failed apoptosis. Alterations in the apoptotic machinery within the CLL cells may stem from a combination of modified presence and interactions of regulatory molecules.

Proteoglycans (PGs) are a large family of protein—glycosaminoglycan (GAG) complexes, basic components of extracellular matrix, capable of interaction with a variety of proteins, as growth factors, enzymes, enzyme inhibitors, extracellular matrix proteins [1]. Based on these interactions, PGs are...
able to influence cell growth, cell proliferation and cell migration of fibroblasts, smooth muscle cells, and vascular endothelial cells. In blood, PGs occur in leukocytes and platelets [2], on the surface of vessel endothelial cells [3] and in plasma [4]. The carbohydrate moieties of PGs, GAG chains, are heteropolysaccharides containing alternate units of hexuronic acid and aminosugar, glucosamine or galactosamine, usually in sulphated form. In healthy individuals plasma PGs are mainly galactosamine-containing chondroitin sulphates proteoglycans (CS-PGs) [4,5]. The concentration in plasma of glycosaminoglycan chains was shown to be significantly higher in CLL patients than in healthy controls, with no correlation with the number of lymphocytes and no differences among the different stages [6]. PGs are largely involved in tumor progression [7]. Among galactosamine-containing PGs, decorin and biglycan are small leucine-rich chondroitin sulphate/dermatan sulphate PGs [1], also synthesized by endothelial cells; in sprouting endothelial cells decorin expression supports capillary formation and cell survival [8,9]. Decorin may influence the behaviour of several types of cells. On endothelial cells it binds to the insulin-like growth factor-I (IGF-I) receptor [10]. In tumor cells, decorin interacts, via its core protein, with the epithelial growth factor (EGF) receptor or ErbB4 [11,12], causing a substantial low-regulation of the EGF receptor and other ErbB members of receptor tyrosine kinase [12], an action that would negatively affect tumor growth. Decorin is then implicated in cancer development, and “anti-oncogenic” effects have been observed [7,13].

Decorin has never been described in plasma, as well as decorin synthesis in blood cells. Since anti-oncogenic effect of decorin and presence of decorin receptor on endothelial cells, this work was started with the aim to find whether any expression of decorin is present in lymphocytes of patients with CLL, to clarify its role in this proliferative syndrome and to justify the rise of GAG chains concentration in the plasma of CLL patients.

2. Materials and methods

2.1. Materials

Blood samples were obtained by venipuncture from healthy subjects and from patients with chronic lymphocytic leukemia (CLL). Each subject was informed of the research and written authorizations were collected. The cytomorphicologic and immunologic criteria of CLL diagnosis were those recommended by the revised guidelines of the National Cancer Institute (N.C.I.) [14]. The patients were investigated either at the time of diagnosis before any treatment or after they had been “off treatment” for at least 45 days. Patients had no indication of inflammatory liver or kidney or connective tissue diseases. Patients were grouped according to the staging system of Rai in stage 0 (low-risk), stage I and stage II (intermediate risk), stage III and stage IV (high-risk). Furthermore, patients belonging to each single stage were again divided, according to the time of the initial diagnosis, into two subgroups: patients with diagnosis made 5 years or more from analysis (old diagnosis) and patients with more recent diagnosis (recent diagnosis). A total number of 61 CLL patients, 27 men and 34 women, mainly of age between 55 and 80 years, were involved. Patients with “old diagnosis” were 23 in the five (0, I, II, III and IV) Rai stages, 5, 5, 11, 1 and 1, respectively; patients with “recent diagnosis” were 38 in the five Rai stages, 12, 10, 7, 8 and 1, respectively.

Two groups of normal controls were utilized: a group of 12 university students aged 20–21 years; and a group of 12 individuals, both university students and university staff components, of different ages, from 20 to 55 years. This second group was utilized as control for the comparative evaluation of patients, both in PGs and Bcl-2 expression and in plasma PG concentration determinations. The first group was only used as internal control of the second one in plasma PG concentration measurements.

Blood samples were collected early in the morning into tubes with heparin as anticoagulant for lymphocyte isolation, and into tubes containing EDTA as anticoagulant for plasma PGs isolation.

2.2. Lymphocyte isolation

Blood samples were diluted 1:4 with culture medium RPM 1640 (Euroclone, UK) and lymphocytes isolated by centrifugation at 2000 rpm for 30 min at room temperature on Ficol solution at 1.077 density (Lympholyte, Cederlane, Canada). The isolated lymphocytes were washed three times with culture medium and stored at −80 °C.

2.3. Quantification of decorin and biglycan mRNA

Total RNA was extracted from the isolated lymphocytes by a commercial kit (Trizol Reagent, Invitrogen, USA). After precipitation and cold ethanol washing, RNA was dried and dissolved in appropriate volume of TE buffer (10 mM Tris–HCl pH 8.0 and 1 mM EDTA). The RNA solution was quantified at 260 nm by spectrophotometer reading (Biomate 3, Thermo Electron Corporation, USA) and its purity was assessed by the ratio at 260 and 280 nm readings.

By using hexameric random primers and the High Capacity cDNA archive kit (Applied Biosystems, USA), 2.5 µg of total RNA were reverse-transcribed in cDNA according to the manufacturer’s instructions. To quantify decorin and biglycan mRNA, 0.25 µg of total cDNA, for each one, was used by PCR real time method, as well as β-actin cDNA as housekeeping control. In order to obtain a standard curve for relative quantitation method, a series of dilutions of total RNA from human fibroblast cultures was performed (Cell line DPK- SKDF-H, Pharmakin, Spain). The biplex reactions were carried out by the TaqMan Universal PCR Mater Mix and the ready to use Assays on Demand (decorin products ID Hs00266491_m1 and Hs00370383_m1, biglycan product ID Hs00156076_m1) and human β-actin endogenous control, with different reporter dies. The probes resulted to anneal splice-junction regions. Analysis was performed in triplicate with the mod. 7500 PCR real time System (Applied
Biosystems, USA). Quantitation was determined by the relative standard curve method for both target and endogenous control. After normalization, the results were expressed as an n-fold difference to the median levels of normal subjects (relative expression). All the PCR reagents were purchased from Applied Biosystems, USA.

2.4. Quantitation of Bcl-2 intracellular protein

The intracellular level of Bcl-2 protein was assessed by a commercial ELISA kit. The results are expressed as relative amounts to normal levels. Bcl-2 analysis was performed in the supernatant of 4–10 × 10^6 cell samples. The assay was carried out by using a specific enzyme-linked binding protein assay test kit (cat. N. KHO 0301, Bio Source, USA). Briefly, samples were added into a microtiter well together with a primary antibody and incubated at room temperature for 60 min. After washing, a secondary antibody (HRP-conjugated) was added into well and incubated at room temperature for 30 min. Then, after the addition of a chromogen solution, and further incubation, at room temperature, for 30 min, the reaction was stopped and the absorbance was read at 405 nm by using a microplate reader (DAS srl, Rome, Italy). The concentration of Bcl-2 was determined by interpolation from a standard curve.

2.5. Decorin and biglycan protein expression

The protein expression of both decorin and biglycan was measured by Western blotting. About 10^7 cells were lysed by cell extraction buffer (Bio Source) in the presence of 1 mM PMSF and protease inhibitors cocktail. Following centrifugation at 13,000 rpm for 10 min at 4°C, the protein content of the supernatant was measured by DC protein assay kit (Biorad). A volume of supernatant containing 40 μg of protein was diluted with the same volume of Laemmli Sample buffer (Biorad). Each sample was treated with chondroitinase ABC, one unit dissolved in 20 μl of Tris–HCl, pH 8. Digestion was carried out at 37°C for 2 h and the enzymes were inactivated by heating in a boiling water bath for 10 min. Following denaturation, the samples were applied to a 10% polyacrylamide gel. Electrophoresis was carried out at constant voltage (160 V). Following electrophoretic separation, proteins were electroblotted on nitrocellulose PVDF (Amersham) overnight at 30 V. Following treatment of the membrane, at room temperature for 1 h, with a 10 mM Tris, 50 mM NaCl buffer pH 7.4, containing 7% non-fat-dried milk and 0.1% Tween-20, the membrane was incubated for 2 h at room temperature with the same TBS/Tween-20 buffer, containing 5% non-fat-dried milk. After several washings, the membrane was treated with ECL (Amersham Biosciences) for 5 min, and chemiluminescence was measured by autoradiography on Fuji Medical X-ray film. Results are expressed as semiquantitative relative amounts, using β-actin as reference structure, detected on membrane by specific antibody and revealed by chemiluminescence. Standard decorin utilized in line 1 is recombinant decorin protein kindly provided by Prof. Ruggero Tenni, Pavia, Italy. Two chimaeric molecules are present.

In a few plasma PG preparations, the occurrence of decorin was tested by the same method.

2.6. Measurement of plasma PGs

The isolation of plasma PGs fraction in each plasma sample was performed [15] by filtering through an Ecteola-cellulose column (Fluka, Sigma–Aldrich, Milan, Italy, chloride form, diameter 0.7 cm, height from 2 to 4 cm according to the sample volume) a known amount (from 1 to 5 ml) of untreated (only diluted 1 to 1 with distilled water) plasma. The resin was subsequently washed with 50 ml of 0.15 M NaCl solution, and acid PGs were eluted by 4 ml of 2 M NaCl solution. Hexuronic acid was determined in each eluting solution. This solution was then reduced in volume and desalted and purified on Bio-Gel P-2, 0.7 × 50 cm column packed and eluted with distilled water. The hexuronic acid measurement was repeated and aminosugar hydrolysis was performed on the desalted solution in 4 M HCl at 100°C for 6 h. Finally hydrolysed samples were taken to dryness under vacuum (Rotovapor-R, Buchi Instrumentation, Switzerland). Samples were then analysed for glucosamine and galactosamine content as described below. The intra-assay and inter-assay coefficient of variation was less than 5%.

2.7. Analytical methods in PGs determination

Hexuronic acid was determined by colorimetric method of Bitter and Muir [16], using glucuronic acid (Sigma–Aldrich, Milan, Italy) as a standard. Glucosamine and galactosamine were determined, following acid hydrolysis and HCl elimination as described before, by using HPLC separation on anion exchange resin CarboPac PA10 (Dionex, Sunnyvale, CA, USA), eluting with 0.020 M NaOH, with pulsed amperometric detection (Dionex, Sunnyvale, CA, USA) [17]. Each determination was made in duplicate.

2.8. Statistics

Data shown are mean of duplicate analyses for each sample. Data are presented as mean ± standard deviation. T-test for paired samples was used to examine whether the mean PG values in patients were different from controls, or values in patients with “old diagnosis” from those of patients with “recent diagnosis”. The difference was considered significant when p < 0.05.
3. Results

Lymphocytes obtained from healthy controls showed no or very low levels of mRNA expression of both decorin and biglycan. The values obtained following normalization vs β-actin expression were 0.49 ± 0.09 for decorin, and 0.38 ± 0.14 for biglycan. These mean values were utilized to express mRNA level of both decorin and biglycan in patients.

Figs. 1 and 2 show the mRNA expression of decorin and biglycan, respectively, in CLL patients. In lymphocytes from the 17 patients of Rai stage 0 the mRNA mean decorin expression resulted 9.3 ± 6.22 times higher than the very low mean value found in lymphocytes from healthy controls, with a range from 3 to 22 times. The highest values (range 13.8–22) were observed in the five patients with “old diagnosis”, with a mean value 18.16 ± 3.10 times higher compared to the normal mean value. The mean value relative to the other 12 patients with “recent diagnosis” resulted 5.62 ± 1.65 (range 3–7.8), and difference with the “old diagnosis” group mean value was statistically highly significant (p < 0.0005). In lymphocytes from patient of Rai stage I the mRNA mean decorin expression resulted 4.24 ± 1.95 times higher than the mean value found in lymphocytes from healthy controls, again with statistically significant (p < 0.01) difference between “old” (5.26 ± 0.26 mean value) and “recent” (3.74 ± 1.46 mean value) diagnosis patients. The difference in decorin expression mean level between the group of patients with stage I and the group of patients with stage 0 resulted statistically significant (p < 0.005). A mean value lower than those of stages 0 and I was found in patients with stage II (2.95 ± 1.09 times vs the control mean value), with quite low, although close to significance, difference (3.30 ± 1.07 vs 2.41 ± 0.71) for the time of diagnosis, and very low mean value (1.26 ± 0.48) in the patients of stages III and IV.

Results of biglycan mRNA expression (Fig. 2) in B-lymphocytes of patients were completely different from those of decorin, since low expression was usually measured, with no differences between early and advanced stages of disease. The highest value, 3.20 ± 1.12, always as ratio to the mean value obtained in healthy controls, was found in patients with CLL at stage I, with no differences between “old” and “recent” diagnosis.

Fig. 3 shows the proteoglycan decorin expression, by Western blotting, in lymphocytes from CLL patients and normal controls.

Fig. 4 shows the intracellular levels of Bcl-2 protein in patients and controls. Level expression in patients was relative to mean value of controls and patient’s groupings was as described. As expected, a value about five times (4.77 ± 1.67) higher than the control value was detected in patients with the severe stage of the disease. While in patients with Rai
stages 0 and I, when decorin is synthesized, the amount of Bcl-2 protein is only twice as much with respect to normal value (2.49/1.03 and 2.45/0.79, respectively). However, among the patients with stage 0, the mean value of patients with “old diagnosis” (1.80/1.05) was significantly \((p < 0.005)\) lower than the mean value of patients with “recent diagnosis” (2.77/0.94).

Table 1 shows the amount of isolated and purified plasma PGs in terms of hexuronic acid (mg/l) and in terms of galactosamine-to-glucosamine molar ratio in CLL patients, in the same patients grouped according to the Rai staging system, and in controls. Table 2 shows the value of the same biochemical parameters in CLL patients of each different Rai stage separated according to the time of diagnosis. Data in Table 2 show that the plasma level of hexuronic acid and galactosamine-containing PGs resulted significantly higher in patients stage 0 with “old diagnosis” than in patients of similar stage 0 with “recent diagnosis”. Since decorin GAGs are hexuronic acid and galactosamine-containing chains, the significance of the difference between stage 0 “old diagnosis” and stage 0 “recent diagnosis” in Table 2 gives support to the decorin expression data.

4. Discussion

Chronic lymphocytic leukaemia (CLL) is a clinically and biologically heterogeneous disease, characterized by the clonal proliferation of mature-appearing B-lymphocytes. In early stages of disease, CLL B-lymphocytes can be found primarily in peripheral blood and bone-marrow; however, disease progression is associated with infiltration of lymphoid and non-lymphoid organs, suggesting that microenvironmental factors might change during the disease course, thus affecting tumor cell adhesion and patterns of dissemination. Although several investigators have focused on the expression and function of cell adhesion molecules in chronic B-cell leukemias, few reports have correlated some molecules expression with disease stage and progression.

In this study, the mRNA expression of decorin resulted almost absent in B-lymphocytes from healthy controls, while it was clearly evident in B-lymphocytes from the majority (90%) of CLL patients, and it resulted especially high in most patients at Rai stage 0 or I. This evidence suggests that decorin may be synthesized in CLL lymphocytes, and the synthesis appears especially feasible in the early stage of the disease. Furthermore, patients at stage 0 who had diagnosis 5 years or more before the analysis showed always high value of mRNA decorin expression, with a mean value three times higher with respect of patients at stage 0 with diagnosis made more recently. A similar result was observed also in CLL patients at stage I. Since the difference between the two values, “old diagnosis” and “recent diagnosis”, is statistically significant in both 0 and I stages, the result clearly suggests that decorin synthesis is significantly increased in low-progressive mild expression of the disease. And that correlates well with the anti-oncogenic role of decorin.
mRNA expression of decorin is indeed decreased in patients with stage II, with still difference between “old diagnosis” and “recent diagnosis”, and it is very low in B-lymphocytes of CLL patient’s stages III and IV.

On the contrary, the mRNA expression of biglycan, the other small leucine-reach proteoglycan of endothelial cells, turned out to be low in B-lymphocytes of CLL patients.

Western blot analysis validated the results of mRNA expression, and decorin occurrence in B-lymphocytes of CLL patients at stage 0 is documented, at semiquantitative level. Western blot analysis of plasma PG mixture isolated from CLL patients showed less reliable results, and data are not shown. However, decorin presence cannot be excluded. As for the plasma PG mixture composition, the total amount of hexuronic acid containing plasma PGs resulted significantly increased in patients with CLL with respect to normal healthy controls of comparable age. If CLL patients are grouped according to Rai staging system, the difference between mean value of patients and mean value of controls turned out to be statistically significant in the less severe stages of the disease, while not for patients with Rai stages III and IV. Furthermore, the increase is higher in the low progressive mild expression of the disease, as observed for decorin expression. Moreover, significant difference in plasma PGs level exists between patients at stage 0 “old diagnosis” and patients at stage 0 “recent diagnosis”, both in terms of hexuronic acid and of galactosamine.

Since the galactosamine to glucosamine molar ratio in the plasma PG mixture resulted significantly increased in CLL patients, at any stage, with respect to normal control value, the excess plasma PG in CLL patients is supposed to be a galactosamine-containing PG. Decorin is a galactosamine-containing PG, also synthesized in lymphocytes of CLL patients; however, although a contribution of decorin to the plasma PGs mixture may be possible, the main hypothesis is that the net increase in plasma PG level in CLL patients is due to addition of chondroitin-4-sulphate PG, which is the main component of plasma PGs mixture. Plasma PGs express antioxidant activity [26,27] and CLL is a syndrome where free radical injury is present.

The mechanism of the suggested anti-oncogenic effect of decorin in CLL B-lymphocytes has to be investigated. Decorin has a variety of important biological functions that are mediated by its interactions with matrix components, cytokines and cell surface receptors. Decorin can interact via its core protein with the epithelial growth factor (EGF) receptor [12] and the ErbB4 receptor [11], and, with different site of interaction, with the insulin-like growth factor-I (IGF-I) receptor [10]. Decorin causes differentiation in cells, and may protect cells from programmed apoptosis, while in tumor cells lead to apoptosis [18]; it induces reduction of tumor growth in breast carcinoma [19]. How decorin affects differentiation and apoptosis is not completely understood. Patients with B-CLL express fibromodulin [20]. Fibromodulin and, to a lower extent, decorin and biglycan, interact with transforming growth factor (TGF)-beta, sequestering it into extracellular matrix [21]. TGF-beta is an important regulator of the immune system, and controls proliferation, differentiation and survival of lymphocytes [22]. The alteration of B-CLL cell response to TGF-beta signal (inhibition of proliferation and induction of apoptosis) [23] may be related to reduction in the effect of the factor by interactions with sequestering molecules, as decorin and fibromodulin, both structures peculiarly synthesized in B-CLL lymphocytes.

Most CLL cells contain high level of the antiapoptotic protein Bcl-2, which is considered to have a role in the extended survival of B-lymphocytes and on their resistance to cytotoxic effects [24,25]. In our study, although the mean values of Bcl-2 protein content are one half in patients with Rai stages 0 and I than in patients with Rai stages III and IV, and although the patients with “old diagnosis” show less Bcl-2 protein content than patients with “recent diagnosis”, intracellular Bcl-2 levels basically fail to correlate with decorin and biglycan expression. The data suggest that decorin could act with Bcl-2 independent anti-cancer mechanism.

5. Conclusion

Small leucine-rich PG decorin synthesis in B-lymphocytes appears to be characteristic of B-CLL patients, and able to discern differences between low-risk and high-risk CLL patients. Furthermore, decorin synthesis is especially raised in low progressive mild expression of the disease. It might be then suggested as a gene expression signature that could associate with disease progression, although a more detailed clinicopathologic
study must be performed in order to prove this suggestion. Changes in decorin expression do not correlate with those of Bcl-2 expression, suggesting a decorin effect not dependent upon Bcl-2 antiapoptotic mechanism. Recent literature data on anti-oncogenic role of decorin suggest that the effect of B-lymphocytes decorin in CLL patients could be exerted by influence on TGF-beta activity, along with interference with growth factors receptors.

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References


