Kinetics of versican-expressing macrophages in bone marrow after cord blood stem cell transplantation for treatment of acute myelogenous leukaemia

Miho Senda,1,2 Ryuichi Fukuyama,3 Tetsuro Nagasaka2

ABSTRACT

Aims To determine versican-producing cells in normocellular bone marrow and to evaluate chronological alteration in the number of versican-producing macrophages in bone marrow of patients with acute myelogenous leukaemia (AML) after cord blood stem cell transplantation (CBSCT) to gain insight in the significance of versican in recovery of haematopoiesis.

Methods We enrolled seven age-matched unrelated patients with normocellular bone marrow for determining versican-producing cells in bone marrow, CBSCT-treated patients with AML, 18 with fine and other four with poor engrafment, for determining chronological alteration of versican-expressing and CD68-expressing cells in transplanted bone marrow in reference to the total cells. Clot samples of patients with AML were collected from the +16 to +55 day after transplantation and separated into four groups. We included an AML case whose specimen was obtained on the +9 day. Cells positive in immunohistochemistry using antibodies to versican and CD68 were counted to obtain the mean±SD in a unit area of the bone marrow, plotted chronologically and compared with the numbers from the age-matched normocellular group.

Results We determined by a double immunohistochemistry that the versican-expressing cells in bone marrow are macrophages. The time-course curve demonstrated an inverse relationship between the versican-positive macrophages and the total cells in the transplanted bone marrow for over 55 days. In bone marrow of poor engrafment cases, versican-positive macrophages appeared to be decreased in comparison with age-matched and sampling day-matched patients.

Conclusions These results suggest that versican and/or versican-expressing macrophages positively contribute to bone marrow regeneration of patients with AML after CBSCT.

INTRODUCTION

Versican/PG-M is a type of large chondroitin sulfate proteoglycan belonging to the aggregcan family, and plays important roles in cell adhesion, migration and differentiation as a molecule of extracellular matrix (ECM).1-4 Versican is first identified in culture medium of fibroblasts and its wide-range distribution is subsequently revealed in the smooth muscle cells, cartilage, skin and blood vessels.5 Versican is also expressed at the ECM of malignant tumors,6-12 and developing embryos.8-13

The main cell type that produces versican in inflammatory lesions has been revealed to be macrophages.14 Many other reports also demonstrated that macrophages express versican and that it is overexpressed when they are activated by granulocyte-macrophage-colony-stimulating factor (GM-CSF),15 lipopolysaccharide16 and hypoxia.17 At ECM, it binds to hyaluronan and other ECM molecules such as fibronectin4,17 and several chemokines,18-19 thereby influencing leucocyte function.

Versican reportedly exists in the long-term culture of mouse bone marrow (BM) cells20 and in the ECM of BM after chemotherapy.21 Moreover, Oguri et al22 detected a large amount of proteoglycan with chondroitin 6-sulfate in rabbit BM tissues. Although versican in BM has not been analysed biochemically, proteoglycans at the ECM have been known as binding partners for humoral factors that activate haematopoietic progenitors.23 These reports support the hypothesis that versican may play an important role in the haematopoiesis of BM. Localisation of versican in BM tissue has been analysed immunohistochemically, yet the cells that produce versican in this tissue were not delineated.

Transplantation of cord blood (CB), BM and peripheral blood (PB) stem cells (SGs) has been performed for treatment of haematopoietic diseases such as leukaemia. Down these lines, Nagasaka et al24 showed that the versican level is increased in BM of patients who have undergone chemotherapy. Therefore, it is likely that versican in BM may positively influence haematopoiesis in tissue after transplantation. To date, no study has been conducted to elucidate versican’s overexpression and role in transplanted BM.

The purpose of this study is to identify versican-producing cells in normal BM and to shed light on the significance of versican in transplanted BM.

PATIENTS AND METHODS

Patients

To address the possible significance of versican in BM regeneration, we enrolled 18 patients with acute myelogenous leukaemia (AML) who underwent cord blood stem cell transplantation (CBSCT). As we obtained clot specimens from an AML case 3 times and from 3 AML cases 2 times, the total number of samples in the assessment was 23. Three different stem cell transplantation (SCT) procedures have been performed at our hospital, namely, CBSCT, BMSCT and PBSCT. CBSCT is our current standard procedure because the graft versus host defence is less pronounced with it, and only a part of human leucocytic antigens needs to be matched.24-26 Therefore, we confined our analysis to CBSCT-treated patients. Our preparative regimen for CBSCT was based on previous reports,
which was recently summarised by Arai et al. and was shown in table 1.

BM clot was collected from the +16 to +55 day after transplantation for routine cytological and pathological evaluation of engraftment. We separated patients into four groups based on the duration after transplantation as follows: 16–25, 26–35, 36–45 and 46–55 days. We included a case, whose specimen was obtained at the +9 day to examine a cause of his high fever, because it likely shows a possible tendency of the number of versican-positive/CD68-positive macrophages in the early phase of the recovery. The breakdown of these samples is shown in table 2.

According to the record, no patients experienced recurrence. We identified four other patients with AML who exhibited severe hypocellularity in BM about 3–5 weeks after CBSCT, likely showing engraftment failure. We analysed this age-matched poor engraftment group in the same way and compared with the corresponding 16-25 and 26–33 groups (namely, age-matched and sampling day-matched control). To determine versican-expressing cells in BM, we selected seven patients whose BM was isolated for diagnostic purpose and was normocellular, and then clinicopathologically diagnosed not to have haematological and other significant diseases. We named this an age-matched normocellular group (table 1) and the numbers of the total cells, and versican-positive and CD68-positive cells were used as a baseline for the kinetics assessment. Informed consent was obtained from each patient and the protocol was approved by the ethical committee of Nagoya University.

**Immunohistochemistry**

All samples used in this study were formalin-fixed and paraffin-embedded. Several 4 μm-thick sections were cut from the paraffin blocks, and one was stained with H&E for diagnostic purposes. In immunohistochemistry (IHC), antigen-bound antibodies were visualised using a kit (Bond Polymer Refine Detection, Leica Biosystems Newcastle, UK), and brown colour was developed with diaminobenzidine (DAB).

Since versican-expressing cells were found likely to be macrophages, we performed double IHC using antibodies to versican (Abcam, Cambridge, UK) and to CD68 (PG-M1; Dako, Glostrup, Denmark). We employed this CD68 antibody because it was used to label all macrophages in BM. With this technique, after versican-bound antibody was visualised with a brown colour with DAB, anti-CD68 antibody was reacted to the tissue section, and coloured red using the Bond Polymer Refine Red Detection kit (Leica Biosystems, Newcastle, UK). Nuclei were counterstained by haematoxylin, and immunohistochemical staining was carried out by the automatic IHC device (Leica BOND-MAX, Leica Biosystems).

**Statistical analysis**

All BM samples were divided into four groups based on duration after transplantation, 16–25, 26–35, 36–45 and 46–55 days (table 2). After immunostaining, all cells, versican-positive cells and CD68-positive cells in a 0.15 mm² area of several sections were counted, and the average number and SD of each group were obtained. These data were compared with either each other or those of the age-matched normocellular group (analysis of variance, Bonferroni’s correction). Data of the age-matched poor engraftment group were compared with those from the age-matched and sampling day-matched group (Student’s t test). We used a Stat View program (STAT View for Windows, V5; SAS Institute, Cary, New Castle, USA) and significance was set at p<0.05.

**RESULTS**

**Macrophages are the versican-producing cells in BM**

We selected patients who had no haematopoietic or significant systemic diseases for this purpose (table 2, age-matched normocellular group, figure 1A). Results of the single (figure 1B, C) as well as double (figure 1D) IHC on BM clot samples with anti-versican and anti-CD68 antibodies strongly supported the consideration that versican is produced, partially if not entirely, by macrophages residing at the tissue stroma.

**Kinetics of versican-producing macrophages in repopulating BM after CBSCT**

Average numbers and SD of total cells, versican-positive cells and CD68-positive cells at the unit area in clots from the age-matched normocellular group were approximately 221, 6 and 13 cells/0.15 mm², respectively (figure 2A–C, dotted horizontal bars). Then, BM clots of patients who underwent CBSCT were stained similarly and the total cells were counted and compared with those of this group (figure 2A–C, closed marks). The total haematopoietic cells in BM after pretreatment and transplantation appeared to be decreased on the +9 day (figure 2A) and recovered in number already for the 16–25 day group. Though not statistically significant, the total cell number kept gradually increasing thereafter (figure 2A). A significant increase in the total cell number was observed for the 46–55 day group compared with the 16–25 day group (***p<0.005). On the contrary, the average number of versican-positive cells increased steeply in the 16–25 group to that of the age-matched normocellular group (**p<0.01, figure 2B), and then sharply decreased (***p<0.001, **p<0.001, figure 2B) when the total cell number increased. The number of CD68-positive macrophages was unaltered for the first month but fell significantly to the age-matched normocellular group in the 36–45 day group (**p<0.01, figure 2C). The linearly regressed time-course curves of the number of the total and versican-positive cells are schematically shown in figure 2D, demonstrating their inverse relationship over the examined period.

**Comparison of the poor engraftment group**

The total cell number of the BM in the age-matched poor engraftment group was approximately six times less than that of the control (**p<0.001, figure 3A). The number of versican-positive cells appeared to be less than half of that of the age-matched and sampling day-matched control, yet it did not reach

<table>
<thead>
<tr>
<th>Table 1</th>
<th>General conditioning regimen before and after transplantation of our hospital</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>−7</td>
</tr>
<tr>
<td>TBI</td>
<td>TBI</td>
</tr>
</tbody>
</table>

CA, cytarabine; CY, cyclophosphamide; G-CSF, granulocyte-colony stimulating factor; MTX, methotrexate; TBI, total body irradiation.
statistical significance (p=0.056, figure 3B). Meanwhile, the CD68-positive cell number seemed unaltered between the poor and the control groups (figure 3B).

DISCUSSION

Versican is an ECM molecule known to play an important role in cell adhesion, motility and immobilisation of humoral molecules. Moreover, versican participates intimately in the process of human diseases such as inflammation, atherosclerosis, cardiac infarction, and proliferation and invasion of cancer cells. Versican is a significant ECM molecule as mentioned; hence, it is important to determine the type of versican-producing cells that are active at ECM of various normal and diseased tissues.

Several reports demonstrated that monocytes and macrophages, inflammatory cells, fibroblasts and myofibroblasts express versican. In many normal and disease tissues, however, cells that express versican are still not well established. Moreover, no reports so far have examined the cells in BM, which is known to contain versican. These facts prompted us to delineate cell types that produce versican in BM, while paying particular attention to tissue macrophages (see above). Using the single IHC method, we first stained the normocellular BM and observed that the anti-versican antibody appears to label macrophages. Then, applying double IHC using anti-versican and CD68 antibodies, we stained the same tissues and concluded that the major versican-expressing cells in BM are macrophages.

Macrophages are important players in the process of various diseases such as atherosclerosis, pneumonia, infectious diseases and progression of cancers. Moreover, macrophages are known to play an important role in BM regeneration. Meanwhile, versican functions as an important scaffold ECM molecule, and the haematopoietic restoration in transplanted BM appears to be regulated by proteoglycans of ECM and humoral factors. Therefore, we hypothesised that macrophage-derived versican may contribute to the regeneration process of BM after SCT for haematopoietic diseases.

Haematopoietic SCTs, BM, PB and CB SCT are performed as treatments for haematolymphoid neoplastic diseases including leukemias and lymphomas. Clinically, cytarabine (CA) +cyclophosphamide (CY)+total body irradiation (TBI) (CA +CY+TBI) is generally performed in these transplantsations as pretreatment to kill neoplastic cells as well as patients’ haematopoietic cells and concluded that the major versican-expressing cells in BM are macrophages.

Several reports demonstrated that monocytes and macrophages, inflammatory cells, fibroblasts and myofibroblasts express versican. In many normal and disease tissues, however, cells that express versican are still not well established. Moreover, no reports so far have examined the cells in BM, which is known to contain versican. These facts prompted us to delineate cell types that produce versican in BM, while paying particular attention to tissue macrophages (see above). Using the single IHC method, we first stained the normocellular BM and observed that the anti-versican antibody appears to label macrophages. Then, applying double IHC using anti-versican and CD68 antibodies, we stained the same tissues and concluded that the major versican-expressing cells in BM are macrophages.

Haematopoietic SCTs, BM, PB and CB SCT are performed as treatments for haematolymphoid neoplastic diseases including leukemias and lymphomas. Clinically, cytarabine (CA) +cyclophosphamide (CY)+total body irradiation (TBI) (CA +CY+TBI) is generally performed in these transplantsations as pretreatment to kill neoplastic cells as well as patients’ haematopoietic cells and concluded that the major versican-expressing cells in BM are macrophages.

Several reports demonstrated that monocytes and macrophages, inflammatory cells, fibroblasts and myofibroblasts express versican. In many normal and disease tissues, however, cells that express versican are still not well established. Moreover, no reports so far have examined the cells in BM, which is known to contain versican. These facts prompted us to delineate cell types that produce versican in BM, while paying particular attention to tissue macrophages (see above). Using the single IHC method, we first stained the normocellular BM and observed that the anti-versican antibody appears to label macrophages. Then, applying double IHC using anti-versican and CD68 antibodies, we stained the same tissues and concluded that the major versican-expressing cells in BM are macrophages.

Several reports demonstrated that monocytes and macrophages, inflammatory cells, fibroblasts and myofibroblasts express versican. In many normal and disease tissues, however, cells that express versican are still not well established. Moreover, no reports so far have examined the cells in BM, which is known to contain versican. These facts prompted us to delineate cell types that produce versican in BM, while paying particular attention to tissue macrophages (see above). Using the single IHC method, we first stained the normocellular BM and observed that the anti-versican antibody appears to label macrophages. Then, applying double IHC using anti-versican and CD68 antibodies, we stained the same tissues and concluded that the major versican-expressing cells in BM are macrophages.

Several reports demonstrated that monocytes and macrophages, inflammatory cells, fibroblasts and myofibroblasts express versican. In many normal and disease tissues, however, cells that express versican are still not well established. Moreover, no reports so far have examined the cells in BM, which is known to contain versican. These facts prompted us to delineate cell types that produce versican in BM, while paying particular attention to tissue macrophages (see above). Using the single IHC method, we first stained the normocellular BM and observed that the anti-versican antibody appears to label macrophages. Then, applying double IHC using anti-versican and CD68 antibodies, we stained the same tissues and concluded that the major versican-expressing cells in BM are macrophages.

Several reports demonstrated that monocytes and macrophages, inflammatory cells, fibroblasts and myofibroblasts express versican. In many normal and disease tissues, however, cells that express versican are still not well established. Moreover, no reports so far have examined the cells in BM, which is known to contain versican. These facts prompted us to delineate cell types that produce versican in BM, while paying particular attention to tissue macrophages (see above). Using the single IHC method, we first stained the normocellular BM and observed that the anti-versican antibody appears to label macrophages. Then, applying double IHC using anti-versican and CD68 antibodies, we stained the same tissues and concluded that the major versican-expressing cells in BM are macrophages.

Several reports demonstrated that monocytes and macrophages, inflammatory cells, fibroblasts and myofibroblasts express versican. In many normal and disease tissues, however, cells that express versican are still not well established. Moreover, no reports so far have examined the cells in BM, which is known to contain versican. These facts prompted us to delineate cell types that produce versican in BM, while paying particular attention to tissue macrophages (see above). Using the single IHC method, we first stained the normocellular BM and observed that the anti-versican antibody appears to label macrophages. Then, applying double IHC using anti-versican and CD68 antibodies, we stained the same tissues and concluded that the major versican-expressing cells in BM are macrophages.

Several reports demonstrated that monocytes and macrophages, inflammatory cells, fibroblasts and myofibroblasts express versican. In many normal and disease tissues, however, cells that express versican are still not well established. Moreover, no reports so far have examined the cells in BM, which is known to contain versican. These facts prompted us to delineate cell types that produce versican in BM, while paying particular attention to tissue macrophages (see above). Using the single IHC method, we first stained the normocellular BM and observed that the anti-versican antibody appears to label macrophages. Then, applying double IHC using anti-versican and CD68 antibodies, we stained the same tissues and concluded that the major versican-expressing cells in BM are macrophages.
negative/CD68-positive macrophages start to express versican when BM is damaged by preconditioning and SCs are engrafted. In BM of multiple myeloma patients, after allogenic SCT, it becomes rich in chemokines and other humoral factors. At least, in neuronal cells TNF-α induces versican expression. Perhaps, after preconditioning and CBSCT in BM, a positive feedback loop between versican and macrophage would be in function.

**Figure 1** Determination of versican-expressing cells in bone marrow specimens. (A) H&E stain. (B and C) Immunohistochemical stain with anti-versican (B) and anti-CD68 (C) antibodies, respectively, in the same bone marrow tissue. (D) Double immunohistochemical stain with anti-versican together with the anti-CD68 antibodies in bone marrow tissue. The most versican-positive cells (brown colour) are also CD68-positive (red colour) (open arrows). Nuclei were counterstained with haematoxylin. Original magnification for 3A–3D: x1000.

**Figure 2** The average numbers and SD of the total (A), versican-positive (B) and CD68-positive cells (C) in a 0.15 mm² area of bone marrow tissues. The dotted horizontal bars are an average of total, versican-positive and CD68-positive cells from the age-matched normocellular group. (A) *Indicates a significant difference between the 16–25 day and 46–55 day groups (analysis of variance, *p<0.05). Data were linearly well regressed (r²=0.962). (B) ###Indicates a significant difference between the 16 and 25 day and the age-matched normocellular groups (###p<0.001). ** and *** indicate a significant difference between the 16 and 25 day group and other three groups (**p<0.01, ***p<0.001). Linear regression was applied as in (A) (r²=0.282). (C) and * indicate a significant difference between the 36 and 45 day group and the age-matched normocellular group (##p<0.01) or 16–25 day group (*p<0.05), respectively. A polynomial curve was well regressed (r²=0.974). (D) A schematic presentation of the versican-positive macrophage (closed line) in reference to the total cells (dotted line). Their corresponding baselines from the age-matched normocellular group were overlayered.
Figure 3  Comparison of the total cell numbers (A), versican-positive (B, left) and CD68-positive cells (B, right) of the age-matched and sampling day-matched patients group (open bar) with those of the age-matched poor engraftment group (closed bar). *Indicates a significant difference (t test, **p<0.001). Versican-positive macrophages are approximately half that of the control, yet it does not reach statistical significance (p=0.056).

Although the underlying mechanism behind versican’s contribution to BM regeneration is not clear, given that versican serves as a scaffold molecule that binds several serological factors such as chemokines\(^8\) and midkines,\(^9\) it is likely that versican at the BM stroma binds humoral factors such as granulocyte-colony-stimulating factor. In fact, in our hospital and in many others, it is perfused on the +7 day of transplantation to aid progenitors so as to be differentiated into neutrophils.

Another intriguing unsolved question is the origin of macrophages after CBSCT. Given that versican presents a favourable environment in the BM for donors’ SCs, they should not be eliminated by preconditioning. Comparison of BM clots just before and after preconditioning will be essential to determine the hypothesis above. The use of sex-determining in situ hybridisation on the clot specimens of patients who received CBSCT from sex-unmatched donor should also be performed in future.

In this report, we demonstrated that the predominant versican-expressing cells in BM are macrophages, and evaluated a time course of the number of versican-positive macrophages in BM after CBSCT. Our results suggest that versican and/or versican-expressing macrophages have important roles in BM regeneration by establishing a supportive environment at its ECM for transplanted SCs to be engrafted. We are preparing several more cases of AML with CBSCT and will analyse them to reach statistical significance.

REFERENCES


31 Zimmermann DR, Ruoslahti E. Multiple domains of the large fibroblast proteoglycan, versican. EMBO J 1989;8:2975–81.


目的 骨髄におけるバーシカン産生細胞を同定すること。および骨髄再生におけるバーシカンの役割を明らかにするため、急性骨髄性白血病に対して臍帯血幹細胞移植を行った症例の骨髄再生過程に着目して、バーシカン陽性マクロファージ数の経時的変化を解析すること。

方法 正形成骨髄7例の骨髄クロット検体を用いて、バーシカン産生細胞を同定した。さらに、臍帯血幹細胞移植を施行した急性骨髄性白血病患者（生着例18例、非生着例4例）に対し、骨髄クロット標本における全細胞数、バーシカン陽性細胞数、およびCD68陽性細胞数の変化を経時的に計測した。移植後16日から55日に採取された骨髄クロット検体を4つの時期に分類して検討した。抗バーシカン抗体と抗CD68抗体を用いた免疫組織化学染色を行い、単位面積当たりの陽性細胞数を計測し、平均値±標準偏差を算出した。細胞数の経時的な変化を解析するとともに、年齢をあわせた人の正形成骨髄の細胞数とも比較した。

結果 二重免疫染色を行った結果、骨髄においてバーシカンを発現する細胞はマクロファージであることが分かった。移植後の骨髄における細胞数を経時的に調べると、バーシカン陽性細胞数と全細胞数の間に逆相関が見られた。非生着例では、年齢と採取日をあわせた生着例に比べて、バーシカン陽性マクロファージ数が減少していることが分かった。

まとめ バーシカンには、臍帯血幹細胞移植の患者における造血を亢進する働きがあると考えられた。