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主論文の要旨 (和文)

急性骨髄性白血病、臍帯血幹細胞移植療法後の骨髄におけるバーシカン産生マクロファージの動態

① **目的** バーシカンは、様々な組織の主に細胞外基質 (extra cellular matrix: ECM) に存在するプロテオグリカンで、遊走、接着、分化、増殖などの細胞に関する重要な働きを制御している。以前の研究から、バーシカンは骨髄にも存在し、化学療法後の再生髄において増加することが分かっていた。そこで我々は、骨髄移植後の骨髄再生においてバーシカンが重要な役割を果たすのではないかと考え、以下の目的について検討を行った。1) 骨髄におけるバーシカン産生細胞を同定する。2) 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 (acute myelogenous leukemia: AML) に対して臍帯血幹細胞移植 (cord blood stem cell transplantation: CBSCT) 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变化を解析し、バーシカンの骨髄再生における重要性を明らかにする。

② **対象および方法** 正形成髄 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、CBSCT を施行した AML 患者 (生着例 18 例 23 検体、生着不良例 4 例 4 検体) に対し、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、骨髄クロット標本における全骨髄細胞数、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。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。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 (16-25 日、26-35 日、36-45 日、46-55 日) に分類して検討した。細胞数の経時的な変化を解析するとともに、年齢をマッチングした正形成髄の細胞数とも比較、生着不良検体に対しても、同様の検討を行い、統計学的有意差を検討した。

③ **結果** コントロール検体を用いて、抗バーシカン抗体にて免疫組織化学染色を行ったところ、間質に存在するマクロファージと思われる細胞が陽性に染色されたため、抗 CD68 抗体で二重染色を行い、骨髄におけるバーシカン産生細胞がマクロファージであることを確認した。移植後の骨髄における細胞数を経時的に調べると、全骨髄細胞数は、前処置および移植後に減少するが、移植後 16-25 日には回復し、その後も上昇していた。バーシカン陽性細胞数は、移植後 16-25 日のグループにおいてコントロールと比較して有意に増加したのち、減少していた。CD68 陽性細胞数は、移植後一カ月間変化はみられないが、移植後 36-45 日ではコントロールに対して有意に減少していた。また、バーシカン陽性細胞数と全骨髄細胞数の間には逆相関が見られた。生着不良例では、年齢や採取日をマッチングした生着例に比べて、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。

④ **考察** 以前の研究では、パーシカン[®]は再生髄の細胞外基質に主として陽性となり、骨髄におけるパーシカン[®]産生細胞の候補として骨髄間質細胞、血管内皮細胞、脂肪前駆細胞などを想定していた。今回用いた抗体では、認識するエピトープの違いからかマクロファージの細胞質が陽性となり、マクロファージが産生細胞であることが判明した。マクロファージは生理的あるいは様々な病態においても組織環境のモジュレーターとして重要な役割を果たしていることは知られており、骨髄においても重要な機能を有していることが推測される。化学療法後の骨髄再生においてパーシカン[®]の発現が増加することから、パーシカン[®]が骨髄移植後の骨髄再生においても重要な役割を果たしているのではないかと考え、AMLのCBSCT法の検体を用いて、パーシカン[®]産生マクロファージの経時的動態を解析した。

骨髄移植では、幹細胞移植後2-3週間で造血幹細胞が生着し、その後活発な造血が起こる。移植後3週目の時期は、骨髄移植において、骨髄再生が起こる重要な時期である。今回の検討で、パーシカン[®]陽性細胞数が移植後3週目で急激に増加するにも関わらず、全マクロファージ数に変化が見られなかったことを考えると、骨髄に定常的に存在するマクロファージのうちの一部がパーシカン[®]陽性に変化することが推察された。骨髄移植の前処理としての強力な化学療法によって骨髄が傷害され、その後造血幹細胞の定着と骨髄再生が開始されると、パーシカン[®]陰性マクロファージは陽性に転化するのではないかと考えられる。そして生着後にはパーシカン[®]産生マクロファージが消費され、マクロファージ数も減少し、再び定常状態に回復すると予想された。同様の検討を行った生着不良例では、コントロール症例と比較してマクロファージ数に差はないものの、移植後3週目にパーシカン[®]陽性細胞数が少ない傾向が観察され、パーシカン[®]が骨髄移植における骨髄再生の重要な因子のひとつであると考えられた。

パーシカン[®]が骨髄再生にどのように関与するのか、その詳細は明らかでないが、ケモカインやミッドカイン、GM-CSFなどの造血因子と結合することで、それらの機能を調節し、ECMにおけるさまざまな液性因子の足場として働いている可能性が考えられている。今後、詳細なパーシカン[®]の機能を解明していくことで、臨床に対して有益な情報を提供したいと考える。

⑤ **結語** 今回の我々の検討で、骨髄におけるパーシカン[®]産生細胞がマクロファージであることが明らかとなり、さらにはCBSCT後の骨髄におけるパーシカン[®]陽性マクロファージ数の経時的観察によって、パーシカン[®]やパーシカン[®]を発現するマクロファージが骨髄再生において重要な役割を果たしていることが示唆された。

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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 engraftment, 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 \pm 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 engraftment 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 aggrecan 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[5] and its wide-range distribution is subsequently revealed in the smooth muscle cells[6, 7], cartilage[8], skin[9] and blood vessels[10]. Versican is also expressed at the ECM of malignant tumors[11, 12] and developing embryos[10, 13].

The main cell type that produces versican in inflammatory lesions has been revealed to be macrophages[4]. 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)[14], lipopolysaccharide[15] and hypoxia[16]. At ECM, it binds to hyaluronan and other ECM molecules such as fibronectin[4, 17] and several chemokines[18, 19] thereby influences leukocyte function.

Versican reportedly exists in the long-term culture of mouse bone marrow (BM) cells[20] and in the ECM of BM after chemotherapy[21]. Moreover,

Oguri *et al.*[22] detected a large amount of proteoglycan with chondroitin 6-sulfate in rabbit BM tissues. Although versican in BM has not been analyzed 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 (SCs) has been performed for treatment of haematopoietic diseases such as leukaemia. Down these lines, Nagasaka *et al.*[21] 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 defense is less pronounced with it, and only a part of human leukocytic antigens needs to be matched[24, 25]. Therefore, we confined our analysis to CBSCT-treated patients. Our preparative regimen for CBSCT was based on previous reports, which was recently summarized by Arai *et al.*[26] 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 26-35 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 visualized using a kit (Bond Polymer Refine Detection, Leica Biosystems Newcastle, UK), and brown color 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[27]. With this technique, after versican-bound antibody was visualized with a brown color with DAB, anti-CD68 antibody was reacted to the tissue section, and colored red using the Bond Polymer Refine Red Detection kit (Leica Biosystems, Newcastle, UK). Nuclei were counterstained by haematoxylin, and immunohistochemical stain 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 control (Student's *t* test). We used a Stat View program (STAT View for Windows, V.5; SAS Institute, Cary, New Castle, USA) and significance was set at $p < 0.05$.

RESULTS

Macrophages are the versican-producing cells in BM

(Figure 1 near here)

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

(Figure 2, near here)

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 < 0.05). 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 < 0.001, figure 2B), and then sharply decreased (**p < 0.01, ***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 that of the age-matched and sampling day-matched control, yet it did not reach 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[2, 18, 28].

Moreover, versican participates intimately in the process of human diseases such as inflammation, atherosclerosis, cardiac infarction, and proliferation and invasion of cancer cells[2, 4, 28]. 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[4, 14, 15, 16, 29, 30], inflammatory cells[4], fibroblasts[31] and myofibroblasts[32] 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[21].

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[4]. Moreover, macrophages are known to play an important role in BM regeneration[33-37]. 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[23]. 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 CBSCT are performed as treatments for haematolymphoid neoplastic diseases including leukaemias and lymphomas[38]. Clinically, cytarabine (CA) + cyclophosphamide (CY) + total body irradiation (TBI) (CA+CY+TBI) is generally performed in these transplantations as pretreatment to kill neoplastic cells as well as patients' haematopoietic cells[26, 39] and for the first few weeks, the total cell number

in the BM declines. Then, blood SCs from donors are transfused to patients, engrafted and then start to produce progenitors such as neutrophils. They finally appear in PB, increase for 2-3 weeks and reach the normal level after approximately 40 days. An engraftment is defined by the number of neutrophils in the PB, which should be more than 500/ μ l for three continuous days.

We observed that around 10 days after transplantation the total number of haematopoietic cells in our patients' BM lowered to the minimum and recovered in number after 3-4 weeks, just as mentioned above. On the contrary, the number of versican-positive macrophages was sharply increased for 3 weeks, and then declined thereafter. This result demonstrated an inverse time course of the versican-positive macrophages to that of total cells in the transplanted BM. Meanwhile, the number of CD68-positive macrophages remained unaltered for the first 3 weeks unlike that of versican-positive macrophages, suggesting that not all macrophages but the versican-expressing ones play an important role in regeneration in this period. Given that versican contributes to BM regeneration in a positive fashion, it would be expected that the number of versican-positive

macrophages in BM of the poor engraftment cases is lower than that of fine engraftment cases. Although not statistically significant, we observed a clear trend of lowered versican-positive cells in their BM. It requires more such cases in future. Macrophages are known to exist as resident cells in a steady state of BM[27]. It seems that residential macrophages, which are at first negative in versican expression, become versican-positive for the 3 weeks after transplantation. Perhaps the versican-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 allogeneic SCT, it becomes rich in chemokines and other humoral factors[40]. Interestingly, versican can activate macrophages to upregulate tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6)[41]. At least, in neuronal cells TNF- α induces versican expression[42]. Perhaps, after preconditioning and CBSCT in BM, a positive feedback loop between versican and macrophage would be in function.

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[18] and

midkines[19], 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 presets a favorable 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 in

the near future. Moreover, *in vivo* and *in vitro* experiments on versican induction in macrophages of BM should further be performed to establish the present analysis on human samples.

Legends

day	-7	-6	-5	-4	-3	-2	-1	0	+1	+3	+6	+7
	TBI	TBI	CA	CA	CY	CY	tacrolimus	Transplantation $2.4 \pm 0.5 \times 10^7 / \text{kg}$	MTX	MTX	MTX	G-CSF

CA, cytarabine; CY, cyclophosphamide; MTX, methotrexate; G-CSF, granulocyte-colony stimulating factor; TBI, total body irradiation;

Table 1 General conditioning regimen before and after transplantation of our hospital.

case #	group	days	age	sex	diagnosis	case #	group	days	age	sex	diagnosis
1		9	58	M	MDS/AML	<u>20</u>	46-55	50	54	M	AML
2	16-25	20	40	M	AML	<u>21</u>		52	40	M	AML
3		22	59	F	MDS/AML	22		55	49	F	AML
4		23	57	F	AML	<u>23</u>		55	58	F	AML
5		25	34	F	AML			[†] 53±2	[§] 50±8		
						Age-matched poor engraftment group					
case #	group	days	age	sex	diagnosis	case #	group	days	age	sex	diagnosis
6	26-35	28	37	M	AML	1	poor	20	30	M	AML
7		28	49	F	AML	2		21	31	F	AML
8		29	43	F	AML	3		26	55	M	AML
9		29	54	M	AML	4		34	49	F	AML
10		33	44	M	AML			[†] 25±6	[§] 41±13		
11		34	55	M	MDS/AML	Age-matched normocellular group					
						case #			age	sex	diagnosis
						1			20	F	nephrosis
						2			27	F	lymphadenitis
						3			37	M	lymphadenitis
						4			52	M	hyperthyroidism
						5			68	M	amyloidosis
						6			75	M	COPD
						7			86	F	lymphadenitis
						[§] 52±25					
		[†] 23±2	[§] 48±12								
12	36-45	36	27	F	AML						
13		36	55	M	AML						
14		36	59	M	MDS/AML						
<u>15</u>		36	60	F	MDS/AML						
<u>16</u>		38	57	F	AML						
17		40	54	M	AML						
18		41	55	F	AML						
19		42	30	M	AML						
		[†] 30±3	[§] 47±7								
		[†] 38±3	[§] 50±13								

Patients were grouped based on the day their clots were sampled. The mean age of each group was not significantly different. † indicates the average days and SD after CBSCT in each group. Underlines indicate patients whose bone marrow specimens were obtained multiple times. § indicates the average age and SD in each group.

AML, acute myelogenous leukaemia; CBSCT, cord blood stem cell transplantation; COPD, chronic obstructive pulmonary disease; days, sampling days; F, female; M, male; MDS/AML, myelodysplastic syndrome overt AML.

Table 2 Clinical data of patients enrolled in this study.

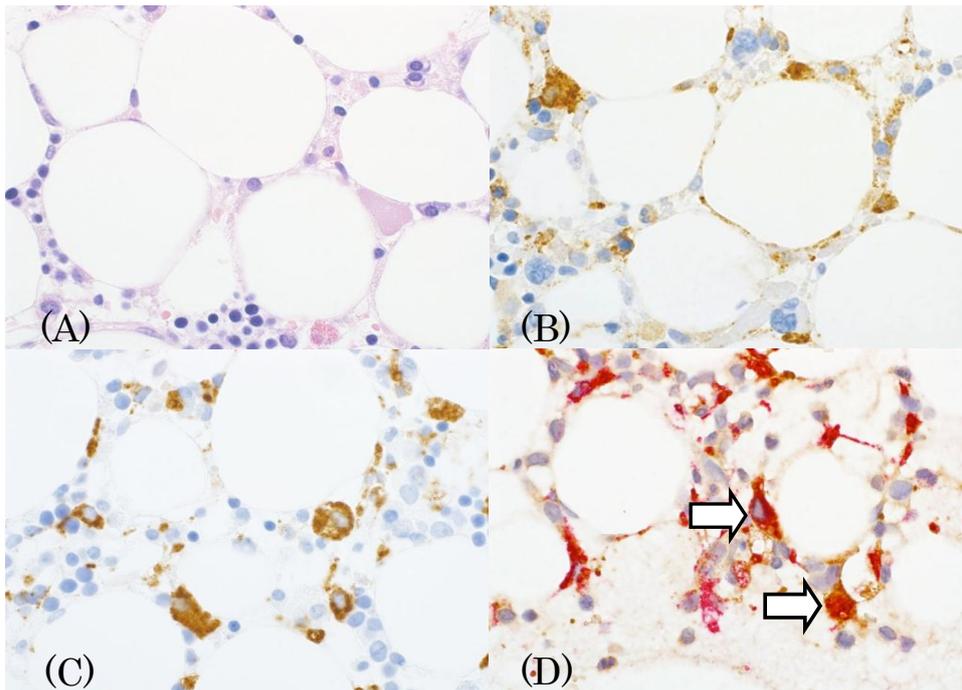


Figure 1 Determination of versican-expressing cells in bone marrow specimens. (A) H&E stain. (B and C) Immunohistochemical stain with anti-versican (B) and anti-CD68 (C) antibodies, respectively, in the same bone marrow tissue. (D) Double immunohistochemical stain with anti-versican together with the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

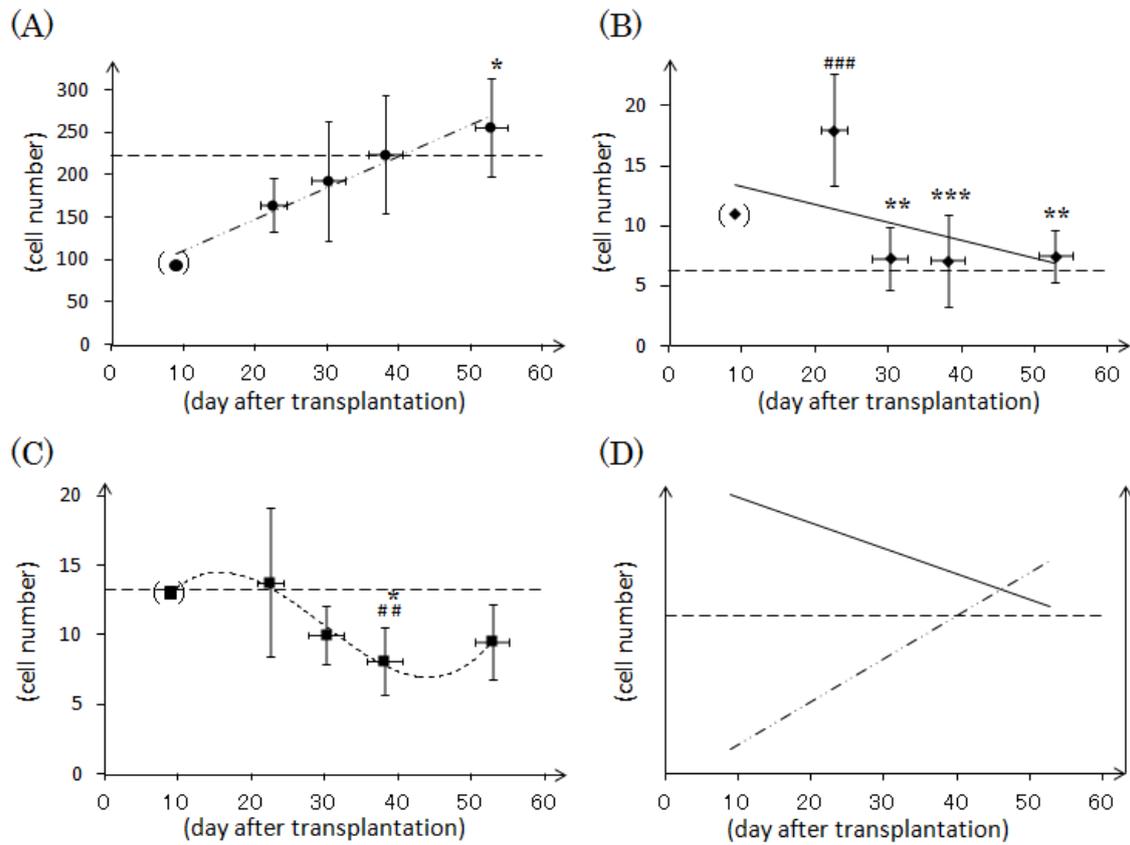


Figure 2 The average numbers and SD of the total (A), versican-positive (B) and CD68-positive cells (C) in a 0.15 mm² area of bone marrow tissues. The dotted horizontal bars are an average of total, versican-positive and CD68-positive cells from the age-matched normocellular group. (A) * indicates a significant difference between the 16-25 day and 46-55 day groups (analysis of variance, * $p < 0.05$). Data were linearly well regressed ($r^2 = 0.962$). (B) ### indicates a significant difference between the 16-25 day and the age-matched normocellular groups (### $p < 0.001$). ** and *** indicate a significant difference between the 16-25 day group and other 3 groups (** $p <$

0.01, *** $p < 0.001$). Linear regression was applied as in (A) ($r^2 = 0.282$). (C) ## and * indicate a significant difference between the 36-45 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 ($r^2 = 0.974$). (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 overlaid.

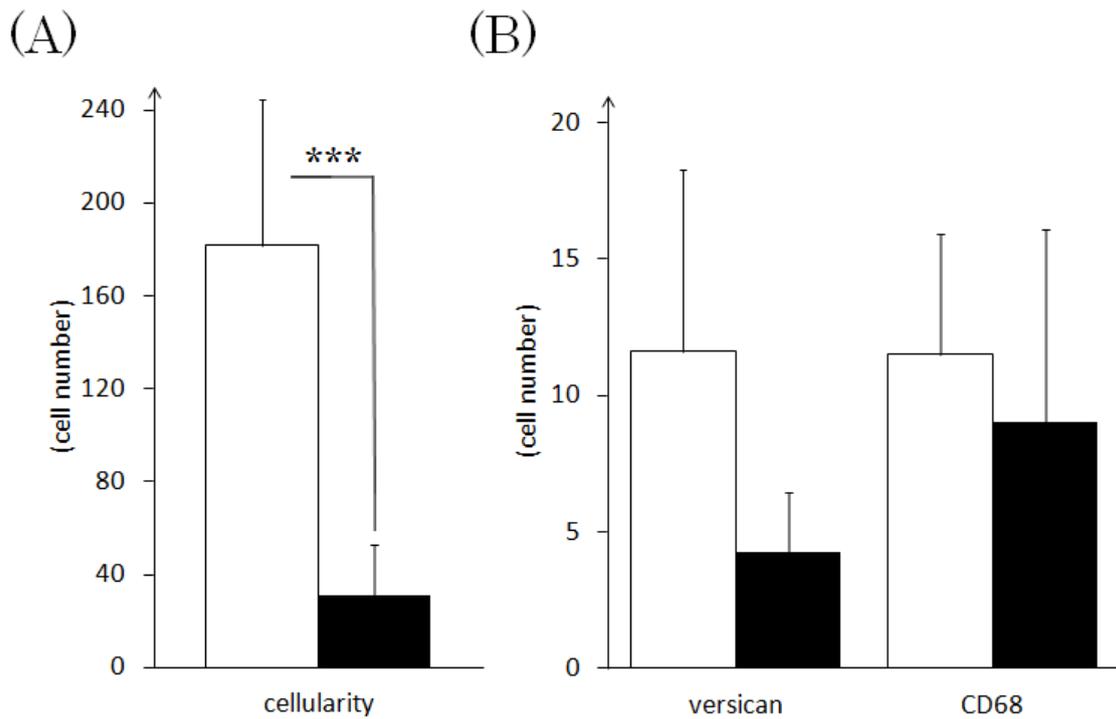


Figure 3 Comparison of the total cell numbers (A), versican-positive (B, left) and CD68-positive cells (B, right) of the age-matched and sampling day-matched patients group (open bar) with those of the age-matched poor engraftment group (closed bar). * indicates a significant difference (*t* test, ****p* < 0.001). Versican-positive macrophages are approximately half that of the control, yet it does not reach statistical significance (*p* = 0.056).

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