above were approved by the Ethics Committee of Tokyo Dental College Ichikawa General Hospital.

Coculture of blasts and stromal cells

Stromal cells of the FL and FBM were irradiated with 15 Gy, while KM101 cells were with 10 Gy, and seeded onto 12-well plates (Corning, Lowell, MA) in 1.6 ml of growth medium. The following day, half of the medium was removed and the same volume of fresh growth medium containing 8 x 10^5 TAM or AML blasts was placed on the confluent monolayer of the adherent stromal cells. In another set of cultures, chambers with a microporous membrane (Transwell, pore size 0.4 μm; Corning) were placed in culture wells and blasts were placed into the chambers. For controls, blasts were cultured without the stromal cell layer in the presence or absence of hematopoietic growth factors. After 7 days of culture, cells were harvested and counted. Blasts cultured without a microporous membrane were treated as follows: 1) blasts that were not attached to the stromal cell layer were harvested as nonadherent cells, and 2) the remaining cells, including the adherent stromal cells and blasts that were attached to them, were trypsinized, harvested together, and the number of blasts was counted, with co-existing stromal cells being easily recognized by their larger size and excluded. Co-existing stromal cells were removed by culturing the cells for 3 h, allowing them to adhere again. The remaining cells were washed and plated in methylcellulose for subsequent colony assay. The number of blast progenitors (clonogenic cells) that had been recovered from these cultures was calculated by multiplying the plating efficiency in the colony assay by the total number of cells after coculture.

Accepted Article

Accepted Article

Suspension culture

TAM blasts were cultured at 2.5-5 x 10^5 cells/mL in 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) or Linbro/Titertek 96-well plates (MP Biomedicals, Solon, OH) in the growth medium with or without hematopoietic growth factors or culture supernatants of FL stromal cells. The recovery of blast progenitors from these cultures was evaluated as described above using subsequent colony assay.

Colony assay

Colony formation of blast progenitors was assessed using methylcellulose culture as previously described [Miyauchi et al., 1987]. Blasts were plated at a concentration of 5 x 10^3 cells per well in 96-well plates in 0.1 mL of growth medium. As a growth stimulant, interleukin-3 (IL-3) was used for TAM blasts since it has been shown to be the most powerful growth stimulator for TAM blasts [Miyauchi et al., 2010], whereas granulocyte-CSF (G-CSF) or IL-3 was used for G-CSF- or IL-3-dependent AML blasts, respectively.

Immunocytochemistry

The stromal cells of the FL and FBM, seeded onto Culture Slides (BD Biosciences, Bedford, MA) and cultured, were air-dried, fixed with buffered formalin-acetone for 30 sec and subjected to immunocytochemistry for analysis of their phenotypic antigen expression. The cell samples were pretreated for antigen retrieval with 0.1 M citrate buffer (pH 6.0 or 9.0) in a microwave oven or a pressure cooker according to the manufacturers' instructions and immunocytochemistry was performed using an automated slide preparation system (Ventana Japan, Yokohama, Japan). The

Accepted Article

antibodies (mouse monoclonal, unless otherwise stated) used are as follows: anti-vimentin (clone V9; Nichirei, Tokyo, Japan), anti-CD146 (clone EPR3208; Abcam, Tokyo, Japan), anti-nestin (clone 2C1.3A11; Abcam), anti-α-smooth muscle actin (α-SMA) (clone 1A4; DAKO, Tokyo, Japan), anti-cytokeratin 8 (CK8) (clone C-43; Abcam), anti-CK18 (clone DC10; DAKO), anti-α-fetoprotein (AFP) (clone ZSA06; Nichirei), anti-E-cadherin (clone NCH-38; DAKO), anti-HepPar1 (clone OCH1E5; DAKO), anti-CD31 (clone JC70A; DAKO), anti-CD34 (clone QBEnd-10; DAKO), anti-CD54 (rabbit polyclonal; Cell Signaling, Boston, MA), anti-CD4 (clone SP35; Roche, Tokyo, Japan), anti-desmin (clone D3; Nichirei), anti-glial fibrillary acidic protein (GFAP) (rabbit polyclonal; DAKO), anti-synaptophysin (rabbit polyclonal; Invitrogen, Tokyo, Japan), anti-CD56 (NCAM) (clone CD564; Leica, Newcastle, UK), anti-lysozyme (rabbit polyclonal; DAKO), anti-CD68 (clone PGM1; DAKO), anti-CD45 (clone PD7/26, 2B11; Nichirei) and anti-CD10 (clone 56C6; Nichirei).

Hematopoietic growth factors

G-CSF was provided by Chugai Pharmaceutical (Tokyo, Japan) and the other hematopoietic growth factors were purchased from various manufacturers: IL-3 (Strathmann Biotech AG, Hamburg, Germany), GM-CSF (Prospec-Tany, Rehovot, Israel), SCF (BioVision, Mountain View, CA), thrombopoietin (TPO) (PeproTech EC, London, UK) and insulin-like growth factor 2 (IGF2) (Abcam). IL-3, GM-CSF, SCF, TPO and G-CSF were used at a final concentration of 50 ng/ml unless otherwise stated, whereas IGF2 was used at 100 ng/mL based on the result of titration experiments (data not shown).

Accepted Article

ELISA assay

Culture supernatants of the FL and FBM stromal cells were harvested 3 days after the cells had become confluent and floating cell debris was removed by centrifugation and microfiltration with a 0.2 μm Acrodisc Syringe Filter (PALL, Ann Arbor, MI). Concentrations of IL-3, GM-CSF, G-CSF, TPO, SCF and IGF2 in the culture supernatants of the FL and FBM stromal cells were measured with ELISA kits (IL-3, GM-CSF, G-CSF, TPO and SCF: R&D Systems, Minneapolis, MN; IGF2: Mediagnost, Reutlingen, Germany) according to the manufacturers' instructions.

Neutralizing antibodies

The antibodies against GM-CSF and G-CSF were purchased from PeproTech (Rocky Hill, NJ) and the antibody against SCF was purchased from Abcam. These antibodies were used at concentrations above the one-half maximal inhibition doses described in the manufacturers' instructions. Before being added to the cultures, these antibodies were incubated with the culture supernatants or hematopoietic growth factors at 4˚C for 4 h.

RESULTS

Characterization of FL and FBM stromal cells

Prior to the experiments using the FL and FBM stromal cells, phenotypic antigen expression of these cells on culture slides was determined by immunocytochemistry (Table 1). Both FL and FBM stromal cells were intensely positive for vimentin (a general mesenchymal cell marker), α-SMA (an antigen expressed in hepatic stellate cells and mesenchymal stem cells (MSCs)) (Fig. 1A, B),

Accepted Article

CD146 and nestin (antigens expressed in perivascular cells/pericytes and MSCs) [Corselli et al., 2013; Covas et al., 2008; Crisan et al., 2008; Gerlach et al., 2012; Mendez-Ferrer et al., 2010] (Fig. 1C, D). In addition, a small fraction of the FL stromal cells weakly expressed CK8 (an epithelial antigen expressed in hepatoblasts and hepatocytes) (Fig. 1E), although they were negative for other epithelial markers such as CK18, E-cadherin, AFP and HepPar1 (antigens expressed in hepatoblasts and/or hepatocytes). The FBM stromal cells were positive for CD10 (an antigen expressed in bone marrow reticular cells) (Fig. 1F), but negative for epithelial markers. Both types of stromal cell were negative for other antigens examined, namely, general endothelial markers (CD34, CD31), markers of hepatic sinusoidal endothelial cells (CD54/ICAM-1, CD4), those of Kupffer cells (lysozyme, CD68) and those of hepatic stellate cells (also called Ito cells) (desmin, GFAP, synaptophysin, CD56/NCAM), except for α-SMA (Table 1). These findings indicate that both the FL and the FBM stromal cells that we used represent the perivascular MSCs, with the former corresponding to hepatic pericytes and the latter being consistent with bone marrow reticular cells. The FL stromal cells also appeared to have some features of cells in epithelial-mesenchymal transition (EMT cells), known to be seen under certain conditions, including developing organs [Choi and Diehl, 2009; Kalluri and Weinberg, 2009] and to support fetal hematopoiesis in the liver [Chagraoui et al., 2003].

Since it has been shown that murine fetal liver EMT cells are induced to differentiate into hepatocytes and lose their hematopoiesis-supporting ability in the presence of oncostatin M (OSM), we tested whether our FL stromal cells also exhibit such changes in response to OSM treatment according to a method previously described [Chagraoui et al., 2003]. Although the in

Accepted Article

vitro growth rate of the FL stromal cells was suppressed by the addition of 1 ng/mL OSM, spindle cell morphology and immunophenotypic features were largely unchanged, with no obvious enhancement or induction of epithelial cell marker expression and only a slight decrease in α-SMA expression.

Coculture of TAM blasts and stromal cells

To examine the influence of cells constituting the fetal hematopoietic microenvironment on the in vitro growth of TAM blasts, we analyzed the growth-supporting ability of stromal cells of the FL and FBM obtained from two human fetuses by coculturing them with TAM blasts obtained from 4 patients. To eliminate the influence of cell-to-cell contact, a transwell coculture system with a microporous membrane, separating blasts from stromal cells and allowing only the passage of humoral factors between the two chambers, was used. To compare the functions of stromal cells of the fetal and postnatal hematopoietic microenvironment, we also examined the effects of an adult human bone marrow-derived stromal cell line, KM101, which is known to be capable of supporting hematopoiesis. Since we found in the preliminary experiments that KM101 cells form colonies in methylcellulose, which makes it difficult to analyze the data of TAM blast colony assay if these cells coexist in the same samples, KM 101 cells were used only in the presence of transwells. When TAM blasts were cocultured with the FL stromal cells in the presence of a transwell, the growth of TAM blast progenitors was potently supported in all patients and the numbers of blast progenitors recovered to levels comparable to those of the cultures with IL-3 in 3 patients (Fig. 2A-C), although it was slightly below the level of IL-3 in one patient (Fig. 2D). After the coculture of TAM blasts and the FL stromal cells in the absence of transwells, numerous viable TAM cells were present in

Accepted Article

culture and the growth of blast progenitors, particularly in non-adherent layers, was efficiently supported although to lower levels than that of the cultures with transwells in all patients (Fig. 2A-D). The immunophenotypic features of the FL stromal cells were unchanged before and after the coculture (data not shown). In contrast, after the culture with FBM stromal cells, TAM blasts dramatically decreased in number and the recovery of TAM blast progenitors was very poor and significantly lower than in the cultures with the FL stromal cells under all culture conditions irrespective of the presence or absence of transwells (Fig. 2A-D). After the coculture of TAM blasts with KM101 cells, the growth of TAM blast progenitors was supported to levels similar to, or slightly lower than, those of the FL stromal cells in the presence of transwells (Fig. 2A-D). These findings indicate that the growth of TAM blast progenitors is dependent on the stromal cells of FL, but not FBM, and supported mainly by humoral factors produced by the FL stromal cells, and that adult bone marrow stromal cells are also capable of supporting the growth of TAM blast progenitors.

Coculture of AML blasts and stromal cells

To determine if the growth-supporting ability of the FL stromal cells is limited to hematopoietic cells of FL origin such as TAM blasts and to test whether FBM stromal cells are capable of supporting the growth of leukemic cells derived from the bone marrow, we next examined the influence of the FL and FBM stromal cells on the growth of AML blasts in adult patients. Since the growth patterns of adult AML blasts in response to hematopoietic growth factors are heterogeneous among patients, two patient samples were selected for this experiment; blast progenitors in patient AML-1 are dependent on IL-3, GM-CSF and SCF as in the case of TAM, whereas those of patient

Accepted Article

AML-2 are dependent almost exclusively on G-CSF and SCF. These AML blasts were cultured with the FL or FBM stromal cells in the same way as for TAM blasts. The growth of blast progenitors in AML-1 and AML-2 was stimulated by IL-3 and G-CSF, respectively, as expected (Fig. 3A, B). In both cases, the FL stromal cells in the presence, but not the absence, of a transwell stimulated the growth of AML blast progenitors more effectively than G-CSF or IL-3 alone. The FBM stromal cells also exhibited some degree of growth-supporting activity on AML blasts in the presence of transwells, particularly for those of AML-1, but the activity was still significantly weaker than that of the FL stromal cells. Hence, it was shown that the growth-supporting ability of the FL stromal cells is not limited to fetal hematopoietic cells but is also valid for adult leukemic blasts originating in the bone marrow, whereas the stromal cells of the FBM that we used, at the gestational ages of 13 and 16 weeks, do not support the growth of either fetal or adult myeloid leukemia cells as efficiently as the FL stromal cells do, possibly due to their functional immaturity.

Hematopoietic growth factors produced by stromal cells

Since the growth of TAM and AML blasts was potently supported by the FL stromal cells in transwell cultures with a microporous membrane hindering cell-to-cell contact between blasts and stromal cells, humoral factors must be secreted from the stromal cells into the culture medium. In order to identify the humoral factors produced by the stromal cells, we measured the concentrations of six major human hematopoietic growth factors: IL-3, GM-CSF, G-CSF, SCF, TPO and IGF2, in culture supernatants of the FL and FBM stromal cells by ELISA. High concentrations of GM-CSF, G-CSF, SCF and IGF2 were detected in the culture supernatants of the FL stromal cells obtained from both of the two fetuses (FL1 and FL2) (Table 2). Compared with the culture supernatants of

Accepted Article

the FL stromal cells, similar or even higher amounts of SCF and IGF2 were detected in those of the FBM stromal cells, but GM-CSF was barely detectable and G-CSF was undetectable. Notably, IGF2 was detected even in the control growth medium containing 10% FCS, but its concentration was lower than in most of the FL and FBM stromal cell culture supernatants. IL-3 and TPO were not detected in any culture supernatants of the stromal cells. Among these hematopoietic growth factors, the key factors that caused the big difference in growth-supporting ability for TAM blast progenitors between the FL and FBM stromal cells should be those that are present at higher concentrations in the stromal cell culture supernatants of FL than those of FBM. Since the concentrations of SCF and IGF2 were not uniformly higher in the FL stromal cell culture supernatants and our previous study showed that G-CSF is not a very active stimulator of the growth of TAM blasts [Miyauchi et al., 2010], we speculated that GM-CSF might be the most likely candidate for such a factor.

Effects of neutralizing antibodies

To ascertain the important hematopoietic growth factor(s) for the growth of TAM blasts that are produced by the FL stromal cells, we tested the effects of neutralizing antibodies against GM-CSF, G-CSF and SCF on the culture supernatants of the FL stromal cells. Antibodies against IL-3, TPO and IGF2 were not included because neither IL-3 nor TPO was detected in any culture supernatants and IGF2 was not shown to be a potent growth stimulator for TAM blasts in our experiments as described below. Prior to the experiments, the neutralizing ability of these antibodies was confirmed using colony assay of three AML cell lines, OCI-AML1, MO7e and KPAM1, the growth of which is dependent on G-CSF, GM-CSF and SCF, respectively. Each antibody

Accepted Article

completely inhibited colony-stimulating activity of the corresponding cytokine (Supplementary Fig. 1A-C), and the antibody against GM-CSF at concentrations higher than 1 μg/mL completely neutralized the activity of 10 ng/mL GM-CSF (Supplementary Fig. 1D). Then, we tested the effects of these antibodies on the culture supernatants of the FL stromal cells (2 samples, FL1 and FL2), using suspension culture and colony assay of TAM blasts obtained from 3 patients. The antibody against GM-CSF alone almost completely inhibited both cell growth in suspension culture (Fig. 4A, C and Supplementary Fig. 2A) and colony formation in methylcellulose culture (Fig. 4B, D and Supplementary Fig. 2B) of TAM blasts with all FL culture supernatants and TAM patient samples. In the colony assay of patient TAM-4, the antibody against GM-CSF inhibited colony formation to levels even below that of the negative control (no added culture supernatants and antibodies) (Fig. 4D), suggesting that TAM blasts of this patient may produce a small amount of GM-CSF in an autocrine fashion and that such activity was also neutralized by the antibody. On the other hand, the antibodies against G-CSF and SCF and the control non-immune IgG did not significantly affect the growth of TAM blasts (Fig. 4 and Supplementary Fig. 2).

Effects of IGF2 on the growth of TAM blasts

IGF2 is produced in fetal organs, including the liver [Pollak, 2008; Zhang and Lodish, 2004], and it has been shown that abnormalities in the IGF2 signaling pathway may be involved in the pathogenesis of TAM and AMKL-DS [Klusmann et al., 2010]. However, our previous study showed that growth medium supplemented with FCS, which contains a high concentration of IGF2 as shown in Table 2, alone does not significantly stimulate the growth of TAM blasts [Miyauchi et al., 2010]. To ascertain if IGF2 has any growth-stimulating activity for TAM blasts, we tested the

Accepted Article

in vitro effects of IGF2 on TAM blasts in 2 patients. Both colony formation in methylcellulose culture (data not shown) and recovery of blast progenitors from suspension culture only slightly increased when IGF2 was added at high concentration (100 ng/mL), but the maximum activity was still much lower than that of other hematopoietic growth factors such as IL-3 and TPO in both patients (Fig. 5). Furthermore, no synergistic effects were detected with any combinations of IGF2 and another hematopoietic growth factor (data not shown). Although the experiments were performed in the presence of 10% FCS, these findings indicate that IGF2 on its own has only modest growth-stimulating activity on TAM blasts.

DISCUSSION

The present coculture experiments demonstrated that in vitro growth of blast progenitors in TAM is dependent on stromal cells of the FL, but not FBM, indicating that the microenvironment of the FL could play an important role in the pathogenesis of TAM. This result is consistent with the data described by Tunstall-Pedoe et al. [2008] that FLs, but not FBMs, in DS patients exhibit perturbed myeloid hematopoiesis with a higher frequency of megakaryocyte-erythroid progenitor cells compared with normal individuals, indicating that trisomy 21 provides a background for leukemogenesis in the FL preceding the acquisition of *GATA1* mutations. These data support the hypothesis previously proposed by us that TAM is a special form of leukemia arising in the FL [Miyauchi et al., 1992]. Although the number of TAM samples used in this study is small, we have previously shown that growth characteristics of TAM blast progenitors in response to hematopoietic growth factors are quite uniform with very little patient heterogeneity [Miyauchi et

Accepted Article

al., 2010]. This could be a reflection of the relatively simple cytogenetic background of TAM, in which the rate of mutations other than *GATA1* is very low [Nikolaev et al., 2013; Yoshida et al., 2013] and a variety of *GATA1* mutations all result in an uniform abnormality of GATA1 protein, namely, generation of GATA1s and abrogation of full-length GATA1 [Gurbuxani et al., 2004]. Therefore, our present findings may represent the general biological nature of TAM blasts. Furthermore, our present in vitro findings are consistent with the in vivo observations of human patient samples that blasts and/or atypical megakaryocytes proliferated predominantly in the liver in autopsy cases of stillborns or liveborn infants with DS and TAM, which corroborate our present data [Becroft and Zwi, 1990; Ishigaki et al., 2011; Miyauchi et al., 1992; Ruchelli et al., 1991].

Although we used TAM samples enriched for >95% blasts morphologically, a minor proportion of normal hematopoietic progenitors may have coexisted in the samples and formed colonies in culture. However, all the patient samples enriched for blasts harbored *GATA1* mutations and one of them (TAM-4) exhibited only mutated *GATA1* sequences both before and after culture with hematopoietic growth factors [Miyauchi et al., 2010], indicating that all colonies of this patient sample should be derived from TAM blasts. Since coculture results of the other patients were very similar to that of this particular patient, the influence of coexisting non-leukemic hematopoietic progenitors, if any, seems to be small and negligible.

MSCs are mesodermal progenitors with the potential to differentiate to multilineage mesenchymal cells [Pittenger et al., 1999]. Perivascular cells, or pericytes, which reside ubiquitously around endothelial cells of capillaries and microvessels in many organs, have been shown to represent ancestors of, or basically the same population as, MSCs [Covas et al., 2008;

Accepted Article

Crisan et al., 2008] and to be capable of supporting hematopoietic stem cells (HSCs) [Corselli et al., 2013]. In addition to the originally detected endosteal niche, the existence of a perivascular niche for HSCs in the bone marrow, such as CXCL2-expressing reticular cells [Sugiyama and Nagasawa, 2012] or nestin-expressing MSCs [Mendez-Ferrer et al., 2010], has been demonstrated. Concerning the liver, perisinusoidal hepatic stellate cells, residing in the space of Disse, express antigens associated with MSCs, produce cytokines, including GM-CSF, and support HSCs [Kordes et al., 2013], suggesting that these cells may be liver-resident perivascular MSCs that form an HSC niche in the liver. The FL stromal cells that we used expressed CD146, nestin and α-SMA, which is consistent with hepatic pericytes/MSCs [Gerlach et al., 2012]. Although these cells might correspond to the immature form of hepatic stellate cells since only one of the stellate cell-associated antigens (α-SMA) was positive, this point needs further confirmation. In addition to the mesenchymal antigens, these cells also expressed CK8, an epithelial cell marker, indicating that they could belong to the unique category of cells called EMT cells. It has been shown that such cells are present in the fetal liver and associated with florid hematopoiesis [Chagraoui et al., 2003]. However, only a minor fraction of our FL stromal cells expressed CK8 and did not express other epithelial markers, even after treatment with OSM, which is different from the results of murine fetal liver stromal cell lines with EMT cell natures described by Chagraoui et al. The exact reason for this discordance is not clear, but it could be due to the difference of species.

Although it has been shown that perivascular MSCs maintain the stemness of HSCs by cell-to-cell contact, cell contact between TAM blasts and FL stromal cells in our study did not enhance, or even reduced, the recovery of TAM blast progenitors, indicating that humoral factors

Accepted Article

produced by the stromal cells stimulated the growth of TAM blasts. This is in agreement with previous findings that fetal HSCs pass through the cell cycle at a higher frequency during fetal development than adult HSCs, which are largely quiescent [Martin and Bhatia, 2005]. However, it is also possible that FL stromal cells provide a hematopoietic niche for a minor population of quiescent leukemic stem cells in TAM and support their long-term survival through cell-to-cell contact in vivo.

Like the case of TAM, the growth of AML blasts in adult patients was supported more efficiently by stromal cells of the FL than those of the FBM, and adult bone marrow stromal cell line KM101 also supported the growth of TAM blasts. Hence, FL stromal cells do not exclusively support the growth of fetal hematopoietic cells and the growth of TAM blasts is not exclusively dependent on FL stromal cells but also supported by adult bone marrow stromal cells. These results can be explained by the present findings that FL stromal cells produce hematopoietic growth factors, including GM-CSF, G-CSF and SCF, that stimulate the growth of adult AML blasts and by the data of other investigators showing that KM101 cells produce GM-CSF [Nakajima et al., 1994] that stimulates the growth of TAM blasts. These data do not support the hypothesis that we previously proposed [Miyauchi et al., 1992] that the cessation of fetal liver hematopoiesis and a shift of major hematopoietic organ from the liver to the bone marrow after birth cause the inhibition of TAM blast growth and spontaneous resolution of TAM because of the loss of a suitable microenvironment. Spontaneous resolution of TAM therefore appears to be more likely associated with a change of the intrinsic genetic program controlling fetal hematopoiesis rather than a shift of the site of hematopoiesis after birth. The FBM stromal cells that we used were not very active in

Accepted Article

supporting the growth of leukemic blasts in either TAM or adult AML, indicating that FBM stromal cells at the 13th and 16th weeks of gestational age may still be immature and incapable of constituting a functional hematopoietic microenvironment. The low concentrations of secreted hematopoietic growth factors, particularly GM-CSF and G-CSF, in the culture supernatants of the FBM stromal cells suggest this possibility.

We showed in the present study that the FL stromal cells produce several hematopoietic growth factors and secrete them into the culture medium. We have previously shown that IL-3, GM-CSF and SCF are the major growth factors for TAM blasts in vitro, all of which directly and potently stimulate the growth of TAM blasts [Miyauchi et al., 2010]. However, IL-3 has not been described to be produced in the liver and was not detected in the culture supernatants of the FL stromal cells. Although low concentrations of SCF were detected in the culture supernatants of FL stromal cells, the anti-SCF antibody did not significantly affect the growth of TAM blasts in the presence of FL stromal cell culture supernatants, indicating that SCF secreted into the culture supernatants at these concentrations did not potently stimulate the growth of TAM blasts. Since the antibody against GM-CSF alone significantly inhibited the growth-stimulating activity of the FL culture supernatants on TAM blasts, it seems highly likely that, among the cytokines produced by FL stromal cells, GM-CSF is the most important hematopoietic growth factor for TAM blasts and might play an important role in the pathogenesis of TAM. However, megakaryoblastic features of TAM blasts are hardly ascribable to the function of GM-CSF alone since GM-CSF is not a potent inducer of megakaryocytic differentiation. We have previously shown that TPO has such an activity for TAM blasts, although its growth-stimulatory effect is weaker than that of GM-CSF

[Miyauchi et al., 2010]. Since hepatocytes are known to be the major source of TPO and to release it into the blood [Sungaran et al., 1997] and TPO was not detected in the culture supernatants of our FL stromal cells, it seems plausible that megakaryocytic differentiation of TAM blasts in vivo is induced by TPO produced by fetal hepatocytes or hepatoblasts, while their growth is stimulated by GM-CSF produced by FL stromal cells. Although it has been shown that IGF2 is produced by fetal hepatocytes and involved in the pathogenesis of TAM and AMKL-DS [Klusmann et al., 2010], it seems unlikely that IGF2 is a major growth regulator of TAM blasts since our data indicate that IGF2 on its own is only a modest growth stimulator of TAM blasts in vitro.

In conclusion, our present study demonstrated that FL stromal cells with unique immunophenotypic features of EMT cells constitute a functional hematopoietic microenvironment that supports the expansion of TAM clones originating from the fetal liver and that GM-CSF produced by FL stromal cells may play an important role in the pathogenesis of TAM.

Accepted Article

ACKNOWLEDGMENT

We thank Dr. Toshio Akashi at Kumakiri Maternal Clinic for providing human fetal tissues, Prof. Kenichi Harigaya at Chiba University for providing KM101 cells, Dr. Tsutomu Toki and Prof. Etsuro Ito at Hirosaki University for providing KPAM1 cells. The authors also thank Drs. Yushi Ito and Keiko Tsukamoto (National Center for Child Health and Development), Hirotaka Takahashi (Tokyo Metropolitan Otsuka Hospital), Kenji Ishikura (Tokyo Metropolitan Kiyose Children's

Hospital), and Kiyoko Sugita (Toyo University) for providing the patient samples. We have no conflict of interest to declare.

Accepted Article

AUTHORSHIP

J.M. designed and performed the research, analyzed the data and wrote the manuscript. H.K. contributed designing of the research and data analysis.

References

Ahmed M, Sternberg A, Hall G, Thomas A, Smith O, O'Marcaigh A, Wynn R, Stevens R, Addison M, King D, Stewart B, Gibson B, Roberts I, Vyas P. 2004. Natural history of GATA1 mutations in Down syndrome. Blood 103:2480-9.

Arai H, Ishida A, Nakajima W, Nishinomiya F, Yamazoe A, Takada G. 1999. Immunohistochemical study on transforming growth factor-beta1 expression in liver fibrosis of Down's syndrome with transient abnormal myelopoiesis. Hum Pathol 30:474-6.

Becroft DM, Zwi LJ. 1990. Perinatal visceral fibrosis accompanying the megakaryoblastic leukemoid reaction of Down syndrome. Pediatric Pathology 10:397-406.

Campagnoli C, Roberts IA, Kumar S, Bennett PR, Bellantuono I, Fisk NM. 2001. Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood 98:2396-402.

Accepted Article

Chagraoui J, Lepage-Noll A, Anjo A, Uzan G, Charbord P. 2003. Fetal liver stroma consists of cells in epithelial-to-mesenchymal transition. Blood 101:2973-82.

Choi SS, Diehl AM. 2009. Epithelial-to-mesenchymal transitions in the liver. Hepatology 50:2007-13.

Corselli M, Chin CJ, Parekh C, Sahaghian A, Wang W, Ge S, Evseenko D, Wang X, Montelatici E, Lazzari L, Crooks GM, Peault B. 2013. Perivascular support of human hematopoietic stem/progenitor cells. Blood 121:2891-901.

Covas DT, Panepucci RA, Fontes AM, Silva WA, Jr., Orellana MD, Freitas MC, Neder L, Santos AR, Peres LC, Jamur MC, Zago MA. 2008. Multipotent mesenchymal stromal cells obtained from diverse human tissues share functional properties and gene-expression profile with CD146+ perivascular cells and fibroblasts. Exp Hematol 36:642-54.

Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng PN, Traas J, Schugar R, Deasy BM, Badylak S, Buhring HJ, Giacobino JP, Lazzari L, Huard J, Peault B. 2008. A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 3:301-13.

Dzierzak E, Speck NA. 2008. Of lineage and legacy: the development of mammalian hematopoietic stem cells. Nat Immunol 9:129-36.

Ford AM, Bennett CA, Price CM, Bruin MC, Van Wering ER, Greaves M. 1998. Fetal origins of the TEL-AML1 fusion gene in identical twins with leukemia. Proc Natl Acad Sci U S A 95:4584-8.

Accepted Article

Ford AM, Ridge SA, Cabrera ME, Mahmoud H, Steel CM, Chan LC, Greaves M. 1993. In utero rearrangements in the trithorax-related oncogene in infant leukaemias. Nature 363:358-60.

Gerlach JC, Over P, Turner ME, Thompson RL, Foka HG, Chen WC, Peault B, Gridelli B, Schmelzer E. 2012. Perivascular mesenchymal progenitors in human fetal and adult liver. Stem Cells Dev 21:3258-69.

Gurbuxani S, Vyas P, Crispino JD. 2004. Recent insights into the mechanisms of myeloid leukemogenesis in Down syndrome. Blood 103:399-406.

Harigaya K, Handa H. 1985. Generation of functional clonal cell lines from human bone marrow stroma. Proc Natl Acad Sci U S A 82:3477-80.

Hitzler JK, Cheung J, Li Y, Scherer SW, Zipursky A. 2003. GATA1 mutations in transient leukemia and acute megakaryoblastic leukemia of Down syndrome. Blood 101:4301-4.

Hitzler JK, Zipursky A. 2005. Origins of leukaemia in children with Down syndrome. Nat Rev Cancer 5:11-20.

Ishigaki H, Miyauchi J, Yokoe A, Nakayama M, Yanagi T, Taga T, Ohta S, Itoh Y, Ogasawara K. 2011. Expression of megakaryocytic and myeloid markers in blasts of transient abnormal myelopoiesis in a stillbirth with Down syndrome: report of histopathological findings of an autopsy case. Hum Pathol 42:141-5.

Kalluri R, Weinberg RA. 2009. The basics of epithelial-mesenchymal transition. J Clin Invest 119:1420-8.

Accepted Article

Klusmann JH, Godinho FJ, Heitmann K, Maroz A, Koch ML, Reinhardt D, Orkin SH, Li Z. 2010. Developmental stage-specific interplay of GATA1 and IGF signaling in fetal megakaryopoiesis and leukemogenesis. Genes Dev 24:1659-72.

Kordes C, Sawitza I, Gotze S, Haussinger D. 2013. Hepatic stellate cells support hematopoiesis and are liver-resident mesenchymal stem cells. Cell Physiol Biochem 31:290-304.

Li Z, Godinho FJ, Klusmann JH, Garriga-Canut M, Yu C, Orkin SH. 2005. Developmental stage-selective effect of somatically mutated leukemogenic transcription factor GATA1. Nature Genetics 37:613-9.

Martin MA, Bhatia M. 2005. Analysis of the human fetal liver hematopoietic microenvironment. Stem Cells Dev 14:493-504.

Mendez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA, Scadden DT, Ma'ayan A, Enikolopov GN, Frenette PS. 2010. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 466:829-34.

Miyauchi J, Ito Y, Kawano T, Tsunematsu Y, Shimizu K. 1992. Unusual diffuse liver fibrosis accompanying transient myeloproliferative disorder in Down's syndrome: a report of four autopsy cases and proposal of a hypothesis. Blood 80:1521-7.

Miyauchi J, Ito Y, Tsukamoto K, Takahashi H, Ishikura K, Sugita K, Miyashita T. 2010. Blasts in transient leukaemia in neonates with Down syndrome differentiate into basophil/mast-cell and megakaryocyte lineages in vitro in association with down-regulation of truncated form of GATA1. British Journal of Haematology 148:898-909.