Animal Model

Nonirradiated *NOD/SCID*-Human Chimeric Animal Model for Primary Human Multiple Myeloma

A Potential in Vivo Culture System

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The *NOD/SCID* human chimeric animal model was generated by implanting of human fetal bones (FBs) into subcutaneous sites of *NOD/SCID* mice (*NOD/SCID-hu*⁺), followed by inoculation of primary bone marrow mononuclear cells (BMNCs) obtained from patients with multiple myeloma (MM) into the FBs. The BMNCs from 30 patients with MM were inoculated, and 28 (93%) of them revealed evidence of tumor growth of myeloma cells (MCs) in the *NOD/SCID-hu*⁺ mice. Intriguingly, 17 (61%) of the 28 patients' BMNCs inoculated developed not only myeloma in the bone marrow of the FBs, but also extramedullary macrotumors (EMTs) along the periosteum of the FBs. The tumor cells in these EMTs had plasmacytoid morphology and preserved antigens and cytogenetics similar, if not identical, to those in the parent MCs. Moreover, small tumor blocks from nine EMTs were transplanted into subcutaneous sites of subsequent recipient *NOD/SCID* mice without human FBs (*NOD/SCID-hu*⁻), and all but one grew successfully. Two of the EMTs have been maintained in the animal model for more than 12 months. The *NOD/SCID-hu*⁺ chimeric animal model is highly efficient for growth of primary MCs and presents clinical features of human MM. The engrafted MCs can be maintained subsequently in *NOD/SCID-hu*⁻ mice as *in vivo* culture. (*Am J Pathol* 2004, 164:747-756)

Multiple myeloma (MM) is a clonal plasma cell neoplasm characterized by proliferation of abnormal plasma cells in bone marrow (BM) that secrete a monoclonal paraprotein (M-protein) in serum and/or urine, and by activation of osteoclasts with related osteolytic bone destruction.

Some patients may have the extramedullary diseases of plasma cell leukemia or plasmacytomas, especially in the terminal stage.

The pathogenesis of MM remains elusive,¹ even though several biological read-out systems, including experimental animal models, have been established. Most xenotransplant severe combined immunodeficiency (SCID) mouse models use human myeloma cell (MC) lines,²⁻⁴ which are of limited relevance to the primary disease state. Attempts to grow primary human MCs from MM patients in the peritoneal cavity of SCID mice have been made. However, typical features of human MM were lacking, although human immunoglobulins (hIgs) were detectable in the sera of these mice.^{5,6} An advance in this field has recently been made by Yaccoby and colleagues,⁷ who generated a chimeric SCID-human model by implanting human fetal bones (FBs) in a subcutaneous site (SC) of *C.B.17/ICr-SCID* mice (*CB17/SCID-hu*),⁸ and then inoculated the primary human MCs directly into the FBs. In their study, 12 (80%) of the 15 inoculated MCs grew in the FBs and presented the clinical features of human MM. It was suggested that the human FBs and their related bone marrow (BM) environment provide the essential condition for growth of these primary MCs. Although the *CB17/SCID-hu* model provided a fair window for observation of the behavior of human MM, the primary MCs only colonized the human FBs in *CB17/SCID-hu* mice and did not migrate to the BM or other compartments of the mice. The primary MCs disappeared after complete resorption of the FBs, indicating that the MCs could not grow without human FBs in the *CB17/SCID* mice.

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More recently, a new SCID strain, nonobese and diabetes-free (*NOD*)/*LtSz-scid/scid* (*NOD/SCID*) mice, are known to be the most immunodeficient SCID variants that lack functional B/T cells and circulating complement, and have low natural killer cell function as well as defective macrophage function.⁹ The *NOD/SCID* mice are the most supportive hosts for normal and malignant human stem cells,^{10–13} and human B-cell differentiation occurs in the absence of supplemental human growth factors,¹⁰ which indicates that the environment and associated growth factors of the *NOD/SCID* mice may be a particularly favorable nonhuman environment for supporting engraftment of human cells, possibly including human MCs.

Pilarski and colleagues¹⁴ recently demonstrated that growth of primary MCs from their 12 patients could be established in BM of 65 (69%) of 94 experimental *NOD/SCID* mice, via either intracardiac or intraosseus injections of MCs. However, Pilarski's model has some limitations because of its high technical level of the intracardiac and intraosseus injections for inoculation of the MCs and a poor window to observe the growth of the inoculated MCs. Some of the established MCs in their model, for example, had to be detected by the polymerase chain reaction (PCR) method rather than by histocytological evaluation. A Matrigel-based, instead of human fetal tissue, xenografting SCID model for primary human MCs has also been reported.¹⁵ Although some solid tumors developed from fresh human MCs injected in the Matrigel, the growth required supplement of large amount of cytokines (interleukin-6 and vascular endothelial growth factor).

In addition to fully mimicking the clinical features of human MM, an ideal and clinically suitable animal model for human MM is one that can be performed and observed more easily, and is able to sustain the growth of MCs by passing tumor cells serially into subsequent recipient mice to preserve the tumor clones. In this study, we demonstrate that the *NOD/SCID-hu* chimeric animal model has high efficacy for growth of primary MCs and presents characteristics of human MM.

Materials and Methods

Patients and Primary Human MCs

Heparinized BM aspirates were obtained from 30 patients with active MM during scheduled clinic visits. The patients' characteristics are listed in Table 1. Among them, four patients had plasma cell leukemia, defined as described,¹ and another six had extramedullary diseases either of soft tissue or spinal plasmacytomas. The bone marrow mononuclear cells (BMNCs) were separated by use of Ficoll-Paque Plus (Amersham Pharmacia Biotech AB, Uppsala, Sweden) centrifugation. The proportion of MCs in the BMNCs was determined by cytospin smear observation and/or by flow-cytometric analysis (EPICS XL-MCL; Beckman Coulter, Miami, FL) by use of phycoerythrin-labeled anti-CD138 (DAKO, Glostrup, Denmark). All of the procedures could be completed within 2 hours after sampling of BM. The mean number of BMNCs inoc-

ulated and the percentage of MCs were $24 \pm 22 \times 10^6$ (range, 2 to 70×10^6) and $50 \pm 26\%$ (range, 2 to 90%), respectively. The study was approved by our Institutional Review Board and Human Ethics Committee. Signed informed consents were obtained from all patients and were retained.

NOD/SCID-Human Chimeric Animal Model

Experimental nonobese and diabetes-free (*NOD*)/*LtSz-Prkdc<scid>/J* (*NOD/SCID*) mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and were housed and monitored in a specific pathogen-free environment with sterile food and water in our animal facility. *NOD/SCID*-human chimeric mice with adoptive implantation of human FBs (*NOD/SCID-hu*⁺) were generated as described.⁸ In short, *NOD/SCID* mice, 4 to 6 weeks of age, underwent implantation of a fragment of human FB (femur or tibia, cut into size of ~ 0.3 cm $\times$ 0.8 cm) at gestational ages of 17 to 24 weeks, into an SC of the right flank region. Four to 6 weeks after the implantation, the *NOD/SCID-hu*⁺ mice were ready for use and light-density BMNCs from the patients, suspended in 50 to 100 μ l of phosphate-buffered saline (PBS), were injected percutaneously into the human FBs. No radiation was applied to the *NOD/SCID-hu*⁺ mice before inoculation of the BMNCs. Blood samples of the mice were obtained periodically by retro-orbital puncture, and sera were prepared and stored at -20°C until analysis. The study was ended either when growth of any visible macro tumor more than 2 cm in diameter occurred or 6 months after inoculation of the BMNCs, depending on whichever occurred first. Either increased levels of circulating isotopic hlg of the M-proteins in mice sera or infiltration of MCs in the FBs with osteolytic bone changes were used as indicators for growth of human MM in the animal model. During all of the procedures, mice were anesthetized with an intraperitoneal injection of thiopental (Abbott Australasia Pty. Ltd., Sydney, Australia). At the end of the experiments, the animals were killed by neck dislocation. All of the experimental procedures were approved by our Human Ethics Committee and Animal Care and Use Committee.

Quantification of Levels of Human Immunoglobulins by Enzyme-Linked Immunosorbent Assay (ELISA)

Levels of hlg in mouse sera were assessed by the Human Ig ELISA Quantitation kit (Bethyl Laboratories, Inc., Montgomery, TX) according to the manufacturer's instructions. Briefly, 96-well Nunc C-bottom immunoplates were separately coated with 1 μ l per well of 10 μ g/ml goat F(ab)₂ anti-human IgG, IgA, κ and λ chains, supplemented with 99 μ l of coating buffer for 60 minutes at room temperature. Plates were then washed with Tris-buffered saline (TBS) containing 0.05% Tween 20, and were blocked by TBS with 1% bovine serum albumin (Sigma Chemical, St. Louis, MO). Serially diluted mouse

Table 1. Patients' Characteristics and the Experimental Parameters for the *NOD/SCID-hu* Animal Model

Patient no.	Stage*	Status	Isotype	BMNCs ($\times 10^6$) [†]	MCs (%) [‡]	Time to end of experiment (m)	Final hlg levels ($\mu\text{g/ml}$)	Myeloma growth	EMT [§]	Time to EMT formation (m)	Subsequent transplantation	EBV PCR
1	IIIA (PCL)	U	G/λ	7	70	3.5	IgG (223), λ (395)	Y	Y	3	ND	—
2	IIIB	U	A/κ	12	80	6	IgA (23), κ (426)	Y	N	—	—	ND
3	IIIA	R	G/λ	6	80	5	IgG (626), λ (381)	Y	Y	4	ND	—
4	IIIA	U	G/κ	5	30	2.5	IgG (59), κ (547)	Y	Y	1	ND	—
5**	IIIB (PCL)	R	κ	6	85	2.5	κ (259)	Y	Y	2	Y	—
6**	IIIA ^{††}	R	G/λ	22	82	4.7	IgG (487), λ (177)	Y	Y	1	Y	—
7	IIA ^{††}	R	λ	4	35	6	λ (396)	Y	N	—	—	ND
8	IIIA	U	G/λ	4	50	3	IgG (365), λ (15)	Y	Y ^{††}	1.7	ND	—
9**	IIA ^{††}	R	G/λ	2	27	4.3	IgG (481), λ (211)	Y	N	—	—	ND
10	IIA ^{††}	U	κ	26	49	5.7	κ (103)	Y	Y	4.5	ND	—
11**	IIIA ^{††}	U	G/κ	70	77	2.7	IgG (30), κ (90)	Y	Y ^{††}	1.4	ND	—
12**	IIIB	U	κ	65	30	4.9	κ (156)	Y	Y ^{††}	1	ND	—
13**	IIA	R	G/λ	14	28	6	IgG (427), λ (488)	Y	Y	3	ND	—
14	IIA	U	G/λ	53	2	3	IgG (468), λ (265)	Y	Y ^{††}	2.3	ND	—
15	IIIB ^{††}	R	A/λ	37	97	2.3	IgA (680), λ (744)	Y	Y	0.5	Y	—
16	IIIA	U	G/κ	10	45	2	IgG (419), κ (204)	Y	Y ^{††}	0.5	Y	+
17	IIIA	U	G/κ	43	2	6	IgG (939), κ (463)	Y	Y ^{††}	2.5	Y	—
18	IIIB	U	λ	10	89	3.5	λ (406)	Y	Y ^{††}	2.7	ND	—
19	IIIB	U	G/κ	5	70	3	IgG (258), κ (300)	Y	N	—	—	ND
20**	IIIA (PCL)	U	D/λ	70	40	6	IgD (ND), λ (—)	N	N	—	—	ND
21**	IIIB	U	G/λ	66	56	2	IgG (334), λ (158)	Y	Y ^{††}	1.5	Y	+
22	IIIA	U	G/κ	34	50	2.5	IgG (290), κ (455)	Y	Y ^{††}	2	Y	—
23	IIIA	R	G/κ	14	40	4.5	IgG (191), κ (297)	Y	N	—	—	ND
24	IIIB	U	G/κ	12	72	3.5	IgG (200), κ (244)	Y	Y ^{††}	2	Y	+
25	IIIB (PCL)	U	G/κ	23	63	4	IgG (481), κ (503)	Y	Y ^{††}	2	Y	—
26	IIIB	U	K	13	50	6	κ (390)	Y	N	—	—	ND
27	IIA	U	A/λ	3	10	6	IgA (—), λ (—)	N	N	—	—	ND
28	IIIB	R	λ	26	31	6	λ (229)	Y	N	—	—	ND
29	IIIA	U	A/κ	22	35	6	IgA (22), κ (41)	Y	N	—	—	ND
30	IIIA	U	A/λ	26	95	6	IgA (147), λ (168)	Y	N	—	—	ND

* Clinical stage at diagnosis according to the Durie-Salmon staging system.

† Total number of unsorted BMNCs inoculated.

‡ Percentage of myeloma cells (MCs) in inoculates as determined by forward, side scatters, and CD138-PE on flow cytometry, as well as by cytopsin smear evaluation.

§ The human immunoglobulins (hlg) were detected by ELISA, and the final levels were detected at the end of the experiments.

¶ Formation of any visible extramedullary macro-tumor (EMT) outside the fetal bone.

|| We tried to transplant the EMTs into SC of secondary and tertiary *NOD/SCID-hu*[−] mice.

** Equal amounts of BMNCs were inoculated into subcutaneous site (SC) of a *NOD/SCID-hu*[−] mouse as a control and none was engrafted.

†† Extramedullary disease with either soft-tissue or spinal plasmacytomas at the sampling of their BMNCs.

‡‡ The EMTs consisted of not only monoclonal plasmacytoid cells, but also clonal CD20⁺ immunoblast-like B cells.

Abbreviations: BM, bone marrow; BMNCs, bone marrow mononuclear cells; EBV, Epstein-Barr virus; EMT, extramedullary macro-tumor; M, months; MCs, myeloma cells; N, no; ND, not done; PCL, plasma cell leukemia; PCR, polymerase chain reaction; R, treated and refractory disease; U, before treatment; Y, yes.

sera were incubated for 60 minutes at 37°C and then washed. The bound hlg were then detected with goat anti-human Igs conjugated with horseradish peroxidase. After washing, substrate (TMB and peroxidase solution B) was added and color development was read at 450 nm. Standard curves were constructed with purified hlg (IgG, IgA, κ, λ) ranging from 500 to 7.8125 ng/ml. All reactions, including pooled normal mouse serum, were repeated at least three times, and mean values were obtained.

Cytospin Cytology and Immunostaining

BMNCs from the patients and any tumors recovered from the animal model were prepared and disaggregated into single-cell suspensions by means of a pipette, and then were washed and resuspended in PBS. These cells were cytopspun down onto slides (450 rpm, 4 minutes; Cytospin 3; Shandon, Pittsburgh, PA), which were air-dried and stained. Additional cytopsin smears were also prepared for immunostaining of the tumor cells. The antibodies

used were monoclonal mouse anti-human CD59 (hCD59), a pan-marker of human cells⁸ (PharMingen, Becton Dickinson Co., San Diego, CA), monoclonal mouse anti-human CD38 (hCD38), κ, λ (DAKO, Glostrup, Denmark), and monoclonal rat anti-mouse CD45R (mCD45), a common leukocyte antigen of the mouse⁸ (Immunotech, Marseille, France).

Histology, Histochemistry, and Immunohistochemistry

Tissues and organs recovered from the animal model were fixed in 10% neutral buffered formaldehyde followed by routine paraffin embedding and sectioning. The human FBs were fixed in 10% neutral buffered formaldehyde for at least 24 hours, decalcified with TBO-2 decalcifier (Shandon) for 2 hours, and embedded in paraffin. Sections measuring 4 to 5 μm were deparaffinized in xylene, rehydrated with ethanol, and rinsed in PBS. The

deparaffinized sections were stained with hematoxylin and eosin for accessing the morphology. To identify the osteoclasts, deparaffinized sections were stained for tartrate-resistant acid phosphatase.¹⁶ Briefly, the deparaffinized bone sections were immersed in fixative of 60% acetone in citrate at pH 5.0 for 30 seconds, and then were incubated with naphthol-AS-BI phosphoric acid, dimethylformamide (Sigma Chemical), acetate buffer, and tartaric acid at pH 5.3, 36°C for 90 minutes. Hematoxylin was used as a nuclear counterstain. Red granules in the cytoplasm indicate the tartrate-resistant acid phosphatase activity. An immunohistochemistry stain was performed on deparaffinized sections with an avidin-biotin complex detection system (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. The primary antibodies used for immunohistochemistry study were monoclonal mouse anti-human CD34 (hCD34), CD54 (ICAM-1), CD106 (VCAM-1), and monoclonal rat anti-mouse CD34 (mCD34) (PharMingen, Becton Dickinson Co.).

Flow Cytometry

BMNCs from the patients, MCs from the human FBs in *NOD/SCID* mice, and tumor cells from any visible macro-tumors that developed in the animal model were prepared and stained as previously described.¹⁷ Flow cytometry was analyzed in an EPICS XL-MCL flow cytometer (Beckman Coulter) as described previously.¹⁷ The antibodies used for flow cytometry were dual-colored phycoerythrin-conjugated CD19/fluorescein isothiocyanate-conjugated κ , phycoerythrin-conjugated CD19/fluorescein isothiocyanate-conjugated λ , fluorescein isothiocyanate-conjugated CD20 and CD28, and phycoerythrin-conjugated CD56 and CD138 (DAKO).

Cytogenetics, PCR, and Direct Sequencing

The cytogenetic analysis was performed as previously described.¹⁸ Genomic DNA was extracted from BMNCs, tumor tissues, or cells recovered from the animal model by use of the DNeasy Tissue Kit (Qiagen, Valencia, CA) according to instructions from the supplier. To amplify the complementarity-determining region III (CDRIII) of myeloma clones, the consensus primers to V_H and J_H were used as described previously.^{19–21} One μ g of genomic DNA was used in each PCR reaction, and the PCR mixture contained 5 μ l of 10 $\times$ PCR buffer (1 $\times$ is 10 mmol/L Tris-HCl, pH 8.8, at 25°C, 1.5 mmol/L MgCl₂, 50 mmol/L KCl, and 0.1% Triton X-100), 1 μ l of 10 mmol/L each deoxynucleoside triphosphate, 1 U of cloned *Taq*DNA polymerase (Promega Corp., Madison, WI), and 1 μ l of 100 pmol/L each primer at a final volume of 50 μ l. Cycling parameters were: initial denaturation at 95°C for 7 minutes for one cycle, followed by five cycles of 95°C for 30 seconds, 45°C for 30 seconds, and 75°C for 1 minute, which was followed by 30 cycles of 95°C for 30 seconds, 53°C for 1 minute, 75°C for 2 minutes; then a final extension at 75°C for 10 minutes. The final PCR products were electrophoresed in a 2% agarose gel and visualized with

ethidium bromide under a UV lamp. The human MC line, IM-9, from the American Tissue Type Culture Collection (Rockville, MD) with a J_H rearrangement¹⁹ served as a positive control, and the human lung cancer cell line, CL-1 (kindly provided by Dr. PC Yang, National Taiwan University, Taipei, Taiwan), and distilled water without template were used as negative controls. Amplified PCR (CDRIII) products were directly sequenced by the CEQ2000XL system with a CEQ Dye Terminator Cycle Sequencer Start Kit (DTLS, Protech Technology Enterprise, Co., Ltd., Taipei, Taiwan). For screening for Epstein-Barr virus (EBV), EBV-specific primers were used as previously described.²²

Results

The NOD/SCID-hu⁺ Model Has High Efficacy for Engraftment of Primary Human MCs and Presents Characteristic Features of Human MM

The BMNCs from all but two MM patients (93%) were successfully engrafted into the *NOD/SCID-hu⁺* mice, with typical clinical features of human MM, including osteolytic lesions of FBs with activation of multinucleated osteoclasts. Infiltration of MCs in marrow cavities of FBs (Figure 1; A to H) could be demonstrated in all of them. The infiltrated cells recovered from FBs had plasmacytoid morphology on cytospin smear (Figure 1G) and strong expression of the plasma cell markers CD38 and CD138, as well as the same light-chain restriction expression as that in the patients. Isotypic hlg of M-proteins of the MM patients were also detectable in mouse sera by ELISA (Figure 2 and Table 1). The two patients (nos. 20 and 27) whose MCs failed to grow in the model had IgD/ λ MM and osteoblastic MM with POEMS (collection of polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes) syndrome,¹ respectively. Thus, in this study, the highly successful rate of MM engrafting was not entirely attributable to an advanced clinical stage or refractory disease, or to the number of tumors inoculated.

We also injected the BMNCs from 8 of the 30 MM patients directly into an SC of *NOD/SCID* mice without preceding implantation of human FBs (*NOD/SCID-hu⁻*) as controls (Table 1). There was no evidence of growth of MCs in the mice up to the end of the experiments.

The Neovascularization in FBs Was of Human Origin; a Few Murine CD34⁺ Spindle Cells and Formed Tubular Structures Were Also Visible within FBs

Anti-human CD34 antibody (hCD34) and anti-mouse CD34 (mCD34) were used for determining the nature of the neovascularization in FBs of the *NOD/SCID-hu⁺* mice inoculated with primary human MCs. Most of the endothelial cells seen within the myeloma tissues were of human origin (Figure 1, I and J). Only very few spindle

Figure 1. Typical features of human MM are shown in the *NOD/SCID-hu*⁺ animal model. Gross picture of resorptive human FB, 12 weeks from inoculation of primary BMNCs of the MM patient (no. 19), is shown in **A**. A radiograph of the FB (**arrow**) taken at the same time is shown in **B**. The marrow cavity was heavily infiltrated by monotypic MCs (**E** and **F**, for patients 19 and 26, respectively). A cytospin smear of these plasma cells is illustrated in **G**. Evidence of osteolysis and formation of multinucleated osteoclasts (**arrows**) invaded the sequestered trabecular bone (patient 26) (presented in **H**). In all cases, the MCs stained positively for human plasma cell markers (CD38 and CD138). They expressed monotypic light chain on flow-cytometric analysis (not shown). It is important to note that a neovascularization of the FB in myeloma-bearing *NOD/SCID-hu*⁺ mice was of human origin. The vascular endothelium in FB was positively stained for human CD34 (**arrows**) (**I**), but only rarely for murine CD34 (**J**). For comparison, we included the gross picture (**C**) and radiograph of the FB (**D**), which was not inoculated with BMNCs, in a control, age-matched mouse. Original magnifications: $\times 400$ (**E**, **I**, **J**); $\times 1000$ (**F**, **G**, **H**).

Figure 2. Monotypic human immunoglobulins (Igs) were detected in mouse sera by ELISA. Note a marked increase in hIgs 12 weeks after MC inoculation.

cells and several near- or well-formed tubular structures (neovasculature ?), were positively stained for mCD34.

The Primary Human MCs Inoculated in the NOD/SCID-hu⁺ Model Not Only Infiltrated the FBs, but Also Frequently Developed Extramedullary Macrotumors (EMTs)

In addition to MCs infiltrating marrow cavities of the FBs, 17 (61%) of the 28 patients' BMNCs inoculated in the *NOD/SCID-hu⁺* mice developed EMTs along the periosteum of FBs (Figure 3; A to C) after a median of 2 months (range, 0.5 to 4.5 months) from inoculation of the BMNCs (Table 1). The neovasculature in those EMTs were dominantly of human origin, and similar to that in the marrow cavities of the FBs. Among these 17 patients whose BMNCs developed EMTs in the animal model, only 7 had clinical extramedullary disease at the time of sampling of their BMNCs, including the 3 with PCL and 4 with either soft-tissue or spinal plasmacytomas, and the remaining 10 patients had their disease limited to the BM (Table 1). Only 5 of the 17 patients were treated and had refractory disease; the remaining 12 were untreated before BM sampling (Table 1).

The tumor cells in the EMTs showed more immature cytomorphology than those in the marrow cavities of FBs (Figure 3, D and E), but they expressed the same surface antigens. In 11 (65%) of the 17 EMTs, there were also clonal (light chain-restricted) CD20⁺ immunoblast-like B cells (~12%) on flow cytometry, which were also readily demonstrated on tissue sections and cytospin smears. Concerning infection or reactivation of EBV in these EMTs, we screened for the EBV genome by PCR of the 17 EMTs and found positive results in only 3 of them (18%) (Table 1).

The EMTs Can Be Maintained in the Animal Model by Repeated Transplantation of Very Small Tumor Blocks into SC of NOD/SCID Mice without Human FBs (NOD/SCID-hu⁻)

Serial transplantation was performed with a small piece of tumor (~5 mm³) harvested from 9 of the 17 EMTs into SC

of the subsequent recipient *NOD/SCID-hu⁻* mice, and all but one grew successfully. With this approach, four EMTs (from patients 5, 15, 16, and 22) had been passed serially from one *NOD/SCID-hu⁻* mouse to another for more than three generations, and two of the EMTs (patients 5 and 15) had been maintained in the system for more than 12 months. The tumor cells recovered from the subsequent recipient mice preserved a plasmacytoid morphology, light-chain restriction, and the same surface markers as the original MCs in the patients. These tumor cells were negative for mCD45, and were positive for hCD59 and hCD38.

The Transplanted EMTs Preserved Original Cytogenetic Markers and Had Limited Clonal Evolution, as Shown by Changes in the Nucleotide Sequence of CDRIII

Compared with their parent BMNCs, the clonal cytogenetic aberrations of the successfully transplanted EMTs remained similar, if not identical, after repeated transplantations in the animal model (Table 2). However, on analysis of nucleotide sequence of the CDRIII of immunoglobulin heavy chains, several additional mutations, deletions, and insertions of the nucleotides occurred in the subsequently transplanted EMTs (Table 2).

The Neovascularization of the Transplanted EMTs in the SC of NOD/SCID-hu⁻ Mice Was a Chimera of Dominant Murine and Remnant Human Vessels

The origin of the neovascularization in the subcutaneously transplanted EMTs in the subsequent recipient *NOD/SCID-hu⁻* mice was predominantly from the murine host (mCD34⁺), quite different from that in the human FBs. However, several residual blood vessels reactive with the anti-human CD34, CD54 (ICAM), and CD106 (VCAM-1) were still visible.

The Plasmacytoid Cells in the EMTs Could Move into the Circulation of the Mouse and Develop Distant Metastatic Tumors

Although the subcutaneously transplanted EMTs (patients 5 and 15) grew to large sizes, several distant metastatic tumors far from the EMT at the transplanted sites could be observed (Figure 3, F to I). In addition, the presence of plasmacytoid cells reactive with hCD138 could be demonstrated by flow cytometry in PB and BM from recipient mice. PCR amplification of CDRIII of the cells from mouse PB and BM further confirmed the same origin of those plasmacytoid cells in mouse PB/BM and EMT.

Discussion

In this study, primary human MCs from 28 of the 30 MM patients engrafted successfully in the *NOD/SCID-hu⁺*

Figure 3. Primary MCs inoculated in *NOD/SCID-hu*⁺ mice not only infiltrated marrow cavities of FB, but also developed EMTs (arrow) along the periosteum (A). B and C: Radiographical pictures of the FB before inoculation of human MCs are shown on the left, and the nearly resorptive FB (arrows) and formation of EMTs are shown on the right (patients 5 and 6, respectively). It is noteworthy that the morphology of the MCs in EMT (D) was more immature than that in marrow of FB (E). Several mice had developed metastatic tumors. F: Gross view of a *NOD/SCID-hu*⁺ mouse with a metastatic tumor at the left axilla (Ax, arrow) and an enlarged EMT. In this case, the tumor cells from EMT (G and H) and metastatic tumor (I) lost their initial plasmacytoid features and became large cells or immunoblastoid cells. Original magnifications: $\times 400$ (D, E); $\times 1000$ (G, H, I).

Table 2. Cytogenetic Analysis and Sequence of Nucleotides of CDRIII of Four Primary MM Cells Studied in the *NOD/SCID-hu* Model

Patient number	Sample	Cytogenetics	Sequence of nucleotides of CDRIII of Ig heavy chain (5' → 3')*
5	P ⁰	†	CTCCTTGAGAGTGTCTAGAAACTATGC
	1 ⁰	†	CTCCTTGAGAGATGTCTAGAAACTATGC
	2 ⁰	†	CTCCTTGAGAGATGTCTAGAAAGC_ATGC
6	P ⁰	46,XY	CTCCTTGAGAGTGTCTAGAAACTATGC
	1 ⁰	46,XY	CTCCTTGAGAGTGTCTAGAAAGCAT_GC
15	P ⁰	‡	CTCCTTGAGAGTGTCTAGAAAGCATGC
	1 ⁰	‡	CTCCTTGAGAGTGTCTAGAAAGCATGC
	2 ⁰	‡	CTCCTTGAGAGTGTCTAGAAAGCATGC
16	P ⁰	46,XY	TCCTGAGAGTGTCTAGAAACTTGC
	1 ⁰	46,XY	TCCTGAGAGTGTCTAGAAACTTGC
	2 ⁰	46,XY	TCCTGAGAGTGTCTAGAAACTTGC

* The mutation of nucleotide between the relevant samples of the same patient was indicated in boldface, insertion bold and underlined, deletion underlined alone. V_H consensus sequence: 5'-AACCCACA-3'; J_H consensus sequences are shown and the primer binding sequences are underlined: 5'-AGGGAGGAGGAGCTGTGTTCCGATGACAGGGAAGATGAGGTTAAAGTTGTTAGAAAATGGTTCAAGGGATTCTCTGGGGCCCAAGGATCCCTGGTCA CCGA-3'.

† 46, XY, t(1)(q12q32), der(14)t(11;14)(q13;q32).

‡ 47~50, XY, +add(1)(p13), dic(1;17)(p13;p11), inv(2)(p25q31), -4, +5, +del(7)(p11p22), add(14)(q24), der(16)t(9;16)(q12;p11), +19, +21.

Abbreviations: CDRIII, complementarity-determining region III; P⁰, unsorted BMNCs of the patient; 1⁰, myeloma cells from human fetal bone in the *NOD/SCID-hu*⁺ mouse inoculated with BMNCs of the patient; 2⁰, the tumor cells from the EMTs in the secondary recipient *NOD/SCID-hu*⁻ mouse; Ig, immunoglobulin.

mice. The characteristic features of human MM could also be demonstrated in this model. It is noteworthy that in 17 (61%) of them, the BMNCs inoculated developed not only myeloma in marrow cavities of FBs, but also EMTs along the periosteum of resorptive FBs. Interestingly, very small tumor blocks from most EMTs could be further maintained in the new recipient *NOD/SCID* mice without human FBs.

This new model presents several advantages on study of the pathogenesis and treatment efficacy of MM. First, the SC localization enables early detection of tumor take, easy-to-follow tumor growth, and precise measurement of the tumor size in evaluating the potential effects of experimental treatments. Second, growth of EMTs, and any metastatic tumor(s), to a large size before mouse discomfort and sacrifice makes a large number of tumor cells available for immunotherapeutic studies and provides enough material for molecular-biology experiments, as well as exploring the mechanisms of malignant spread. Third, important genetic aberrations of the MM clones, if any, such as t(11;14) in one of our patients (no. 5), will be maintained in this model as an *in vivo* culture system, and will be helpful for further studies on the pathogenesis of these genetic insults.

Compared with the results from other studies,^{7,14} the high efficacy of our model in allowing engraftment of primary MCs was contributed by several factors: first, in parallel with the study performed by Yaccoby and colleagues,⁷ the human FB microenvironment was essential, at least initially, for engraftment of primary MCs. None of the mice without implantation of human FBs in our control group showed growth of primary human MCs. Furthermore, in our model, because no radiation was applied to the *NOD/SCID-hu*⁺ mice before inoculation of MCs, the human hematopoietic environment, especially the cellular components, within the FBs could be better preserved.⁸ Second, the extreme multiple immune defect *NOD/SCID* mouse has been shown to be an ideal animal for transplantation of normal and malignant human hema-

topoietic cells.^{12,14} It is also possible that such a murine environment and its related stromal cells/growth factors could, to some degree, take the place of the human environment for growth of human MCs, and perhaps other cells, including human endothelial cells. The neovascularization of human origin that developed in the FBs and EMTs is of importance. It may play some roles in the initial grafting of MMs, frequent EMT growth, and growth of MM after absorption of FBs. The presence of microvessels of human origin in engrafted MM and EMTs is of interest. MCs are proangiogenic by their ability to produce a variety of cytokines, including vascular endothelial growth factor and angiopoietin-1.²³⁻²⁵

There were no useful parameters for predicting the formation of EMTs after engraftment of the primary MCs in our study. All of the EMTs developed along the periosteum of resorptive FBs, and the EMTs and their transplanted progeny had a more primitive, undifferentiated morphology than did the primary MCs seen in the human FBs, despite sharing the same light chain-restricted expression and marker (eg, CD138) expression with the original MCs. Furthermore, MCs in the FBs, EMTs, and the subsequent transplanted tumors shared preserved cytogenetic markers, and had only limited changes in nucleotide sequence of CDRIII in subsequently transplanted MM clones. There is increasing evidence that the development and progression of myeloma involve a multistep transformation process, and the growth pattern of MCs is influenced by the genetic changes of MCs and the microenvironment.²⁶ The EMT plasmacytoid and/or large transformed cells may result from a self-cloning outgrowth from MCs with heterogeneity, selected or elicited by such SCID-hu chimeric microenvironment. On the other hand, additional mutations or genetic activation/dysregulation may also have biological effects on MC proliferation during tumor propagation in mice. Our model provides opportunities for further studies of the molecular mechanisms responsible for the progression and transformation in EMTs and their transplanted progeny.

It is known that EBV, when it was introduced along with human B cells simultaneously, can induce aggressive lymphoproliferative disorders of human B cells in SCID-hu chimeric mice, mostly large B-cell immunoblastic lymphomas with prominent plasmacytoid differentiation.²⁷ In our study, it was unlikely that the EMTs were triggered or selected by EBV infection, for its genome amplified by PCR was detected in only a minority (18%) of our EMTs.

It is known that the MM clone is morphologically heterogeneous and includes not only plasma cells, but also CD19⁺ B cells.²⁸ The MM-related CD19⁺ B cells were found to express interleukin-6, interleukin-6 receptor,²⁹ and P-glycoprotein,³⁰ which could participate in the disease process and drug resistance of MM, respectively. The persistent preswitch clonotypic MM B cells after transplantation also was correlated with decreased survival.³¹ However, the role of the clonally related B cells in MM is unclear, and whether the clonotypic B cells in MM were myelomagenic is still controversial in xenografted mice.^{32,33} In addition to the plasmacytoid cells, the expanded isotypic CD20⁺ immunoblastic B cells detected in some EMTs might have originated from the myeloma-associated preplasmacytic B cells; if so, it could provide an opportunity to clarify the relationship between myeloma plasma cells and preplasmacytic B cells.

In summary, we showed here a new animal model that is favorable and highly efficient for engraftment of primary human MCs and also provides a robust and reliable representation of characteristic features of human MM. Intriguingly, frequent development of EMTs along the periosteum of human FBs was noted. The EMTs could be passed from one *NOD/SCID-hu*⁻ mouse to another. The animal model is very useful for investigating the pathogenesis and to approach novel treatment of human MM.

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