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ORIGINAL ARTICLE

Establishment of an HS23 stromal cell-dependent myeloma cell line: fibronectin and IL-6 are critical

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Abstract A multiple myeloma (MM) cell line, MSG1, which depends on HS23 stromal cells for its survival, was established from the pleural effusion of a patient with MM who expressed the M-protein of IgA-λ in his serum. During the first 2 months of culture, the myeloma cells survived on adhesive cells from the pleural effusion and, subsequently, they continued to proliferate on HS23 stromal cells. The phenotype of the established MSG1 cell line was: $CD138^{+}$, $CD38^{++}$, $CD19^{-}$, $CD56^{-}$, VLA-4^{+}, $VEGFR1^{+}$ and $VEGFR2^{+}$. Immunohistochemical staining also demonstrated expression of the IgA and λ chain in MSG1 cytoplasm. Karyotype analysis indicated complex chromosomal abnormalities; hypertriploidy, including the deletion of chromosomes 13 and 17, and *c-myc* translocation. MSG1 cells continued to proliferate, not only when co-cultured with HS23 cells, but also when cultured only on fibronectin-coated plates with the supernatant of HS23 cells or with control medium containing IL-6. Tocilizumab, an anti-IL-6 receptor antibody, inhibited MSG1 survival under these conditions. Therefore, MSG1 may be a unique myeloma cell line that is useful for the study of cell adhesion-mediated drug resistance induced by adhesion molecules and IL-6 stimulation of myeloma cells.

Keywords Multiple myeloma · Stromal cell · VLA-4 · Fibronectin · IL-6

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1 Introduction

Adhesion of multiple myeloma (MM) cells to bone marrow stromal cells triggers cytokine-mediated tumor cell growth, survival, and drug resistance [1]. In particular, integrin $\alpha_4\beta_1$ (very late antigen 4, VLA-4)-mediated fibronectin adhesion confers a survival advantage to myeloma cells [2]. Furthermore, osteoclasts enhance myeloma cell growth and survival via cell–cell contact [3]. Therefore, knowledge of the bone marrow (BM) microenvironment surrounding myeloma cells is crucial for understanding the pathophysiology of MM and for devising a new strategy for the treatment of MM.

Even today, MM is an incurable disease. One of the problems in treating this disease is that it is very hard to eliminate residual myeloma cells, even following high-dose chemotherapy followed by auto-stem cell transplantation. Thus, cell adhesion-mediated drug resistance (CAM-DR) must be overcome in order to eliminate the minimal residual disease of MM. Here, we established a myeloma cell line, the survival of which depends on adhesion to HS23 stromal cells. We show that this cell line does not require the HS23 stromal cells themselves, but can survive by binding to fibronectin in the presence of the supernatant of these stromal cells, or to fibronectin plus IL-6 alone. We, therefore, suggest that this cell line will be a useful resource for the study of the relationship between myeloma cells and the microenvironment.

2 Materials and methods

2.1 Patient

A 67-year-old man was admitted to the hospital because of lumbago and orthopnea. Blood tests showed severe anemia (Hb 7.9 g/dl), and plasma cells were not detected in his peripheral blood. Chest X-rays showed bilateral pleural effusion, a compression fracture of a thoracic vertebra (Th12), and tumor formation on the 5th right rib. Laboratory tests indicated the presence of the IgA-λ type M-protein in the serum (IgA 1970 mg/dl). Pathological examination of a bone marrow (BM) aspirate showed that 32.5% of the cells of the BM were plasma cells and cytological examination of the pleural effusion showed atypical plasma cell proliferation (Fig. 1a). Based on these data the patient was diagnosed with symptomatic multiple myeloma, stage IIIA (Durie & salmon), ISS 3.

2.2 Phenotype analysis

Three-color flow cytometry was performed on cells from the pleural effusion using the CD38 Plasma Gating Method (Marrow plasma 38 (BML, Tokyo, Japan)). Mononuclear cells in the pleural effusion were isolated using the same method described previously for BM samples [4]. The isolated cells were stained using the following combinations of fluorescent-labeled antibodies: (1) FITC-CD38 (Pharmingen, San Diego, CA, USA), PerCP-CD45 (Becton–Dickinson, Franklin Lakes, NJ, USA), APC-CD19 (Immunotech, Marseille, France), and PE-CD56 (Pharmingen); (2) FITC-CD38, PE-MPC1 (JIMR, Tokyo, Japan), and PerCP-CD45; (3) FITC-CD38, PE-CD49e (VLA-5) (Pharmingen), and PerCP-CD45; (4) FITC-CD38, PE-CD138 (Immunotech), and PerCP-CD45; (5) FITC-CD38, PE-CD13 (Immunotech), and PerCP-CD45; and (6) FITC-CD38, PE-CD33 (Immunotech), and PerCP-CD45. Immunofluorescence of the labeled cell membrane was

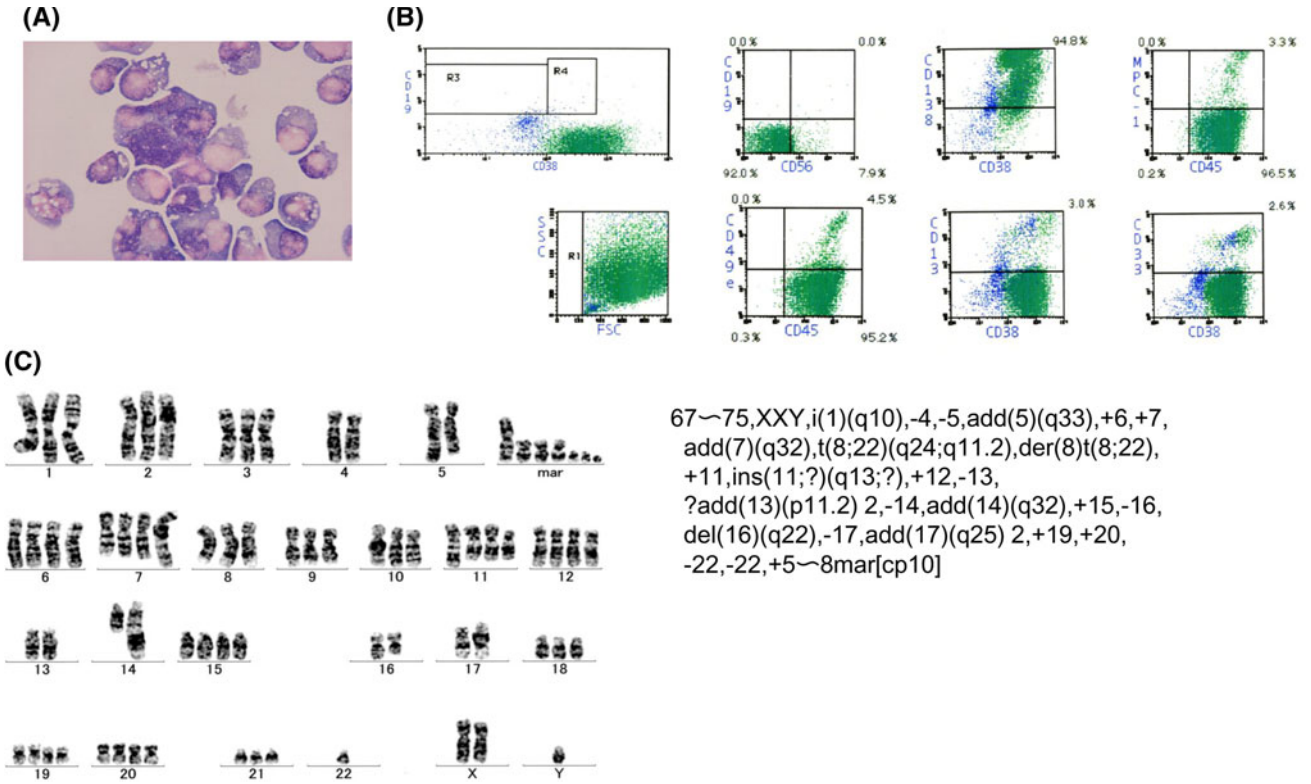

Fig. 1 Characteristics of myeloma cells in the pleural effusion of an MM patient. **a** Myeloma cells stained with May–Giemsa showed atypical morphology, a large nucleus and vacuoles in the cytoplasm. **b** Flow cytometric analysis of the cell surface antigens of the cell population with a $CD19^{-}/CD38^{++}$ phenotype revealed that the majority of plasma cells were positive for CD138 and CD45, and negative for CD56, MPC-1, CD49e, CD13, and CD33. **c** Karyotype analysis and summary of chromosomal analysis by G-banding. Analysis of a total of 10 cells showed complex chromosomal abnormalities; hypertriploidy including deletion of chromosomes 13 and 17, and t(8;22)(q24;q11.2), which shows a reciprocal translocation between *c-myc* and the *immunoglobulin-light chain gene* (λ chain), are indicated

evaluated using a FACS Calibur flow cytometer (Becton–Dickinson, Franklin Lakes, NJ, USA).

The cell surface expression of the vascular endothelial growth factor receptors (VEGFR1 and VEGER2) on the membrane of the established myeloma cell line (MSG1) and on the HS23 and HS27A stromal cells, and the expression of VLA-4 on MSG1 was also evaluated using flow cytometry after staining with anti-human VEGF R1 (Flt-1), anti-human VEGF R2/KDR-Phycoerythrin (R&D System, Inc., Minneapolis, MN, USA), and FITC-CD49d (Immunotech) antibody, respectively.

2.3 Immunohistochemistry (IHC)

VCAM-1 staining of the paraffin-embedded tissue of HS23 and HS27A tissue was carried out using an anti-VCAM-1 antibody (Santa Cruz biotechnology, Inc., Santa Cruz, CA, USA), and the Ultra Tech HRP streptavidin–biotin detection system (Immunotech) according to the manufacturer's instructions. First, flask-cultured HS23 and HS27A cells were scraped, washed twice with phosphate-buffered saline (PBS), and were subsequently fixed in 5% formaldehyde. The fixed cells were placed in Tris-Buffer and were microwaved for 20 min before VCAM-1 staining. For fibronectin staining, fresh HS23 and HS27A cells were cultured on a Lab-Tek chamber slide (Nalge Nunc International, Rochester, NY, USA) and were fixed using acetone after rinsing with PBS. These cells were stained with an anti-fibronectin antibody (Calbiochem, Darmstadt, Germany) and staining was detected using the Ultra Tech HRP streptavidin–biotin detection system.

2.4 Chromosome (G-banding) and fluorescence in situ hybridization (FISH) analyses

Chromosome and FISH analyses of myeloma cells were performed at Mitsubishi Chemical Medicine Inc. (Tokyo, Japan) and the Center for Molecular Biology and Cytogenetics, SRL, Inc. (Tokyo, Japan), respectively. The target for FISH analysis was a cell with a round nucleus. The Vysis LSI IGH/CCND1 dual color (Abbott Molecular Inc., Des Plaines, IL, USA), dual fusion DNA probe, which hybridizes to chromosome 14q32.3 (IgH SpectrumGreen) and chromosome 11q13 (CCND1 SpectrumOrange), was used to detect the translocation of t(11;14)(q13;q32.3). Similarly, the Vysis LSI IGH/MAF and LSI IGH/FGFR3 dual color, dual fusion DNA probes were used to detect the translocation of t(14;16)(14q32;16q23) and t(4;14)(4p16;14q32), respectively. The Vysis LSI S13S319 and the Vysis LSI TP53 probes were used to detect deletion of chromosomes 13q14.3 and 17p13.1, respectively. The Vysis LSI MYC dual color, break-apart rearrangement probe was used to detect the translocation of *myc*.

2.5 Cell culture

The aspirated pleural effusion was mixed with an equal volume of Roswell Park Memorial Institute (RPMI)-1640 medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS: Invitrogen, Carlsbad, CA, USA), and was cultured in a 75-cm^2 tissue culture flask (Iwaki, Chiba, Japan) After suspension of the myeloma cells that were in contact with adherent cells on the flask using a pipette, the myeloma cells were transferred to a 12-well cell culture plate (Corning, New York, USA) containing a confluent layer of HS23 stromal cells (HS23: a gift from Dr. Beverly Torok-Storb, The Fred Hutchinson Cancer Research Center, Seattle, WA, USA) [5] and were cultured in RPMI-1640 medium supplemented with 10% FBS (control medium). After confirming the proliferation of myeloma cells on HS23, the myeloma cells were cultured in a 12-well cell culture plate under one of the following conditions: (1) control medium, (2) control medium containing IL-6 (10 ng/ml), (3) control medium containing a confluent layer of HS27A stromal cells (HS27A: purchased from the American Type Culture Collection (ATCC), Manassas, VA, USA), or (4) control medium containing a confluent layer of HS23 cells that were separated from myeloma cells by a 0.4 μm mesh (Greiner Bio-One, Frickenhausen, Germany). For each condition, half of the medium was replaced every 3 days.

2.6 Cell culture on HS23 or HS27A cells with HS23 or HS27A supernatants

Myeloma cells (1×10^5/ml) were cultured on a confluent layer of HS23 or HS27A cells together with the conditioned medium (supernatant) of HS23 or HS27A cells. These supernatants were replaced every 3 days with centrifuged conditioned media that was newly obtained from HS23 or HS27A cells that had been cultured for 3 days in control medium.

2.7 Cell culture on fibronectin with HS23 or HS27A supernatants

Tissue culture 12-well plates (Corning) were coated with human fibronectin (5 μg/cm^2: (Becton–Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Subsequently, myeloma cells (1×10^5/ml) were cultured on the coated fibronectin together with HS23 or HS27A supernatants. These supernatants were replaced as described above.

2.8 Cell culture on fibronectin with control medium with or without IL-6

Myeloma cells (1×10^5/ml) were cultured on the coated fibronectin as described above together with control medium with or without IL-6 (10 ng/ml). These supernatants were replaced every 3 days.

2.9 Assay of cell viability

Cell viability was checked using the trypan blue staining method.

2.10 Assay of VEGF and IL-6 concentrations in the supernatant

The concentration of VEGF, and IL-6 in the supernatant of HS23 and HS27A cells cultured in FBS-free medium for 3 days was measured using the Quantikine Human VEGF Immunoassay (R&D System, Inc., Minneapolis, MN, USA) and the Human IL-6 CLEIA Fujirebio (Fujirebio, Tokyo, Japan), respectively.

2.11 Virus detection

DNA was extracted from myeloma cells using a standard method [6]. Epstein–Barr virus (EBV) infection was determined using the polymerase chain reaction (PCR) with primers that directly flank the portion of the EBV lymphocyte-determined membrane antigen (LYDMA) gene that is composed of variable numbers of tandem 33 bp repeats [7]. Human herpesvirus 8 (HHV-8) infection was also determined using PCR [8].

2.12 Evaluation of the effect of bevacizumab on myeloma cells cultured with HS23

Myeloma cells (1×10^5/ml) were cultured on HS23 in tissue culture 12-well plates (Corning) with 1 ml of control medium containing 0, 100, 300, or 500 μg/ml bevacizumab (a gift from Chugai Pharmaceutical Co., LTD., Tokyo, Japan). These media were replaced every 3 days.

2.13 Evaluation of the effect of tocilizumab on myeloma cells cultured on coated fibronectin with control medium containing IL-6 or cultured with HS23

Myeloma cells (1×10^5/ml) were cultured on fibronectin as described above together with 1 ml of control medium containing IL-6 (10 ng/ml) and 0, 10, or 50 μg/ml tocilizumab (a gift from Chugai Pharmaceutical Co., LTD., Tokyo, Japan), or on HS23 in tissue culture 12-well plates (Corning) with 1 ml of control medium containing tocilizumab (0, 10, or 50 μg/ml). These media were replaced every 3 days.

3 Results

3.1 Characteristics of myeloma cells

Clinical observation suggested that the myeloma cells isolated from the patient would have a high capacity for proliferation because the level of the patient's pleural effusion was restored to the original level in a few days after drainage. The phenotype of the majority of the myeloma cells that showed atypical morphology was $CD138^+$, $CD45^+$, $CD38^{++}$, $CD19^-$, $CD56^-$, MPC-1^-, and $CD49e^-$ based on flow cytometric analysis (Fig. 1b), which is consistent with a previously reported immature myeloma cell phenotype [9]. Furthermore, chromosomal analysis using G-banding indicated that three of twenty myeloma cells analyzed had the translocation t(8;22)(q24;q11.2), which involves a reciprocal translocation between the *c-myc* and the *immunoglobulin-light chain* λ genes. Additionally, FISH analysis revealed the presence of myeloma cells with a deletion of chromosome 13q or 17p13 (data not shown).

3.2 Establishment of the HS23 stromal cell-dependent myeloma cell line, MSG1

To establish a myeloma cell line from these myeloma cells, we first cultured the pleural effusion as described in "Materials and methods". Over the first 2 months of culture, the number of myeloma cells decreased and the adhesive cells in the culture detached from the flask. In order to maintain the myeloma cells in culture, we determined whether the myeloma cells could survive by plating them on other adherent cell types. For this purpose, we transferred the detached myeloma cells into a 12-well cell culture plate containing a confluent layer of HS23 and then co-cultured these cells in RPMI-1640 medium supplemented with 10% FBS (control medium). Under these conditions the myeloma cells continued to proliferate for more than 1 year and we named this myeloma cell line, "MSG1". Chromosomal analysis using G-banding indicated complex chromosomal abnormalities, basically hypertriploidy including the deletion of chromosomes 13 and 17, and *c-myc* translocation (Fig. 1c). We confirmed the expression of the immunoglobulin-heavy chain α (IgA) and the light chain λ using IHC and the translocation of *myc* by FISH (data not shown). PCR analysis indicated that MSG1 cells were found to be negative for EBV and HHV-8 (data not shown).

Next, we determined what factors were critical for MSG1 survival in this co-culture. MSG1 was therefore cultured in the absence of HS23 in 12-well cell culture plates in control medium containing IL-6 (10 ng/ml) or plates containing a confluent layer of HS23 in which the medium and the cells were separated by a 0.4-μm mesh which prevents cell–cell contact, but allows the penetration of factors, such as growth factors. We also tested MSG1 survival on a confluent layer of a second stromal cell line HS27A (established by Dr. Beverly Torok-Storb) [5] in the presence of control medium (Fig. 2). MSG1 could not survive under any of these conditions, suggesting that the cells required both contact with stromal cells and some factors in the supernatant of the HS23 cultures. Since we confirmed that detached MSG1 cells were not viable using trypan blue staining assay and suspending MSG1 for analysis of viability decreased the number of MSG1 cells in our preliminary experiments, we did not perform the analysis using trypan blue staining in each condition continuously. Therefore, in order to confirm the MSG1 survival, we directly photographed MSG1 cells that were adhered to, and proliferating on, stromal cells. Interestingly, MSG1 could only survive on HS27A cells if these cells were first irradiated (20 Gy; data not shown), suggesting that HS27A may produce a component that is toxic for MSG1 cells. The combined data suggest that, in order for MSG1 cells to survive, the cells required both contact with stromal cells and some factor in the supernatant of the HS23 cultures.

3.3 Expression of adhesion molecules on MSG1 and stromal cells

We first focused on analysis of the cell contact requirement of MSG1 cells for survival. It has been reported that adhesion of myeloma cells to BM stromal cells is crucial for the survival of myeloma cells in a BM microenvironment [1]. In particular, adhesion between VLA-4 on myeloma cells and its ligands, VCAM-1 or fibronectin, on stromal cells activates proliferation signals in myeloma cells. We confirmed that MSG1 expresses VLA-4 using flow cytometry, and confirmed the previous report that both HS23 and HS27A stromal cells expressed VCAM-1 and fibronectin [5] by using immunohistochemistry (Fig. 3). Therefore, these data suggest that MSG1 may bind to these stromal cells through VLA-4 binding to VCAM-1 and fibronectin.

3.4 HS23 stromal cells maintain the survival of MSG1

Although both the HS27A and the HS23 stromal cells expressed VCAM-1 and fibronectin, MSG1 could not survive in co-culture with HS27A, as shown in Fig. 2, unless HS27A cells were irradiated (20 Gy) (data not shown). However, since it was difficult to maintain HS27A after irradiation, we did not perform further studies using irradiated HS27A. However, the fact that MSG1 could not survive in co-culture with HS27A suggested the possibility that the supernatant of HS27A might be toxic for MSG1.

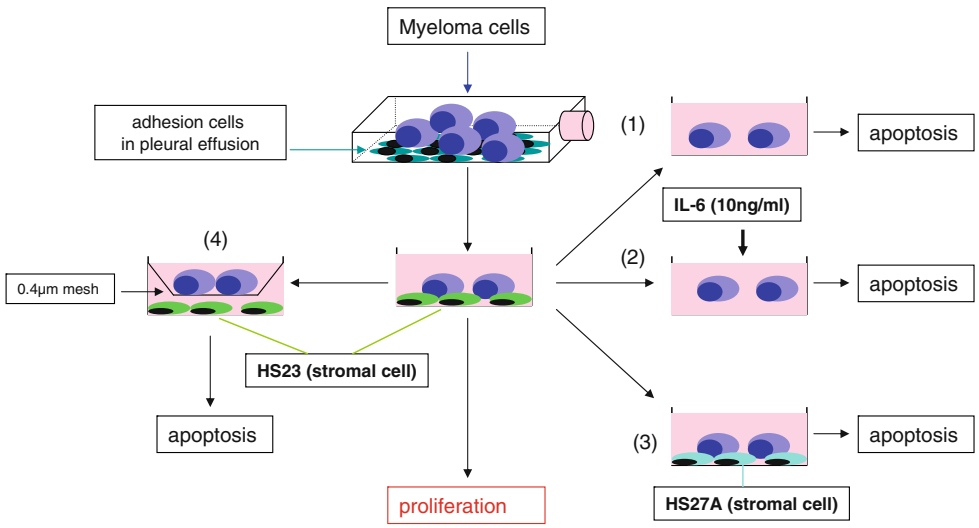

Fig. 2 Establishment of the HS23 stromal cell-dependent myeloma cell line, MSG1. The myeloma cells were first grown on the adhesive cells in the pleural effusion. However, they did not proliferate in this culture for longer than 2 months. Detached myeloma cells were transferred to 12-well cell culture plates containing a confluent layer of HS23 stromal cells (HS23) and were cultured in control medium. After confirming that MSG1 proliferated on HS23, myeloma cells were cultured in 12-well cell culture plates under the following conditions: (1) control medium, (2) control medium containing IL-6 (10 ng/ml), (3) control medium containing a confluent layer of HS27A stromal cells (HS27A), and (4) control medium containing a confluent layer of HS23 separated by a 0.4 μm mesh. None of these conditions could maintain myeloma cells survival. All experiments were performed in triplicate

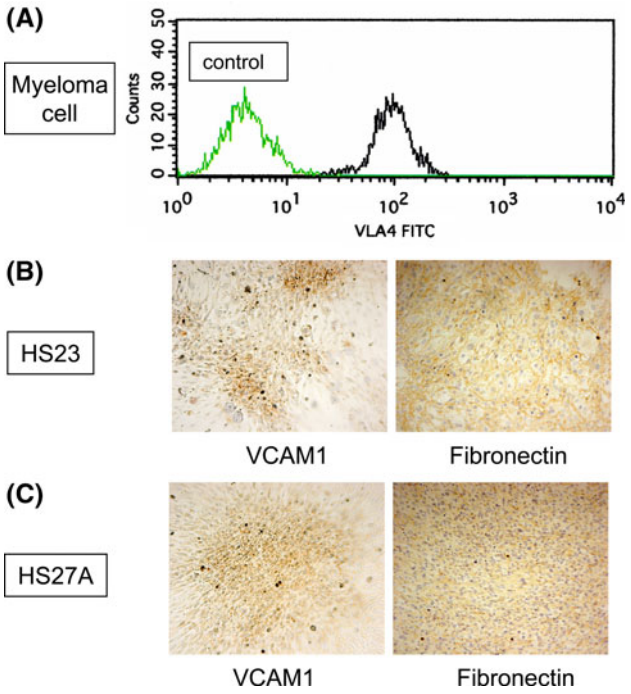

Fig. 3 Expression of adhesion molecules on MSG1 and stromal cells. **a** Flow cytometric analysis of VLA-4 expression on MSG1 cells using FITC-anti-CD49d. Immunoperoxidase staining of the expression of VCAM-1 and fibronectin by HS23 (**b**) and HS27A (**c**) stromal cells

To confirm this possibility, we co-cultured MSG1 on HS23 or HS27A cells in the presence of the conditioned medium (supernatant) of HS23 or HS27A. MSG1 co-cultured with HS23 and the supernatant of HS23, was used as a positive control (Fig. 4a) and MSG1 co-cultured with HS27A and the supernatant of HS27A was used as a negative control (Fig. 4d). MSG1 cells cultured under these conditioned were then photographed to assay their survival. These photographs indicated that, although MSG1 could not survive when co-cultured with HS23 and the supernatant of HS27A (Fig. 4b), MSG1 could survive more than 26 days when co-cultured with HS27A and the supernatant of HS23 (Fig. 4c). These data provided further evidence that the factors that are critical for MSG1 survival may be the binding of MSG1 to stromal cells and factors present in the supernatant of HS23.

3.5 Contact via fibronectin and the supernatant of HS23 maintain the survival of MSG1

Since MSG1 could survive even on HS27A cells if the conditioned medium of HS23 cells was supplied, we assumed that the signals induced by the binding between VLA-4 on MSG1 and its ligands, present both on the stromal cell surface and in the supernatant of HS23 would be critical for MSG1 survival. To test this hypothesis, we cultured MSG1 on tissue culture plates that were coated with fibronectin in the presence of the conditioned supernatant of HS23 or HS27A. As expected based on the above results, MSG1 could not survive when cultured in the control medium alone, or when cultured on plates coated with fibronectin in the presence of the control medium or of the HS27A supernatant. In contrast, MSG1 continued to proliferate for longer than 24 days when cultured on plates coated with fibronectin in the presence of the HS23 supernatant (Fig. 5). Therefore, the critical factors for MSG1 survival may be contact with fibronectin and factors present in the supernatant of HS23.

3.6 Expression of VEGF receptors (VEGFR1 and VEGFR2) in MSG1

We next focused on the analysis of the factors present in the conditioned medium of HS23 cells that are important for MSG1 survival. Based on the analysis of the VEGF concentration of the supernatant of HS23, HS27A, and irradiated HS27A cells (131 ± 5.8, 1666 ± 41, and 2086 ± 715 pg/ml, respectively), we considered that VEGF might be critical for MSG1 survival. In support of this possibility, we also found that the myeloma cells of a third of MM patients weakly expressed VEGFR1 and VEGFR2 (data not shown). The expression of VEGFR1 and VEGFR2 on MSG1, HS23m, and HS27A cells was therefore analyzed using flow cytometry and VEGFR-specific antibodies. MSG1 expressed both VEGFR1 and VEGFR2, while HS23 did not express either of the VEGFRs, and HS27A only weakly expressed VEGFR2 (Fig. 6). Subsequently, we analyzed the effect of 100, 300, or 500 μg/ml bevacizumab, which inhibits the interaction of VEGF with its receptors on MSG1 cell survival. However, there was no significant difference in MSG1 survival between control cells cultured in the absence of bevacizumab, and cells cultured with any concentration of bevacizumab (data not shown). Therefore, even though the myeloma cells express VEGF receptors, VEGF may not be the most critical, or may not be the sole factor, required for myeloma cell survival.

3.7 Contact via fibronectin and IL-6 maintain the survival of MSG1

We next determined if IL-6 may be a component of the HS23 supernatant that is required for MSG1 cell survival. Stromal cells are known to produce IL-6, which is a crucial factor for proliferation of myeloma cells. MSG1 could not survive when cultured in the control medium containing IL-6 (10 ng/ml) (Fig. 2), or when cultured on plates coated with fibronectin in the presence of the control medium alone (Fig. 5). We, therefore determined if the addition of control medium containing IL-6 (10 ng/ml) could

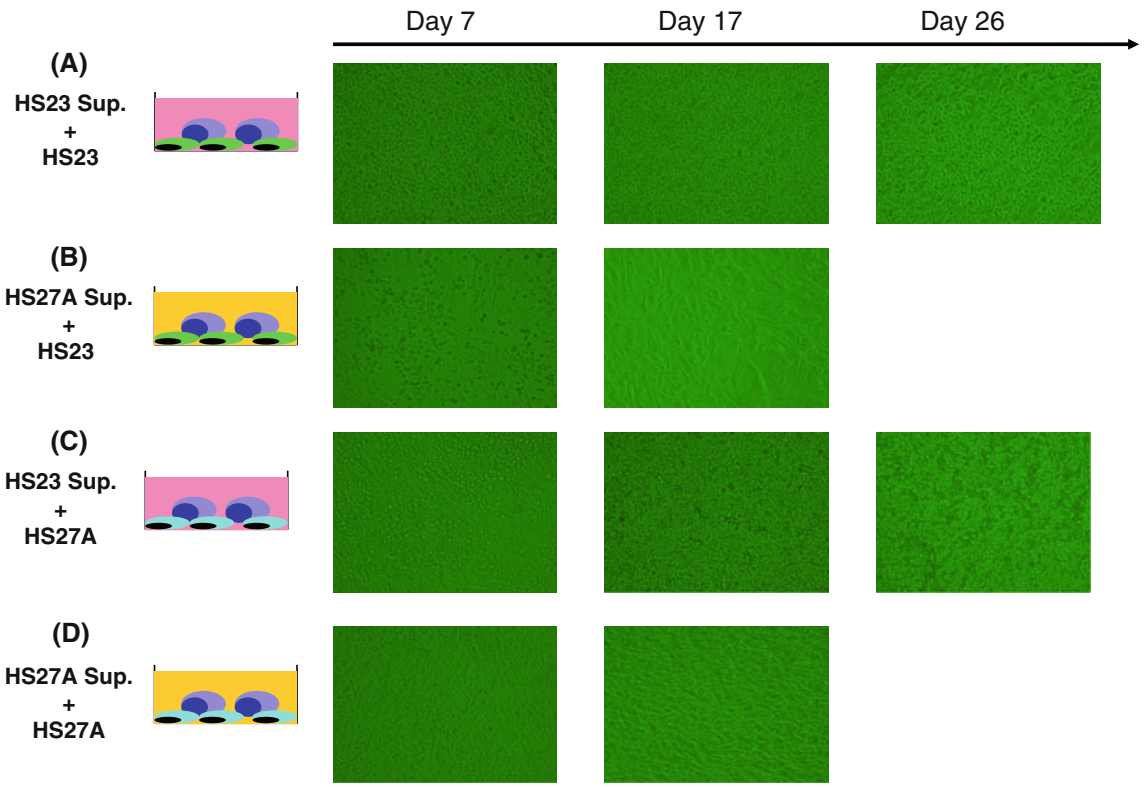

Fig. 4 Culture of MSG1 on HS23 or HS27A stromal cells with the conditioned media of HS23 or HS27A. **a** MSG1 cells were cultured on HS23 cells with the conditioned media of HS23 (**a**) or HS27A (**b**), or were cultured on HS27A cells with the conditioned media of HS23 (**c**) or HS27A (**d**), following which cell proliferation was assayed for up to 26 days. MSG1 cultured on HS23 or 27A cells in the presence of HS23 conditioned media continued to proliferate for up to 26 days (**a**, **c**). MSG1 cultured on HS23 or 27A cells in the presence of HS27A conditioned media survived for only 17 days (**b**, **d**). All experiments were performed in triplicate

modulate survival of MSG1 cultured on fibronectin-coated tissue culture plates. MSG1 continued to proliferate for longer than 13 days under these conditions (Fig. 7). Therefore, the critical factors for MSG1 survival were proven to be contact with fibronectin and IL-6, and control medium containing IL-6 (10 ng/ml) could substitute for HS23 supernatant.

3.8 Inhibition of MSG1 survival by tocilizumab

If IL-6 is a critical factor for MSG1 survival, then tocilizumab, an anti-IL-6 receptor antibody, would be expected to inhibit MSG1 survival when cultured on coated fibronectin with control medium containing IL-6, or when co-cultured with HS23. Indeed, the addition of tocilizumab (0, 10, or 50 μg/ml) under these culture conditions, inhibited MSG1 survival in a dose dependent manner. In particular, MSG1 survival for 13 days was strongly inhibited by 50 μg/ml of tocilizumab under either culture condition (Figs 8, 9, respectively). Combined with the result of Fig. 7, these data indicate that the growth factor present in the HS23 supernatant that is critical for the maintenance of MSG1 survival is IL-6.

4 Discussion

We established an HS23 stromal cell-dependent myeloma cell line, MSG1, from the pleural effusion of a patient with MM. At the time of diagnosis the myeloma cells showed the chromosomal abnormalities including del 13q, del 17p (p53), and translocation of *c-myc*. The deletion of *p53* and the *c-myc* abnormality are familial features of advanced myeloma [10]. The underlying chromosomal abnormality of MSG1 may be hypertriploidy, and the *c-myc* translocation and the deletion of chromosome 17 may induce clonal expansion of myeloma cells. The percentage of plasma cells in the BM of the patient was 32%, and the clinical aspect was not consistent with plasma cell leukemia. However, the bilateral pleural effusion did not decrease in spite of drainage and the administration of high-dose dexamethasone. These findings suggest that the pleural cavity might be a favorable environment for myeloma cell survival.

Our study showed that a cell line could be established from these myeloma cells if the cells were co-cultured with HS23, but not with HS27A stromal cells. We also showed that MSG1 cell-survival depended on interaction with

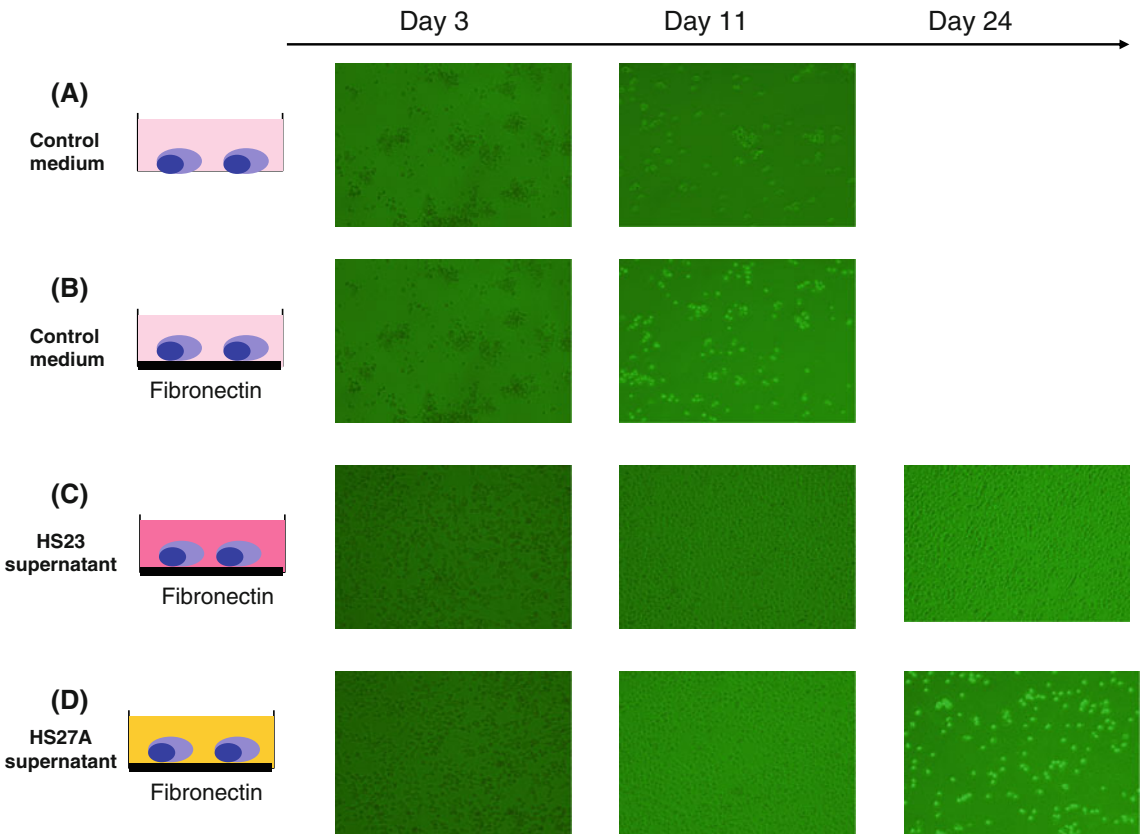

Fig. 5 Culture of MSG1 on the coated fibronectin plates with HS23 or HS27A supernatant. **a**, **b** MSG1 cultured in control medium, on plates with or without coated fibronectin, could not survive until Day 24. **c** MSG1 cultured on coated fibronectin plates with HS23 supernatant could continue to proliferate for at least 24 days. **d** The number of MSG1 cultured on coated fibronectin plates with HS27A supernatant was remarkably decreased on day 24. All experiments were performed in triplicate

fibronectin and on a HS23-produced soluble factor that we identified as IL-6. Since MSG1 could survive on irradiated HS27A cells, and since HS23 and HS27A express similar adhesion molecules [5], these data suggest that HS27A might secrete factors that are inhibitory to MSG1 survival. Identification of such an inhibitory factors may be of interest in terms of the regulation of myeloma proliferation. The HS27A inhibitory factor does not appear to be IFN-γ, which is a well-known inhibitor of myeloma cell growth, since IFN-γ concentration was not significantly different between the supernatant of HS23, HS27A, and irradiated HS27A (2.7, 3.6, and 3.3 IU/ml, respectively). In preliminary experiments, we also tested the effect of different concentrations of the HS27A supernatant (100, 50, 2, and 10%) on the proliferation of myeloma U266, RPMI8226, KMS12BM, or lymphoma (Raji, Jurkat, MT1) cell lines. For this purpose, HS27A culture supernatant was concentrated using a centrifugal filter (Ultracell YM-30, Millipore, Billerica, MA, USA) and was mixed with culture medium supplemented with 10% FBS at different ratios. However, even the concentrated HS27A supernatant (100%) could not suppress the cell growth of Jurkat or MT1, whereas a 50% concentration of the HS27A supernatant could inhibit other cell lines (data not shown). Therefore, the inhibitory effect of the HS27A supernatant may be cell type-dependent.

The adhesion of myeloma cells to stromal cells provides a good microenvironment for myeloma cells because this adhesion induces the proliferation of myeloma cells. Furthermore, stromal cells produce cytokines, such as IL-6 and VEGF [11], which also promote the proliferation of myeloma cells [12] as well as increase angiogenesis in the MM bone marrow [13]. The paracrine role of VEGF in MM was first proposed by Danker et al. [14]. Thus, myeloma cells produce VEGF and stromal cells express the VEGFR. VEGF stimulation induces IL-6 secretion from stromal cells, and IL-6 subsequently promotes myeloma cell growth. Interestingly, we found that MSG1 expresses both VEGFR1 and VEGFR2, HS23 did not express these receptors and HS27A only weakly expressed VEGFR2 (Fig. 6). If VEGF secreted by stromal cells binds to, and modulates MSG1 survival, then it might be expected that VEGF concentration would be higher in HS23 than in HS27A supernatants. However, this was not the case since

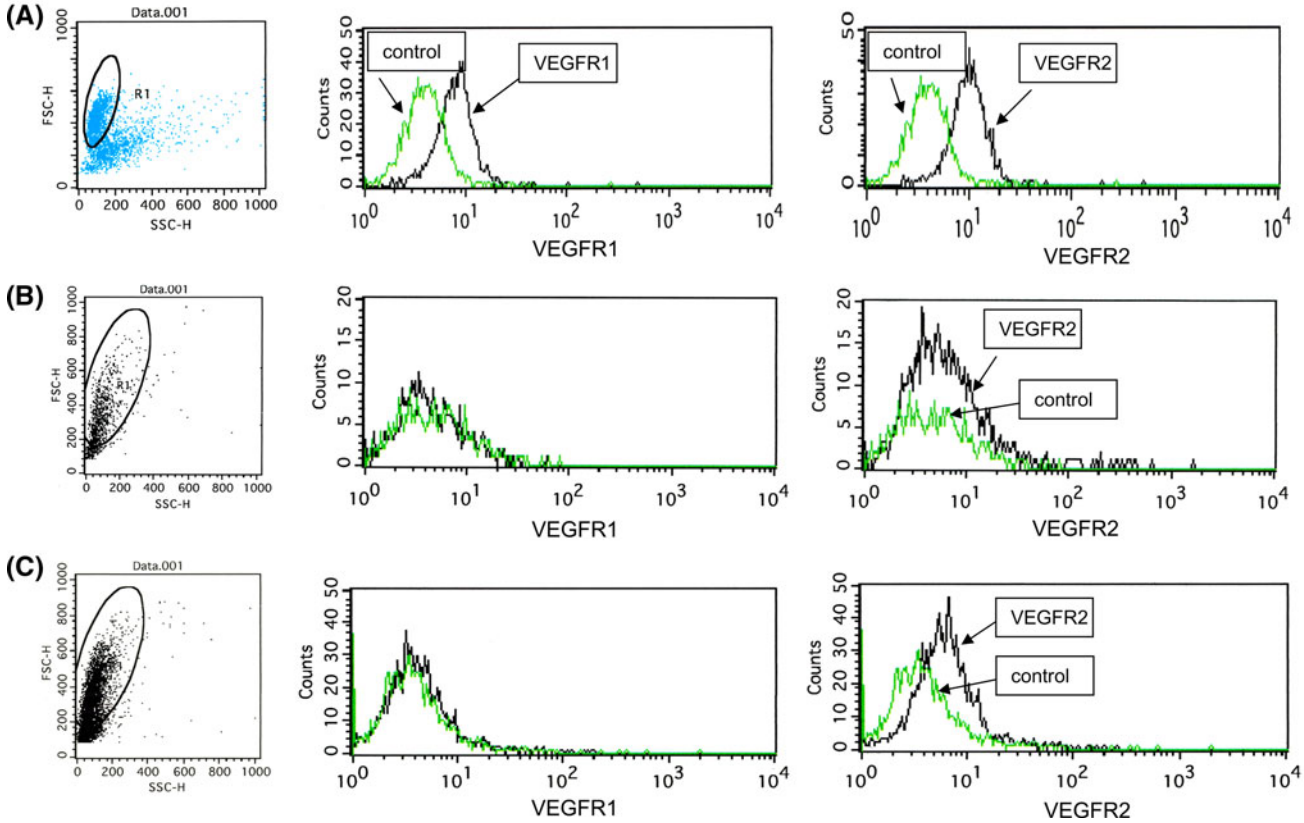

Fig. 6 Expression of VEGF receptors (VEGFR1 and VEGFR2) on MSG1, HS23 and HS27A cells. The expression of VEGFR1 and VEGFR2 on MSG1 (**a**), HS23 (**b**) and HS27A (**c**) cells was analyzed using flow cytometry and VEGFR-specific antibodies. MSG1 expressed both VEGFR1 and VEGFR2, while HS23 did not express either VEGFR, and HS27A only weakly expressed VEGFR2

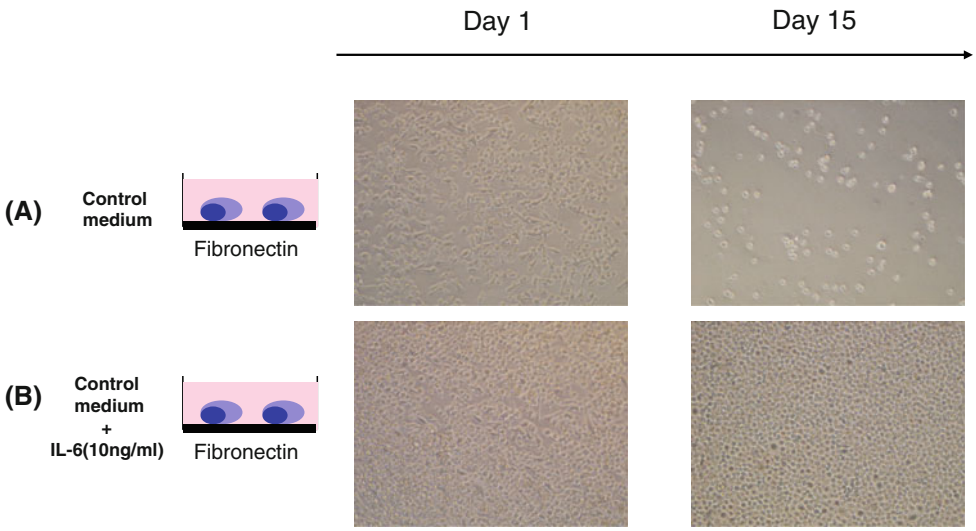

Fig. 7 Culture of MSG1 on coated fibronectin plates with control medium with or without IL-6. **a** MSG1, cultured in control medium without IL-6 could not survive even on coated fibronectin plates as shown in Fig. 5. **b** MSG1 cultured on coated fibronectin plates with control medium containing IL-6 (10 ng/ml) could continue to proliferate for at least 15 days. All experiments were performed in triplicate

the level of VEGF in the HS27A supernatant was about ten times higher than that of the HS23 supernatant (1666 ± 418 pg/ml and 131 ± 5.8 pg/ml, respectively). The lack of effect of the anti-VEGF antibody bevacizumab, on MSG1 survival (data not shown) further suggested that the soluble factor in HS23 conditioned media that was required for MSG1 survival was not VEGF. However, before completely eliminating a contribution of VEGF to MSG1 cells survival, it may be necessary to more completely inhibit VEGF signaling by analyzing the effect of the recently reported anti-VEGF signaling drug sorafenib [15]. In contrast to the lack of effect of VEGF on MSG1 cells, we

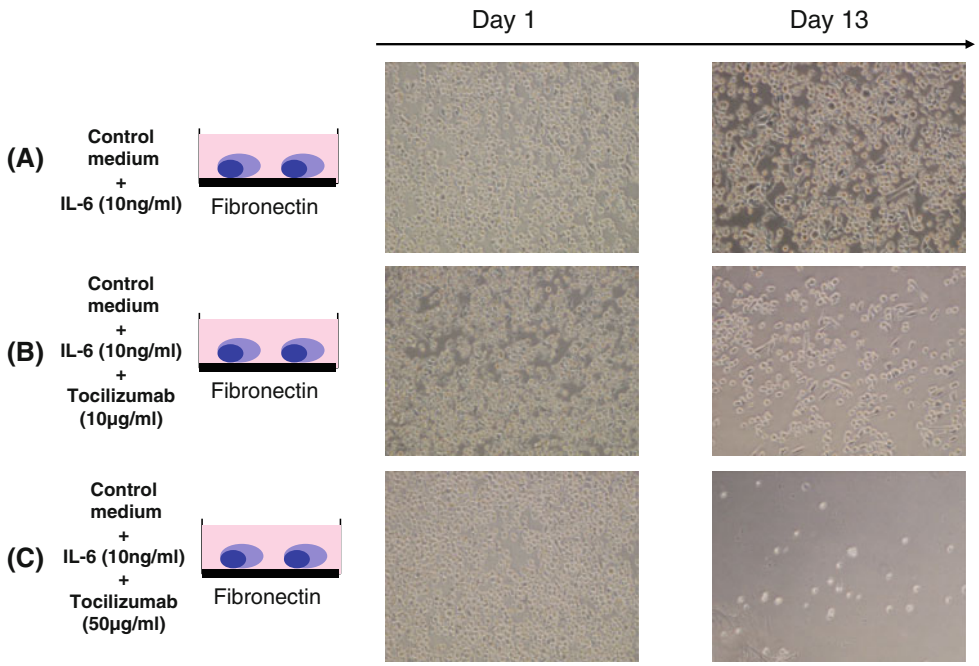

Fig. 8 Effect of tocilizumab on MSG1 survival on coated fibronectin plates with control medium containing IL-6. **a** MSG1 cultured on coated fibronectin plates with control medium containing IL-6 (10 ng/ml) could continue to proliferate for at least 13 days; **b**, **c** Addition of tocilizumab to the culture medium inhibited MSG1 survival in a dose dependent manner (**b** 10 μg/ml; **c** 50 μg/ml). All experiments were performed in triplicate

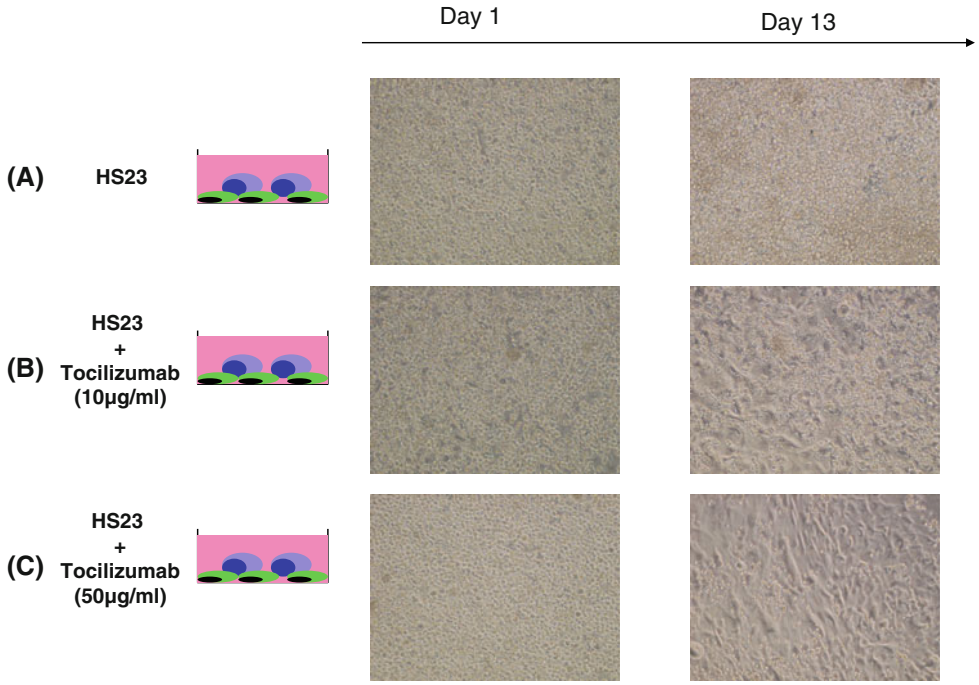

Fig. 9 Effect of tocilizumab on the survival of MSG1 on HS23 stromal cells. **a** Control culture of MSG1 on HS23 stromal cells. **b**, **c** Addition of tocilizumab to the culture medium inhibited MSG1 survival in a dose dependent manner (**b** 10 μg/ml; **c** 50 μg/ml). All experiments were performed in triplicate

found that IL-6 could substitute for HS23 conditioned media in the maintenance of MSG1 survival when cultured on fibronectin-coated plates. This result was consistent with the fact that the level of IL-6 was about three times higher in the HS23 supernatant than in the HS27A supernatant (36.8 ± 4.5, 13.2 ± 1.9 pg/ml, respectively). These data are also in agreement with the ability of tocilizumab, an anti-IL-6 receptor antibody, to inhibit MSG1 survival. This inhibitor has previously been reported to effectively inhibit IL-6 receptor signaling of IL-6-dependent myeloma cells [16]. Indeed, the viability of MSG1 cells cultured on coated fibronectin with control medium containing IL-6 was similar to the viability of MSG1 cells co-cultured with HS23 stromal cells.

Adhesion between VLA-4 on myeloma cells and its ligand, VCAM-1 on stromal cells is believed to play a critical role in cell adhesion-mediated drug resistance (CAM-DR) [2]. Interestingly, a recent study reported that

down-regulation of VLA-4 on myeloma cells using siRNA increased the sensitivity of these cells to the proteasome inhibitor bortezomib [17]. Consistent with this result, in our study, inhibition of the adhesion between the VLA-4 on MSG1 and fibronectin inhibited MSG1 cell viability. Therefore, VLA-4-mediated signal transduction may be a critical target for the treatment of MM. A study suggested that, although adhesion is important for myeloma cells, this adhesion may not necessarily be dependent on the VLA-4/VCAM-1 system. In that study, a human BM stromal-dependent myeloma cell line, MOLP-5, was established from the leukemic phase of MM [18]. MOLP-5 expresses the IL-6 receptor and proliferates following IL-6 stimulation. Although the authors mentioned that adhesion of myeloma cells and BM stromal cells may be critical for MOLP-5 survival, they considered that the mechanism by which BM stromal cells mediate MOLP-5 survival may be distinct from the VLA-4/VCAM-1 system. Since they used BM stromal cells isolated from cryopreserved leukemic bone marrow specimens as feeder cells, we assume that, as long as any stromal cells were present for MOLP-5 to adhere to, specific stromal cells were not required. However, our study suggests a specific requirement for fibronectin binding by myeloma cells.

In summary, we showed that MSG1 requires both adhesion via fibronectin as well as IL-6 in order to survive, and that these conditions can substitute for HS23 stromal cells. Thus, MSG1 cannot survive if one, or the other of these factors is missing. These data suggest that drugs which inhibit the interaction between myeloma and stroma cells, or that inhibit IL-6 signaling in myeloma cells, may be sufficient for myeloma therapy. Future identification of the inhibitors present in HS27A conditioned media may also prove useful, although it may not be so easy to analyze the difference between the culture supernatants of HS23 and HS27A. Nevertheless, the MSG1 cell line characterized in this paper may provide a useful resource for experiments aimed at the development of novel anti-myeloma therapeutic approaches.

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