The 86-kD Subunit of Ku Autoantigen Mediates Homotypic and Heterotypic Adhesion of Multiple Myeloma Cells

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Abstract

Previous studies have shown that triggering multiple myeloma (MM) cells via CD40 induces IL-6–mediated autocrine growth as well as increased expression of cell surface adhesion molecules including CD11a, CD11b, CD11c, and CD18. In this study, we generated the 5E2 mAb which targets an antigen that is induced upon CD40 ligand (CD40L) activation of MM cells. Immunofluorescence, immunoprecipitation, and protein sequencing studies identified the target antigen of 5E2 mAb as the 86-kD subunit of the Ku autoantigen. We demonstrate that increased cell surface expression of Ku on CD40L-treated cells is due to migration of Ku from the cytoplasm to the cell surface membrane. Moreover, cell surface Ku on CD40L-treated MM cells mediates homotypic adhesion of tumor cells, as well as heterotypic adhesion of tumor cells to bone marrow stromal cells and to human fibronectin; and 5E2 mAb abrogates IL-6 secretion triggered by tumor cell adherence to bone marrow stromal cells. These data suggest that CD40L treatment induces a shift of Ku from the cytoplasm to the cell surface, and are the first to show that Ku functions as an adhesion molecule. They further suggest that cell surface Ku may play a role in both autocrine and paracrine IL-6–mediated MM cell growth and survival. (*J. Clin. Invest.* 1998. 101: 1379–1388.) Key words: CD40 • ligands • DNA-dependent protein kinase • cell membrane • IL-6

Introduction

IL-6 is both a growth and survival factor for human multiple myeloma (MM)[1] cells, and the signaling cascades mediating these biological sequelae may be distinct (1–4). Both tumor cells and bone marrow stromal cells (BMSCs) may be sources of IL-6 in patients with MM (5); moreover, recent evidence suggests the presence of Kaposi's sarcoma–associated herpesvirus and related viral IL-6, a homolog of human IL-6, within MM BMSCs (6). Although most MM cells do not spontaneously produce IL-6 and grow in an autocrine mechanism, they can be induced to do so by ligation of their cell surface CD40 (7–9). Similarly, paracrine IL-6 transcription and secretion of IL-6 from BMSCs is triggered by adhesion of MM cells to BMSCs (10–13) or by TGF-β secreted by tumor cells (14). In this case, multiple adhesion molecules on tumor cells and their ligands on extracellular matrix proteins or BMSCs have been identified which localize tumor cells in the bone marrow microenvironment, i.e., very late antigen-4 (CD29/CD49d) on MM cells binding to fibronectin (FN) (15) or to vascular cellular adhesion molecule-1 on BMSCs (10, 16, 17); however, additional adhesion molecules remain to be identified, since blocking known ligand–receptor interactions can only partially abrogate MM-BMSC adhesion and related IL-6 secretion. Although most of these adhesion molecules have been characterized using in vitro laboratory systems, we have described recently an in vivo model of human MM using severe combined immunodeficiency mice implanted with bilateral human fetal bone grafts which provides for the first time a means for identifying adhesion molecules that are responsible for specific homing of human MM cells to the human bone marrow microenvironment, with related induction of IL-6 secretion in human BMSCs (18).

Ligation of cell surface CD40 on human MM cells not only induces IL-6 secretion and proliferation of MM cells, but also upregulates expression of cell surface molecules including CD11a, CD11b, CD11c, CD18, and CD80 on MM cells (8). Therefore, phenotypic changes may correspond with induction of IL-6–mediated autocrine growth. In this study we used CD40 ligand (CD40L)-treated MM cells as immunogens in somatic cell hybridization in an attempt to generate mAbs which would identify such tumor cells. Specifically, the screening strategy used was designed to identify mAbs reactive with CD40L-treated MM cells which were not reactive with unstimulated tumor cells. It was our hypothesis that such molecules may not only represent phenotypic markers of CD40L-activated MM cells, but may also serve as mediators of homotypic adhesion of tumor cells and/or heterotypic adhesion of MM cells to extracellular matrix proteins or BMSCs.

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1. *Abbreviations used in this paper:* Ab, antibody; Ag, antigen; BMSCs, bone marrow stromal cells; CD40L, CD40 ligand; DNA-PKcs, the catalytic subunit of DNA-dependent protein kinase; FN, fibronectin; hrp, horseradish peroxidase; MM, multiple myeloma; NIH3T3/CD40LT, NIH3T3 cells transfected with human CD40L vector; NIH3T3/wt, wild-type NIH3T3 cells; NIH3T3/vt, NIH3T3 cells transfected with vector alone; pAb, polyclonal antibody; PVDF, polyvinylidine difluoride; pRb, retinoblastoma protein.

In this study, we generated the 5E2 mAb which targets an antigen (Ag) that is induced upon CD40L activation of MM cells. The target Ag of 5E2 mAb is the 86-kD subunit of the Ku autoantigen. Ku is primarily a nuclear phosphoprotein composed of 70- and 86-kD subunits which binds DNA and facilitates repair of dsDNA strand breaks and V(D)J recombination (19–24); to date, however, its role when expressed on the cell surface is undefined. Although Ku is expressed on the cell surface in unstimulated MM cells, significant expression is observed only after CD40 ligation; therefore, CD40L induces migration of Ku to the tumor cell surface. Moreover Ku on MM cells mediates not only homotypic adhesion of tumor cells, but also heterotypic adhesion of MM cells to BMSCs and human FN. The latter is confirmed by the ability of 5E2 mAb to abrogate tumor cell–BMSC and tumor cell–FN adhesion and related upregulation of IL-6 secretion. These data suggest that Ku is an adhesion molecule on MM cells and may play a role in both autocrine and paracrine IL-6–mediated tumor cell growth and survival.

Methods

Cell lines and transfectants. ARH-77 (CRL-1621), HS Sultan (CRL-1484), and IM-9 (CCL-159) human MM cells were obtained from American Type Culture Collection (Rockville, MD), and patient (MM1 and MM2) tumor cells (> 99% CD38$^+$ CD45RA$^-$) obtained after informed consent were cultured in 90% RPMI 1640 with L-glutamine medium supplemented with 25 IU/ml penicillin, 25 μg/ml streptomycin, and 5 mM L-glutamine (all from GIBCO BRL, Gaithersburg, MD), and 10% FCS (PAA Laboratories, Inc., Newport Beach, CA). LP101 BMSCs (a kind gift from Dr. Shin Aizawa, Tokyo Medical College, Tokyo, Japan) (25) were cultured in Iscove's modified Dulbecco's medium (Sigma Diagnostics, St. Louis, MO) supplemented with 15% FCS, 25 IU/ml penicillin, 25 μg/ml streptomycin, and 5 mM L-glutamine.

Wild-type NIH3T3 (NIH3T3/wt), NIH3T3 cells transfected with human CD40L vector (NIH3T3/CD40LT), and NIH3T3 cells transfected with vector alone (NIH3T3/vt) were provided by Dr. Gordon Freeman (Dana-Farber Cancer Institute) (8) and cultured in F12/DME medium (45% F12 nutrient mixture [Ham] with L-glutamine medium; 45% DME with 4,500 mg/liter D-glucose and L-glutamine medium [both from GIBCO BRL]; 10% FCS; 2 mM L-glutamine, 10 mM Hepes buffer [Intergen Co., Purchase, NY], and 15 μg/ml gentamicin [Elkins-Sinn, Inc., Cherry Hill, NJ]). NIH3T3/CD40LT and NIH3T3/vt cells were further selected with 400 μg/ml of GENETI-CIN® (G418) (GIBCO BRL). All cells were grown at 37°C in a humidified 5% CO$_2$ atmosphere.

Formalin fixation of NIH3T3 cells and coculture with MM cells. NIH3T3/CD40LT, NIH3T3/vt, and NIH3T3/wt cells were harvested at 70% confluency, washed six times in PBS (Sigma Diagnostics) to remove G418, and fixed in 1 ml of 1% formalin (Sigma Diagnostics) for 10 min at room temperature. After six further washes with PBS, the cells were cultured with MM cells (100 MM cells/1 NIH3T3 cell) for up to 48 h for subsequent assays.

Antibodies (Abs) for flow cytometric analysis, Western blotting, and cell adhesion assays. The following Abs were used: FITC-conjugated anti–human CD40 mAb (PharMingen, San Diego, CA); FITC conjugated anti–human IgG$_1$ mAb, anti-CD3 mAb, and NKH-1 anti-CD56 (neural cell adhesion molecule) mAb (Coulter Corp., Miami, FL); FITC conjugated goat (Fab′)$_2$ anti–mouse IgG mAb (Immunotech, Westbrook, ME); C-19 goat anti–Ku-70 polyclonal antibody (pAb), C-20 goat anti–Ku-86 pAb, C-15-G goat retinoblastoma protein (pRb) pAb, and horseradish peroxidase (hrp)-conjugated donkey anti–goat IgG mAb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); AB-1 antiactin mAb (Oncogene Science, Inc., Cambridge, MA); hrp-conjugated sheep anti–mouse Ig mAb (Amersham Life

Science, Buckinghamshire, England); 4B4 anti-CD29 (β$_1$ integrin) mAb (a kind gift from Dr. Chikao Morimoto, Dana-Farber Cancer Institute) (15); 5E2 mAb; and HB7 anti-CD38 mAbs. Flow cytometric analysis was done using the Coulter Epics XL flow cytometer (Coulter Corp.).

Generation of 5E2 mAb. Balb/c female mice (The Jackson Laboratories, Bar Harbor, ME) were immunized by a combination of subcutaneous and intraperitoneal injection with 10^7 human MM cells in sterile PBS emulsified with an equal volume of CFA (Sigma Diagnostics). Two intraperitoneal booster immunizations of 10^7 MM cells in sterile PBS emulsified with an equal volume of incomplete Freund's adjuvant (Sigma Diagnostics) were then given at 14-d intervals. 10 d after the third immunization, mouse serum was assayed by Western blotting of total cell lysates of MM cells for Abs against novel proteins. Mice with high Ab titers were boosted 4 wk later with an intraperitoneal injection of 10^7 MM cells in sterile PBS.

4 d later, the mouse was killed, the splenocytes removed, fused using polyethylene glycol 1450 (American Type Culture Collection) to SP 2/0 myeloma cells (American Type Culture Collection, CRL-8006) (2 splenocytes/1 myeloma cell) (26, 27), and selected in HAT medium (Sigma Diagnostics). Between 10 and 21 d after fusion, hybridoma clones were screened by Western blotting for reactivity with CD40L-stimulated ARH-77 MM cells and lack of reactivity with unstimulated ARH-77 MM cells. Selected clones were expanded to 24-well cultures and subcloned by limiting dilution to monoclonality. Cells from selected hybridoma subclones were then injected intraperitoneally into mice to produce ascites from which mouse mAbs were purified using a high-salt affinity column.

Homotypic adhesion assay. Homotypic aggregation of ARH-77 MM cells was examined qualitatively as previously described (28). Briefly, ARH-77 MM cells (0.2 × 10^6/well) were first cultured with media or formalin-fixed NIH3T3/CD40LT cells for 16 h, washed twice in RPMI 1640 with L-glutamine medium supplemented with 5 mM Hepes buffer, and then resuspended in complete medium in flat-bottom 96-well tissue culture plates for up to 48 h with media, 5E2, NKH-1 anti-CD56, HB7 anti-CD38, and anti-CD3 mAbs; or C-20 anti–Ku-86 pAb. The final culture volume was 200 μl, and final mAb concentration was 20 μg/ml. Homotypic aggregation was assessed under phase-contrast microscopy (Olympus BX60; Olympus America, Inc., Lake Success, NY) and photographed (CVS Picture Place® 200, Woonsocket, RI). Aggregation was graded as follows: < 10% of cells were in aggregates (+); 10% to < 50% of cells were in aggregates (++); 50–100% of cells were in small, loose aggregates (+++); 50–100% of cells were in larger aggregates (++++); and 50–100% of cells were in large, compact aggregates (+++++).

Heterotypic adhesion assay. The ^{51}Cr binding assay was used to examine adhesion of ARH-77 MM and MM1 cells to LP101 BMSCs as well as ARH-77 MM cells to human FN, as described previously (10, 15). Briefly LP101 BMSCs (0.02 × 10^6/well) were first cultured in 96-well tissue culture plates as a monolayer for 24 h. Human FN (0.04 mg/ml; Collaborative Biomedical Products, Becton-Dickinson Labware, Bedford, MA) was coated onto 96-well tissue culture plates at 37°C overnight, and blocked with 1% BSA at 4°C for 2 h. ARH-77 MM and MM1 cells were cultured with media or formalin-fixed NIH3T3/CD40LT cells for 16 h, and then incubated either with PBS, 5E2 (20 μg/ml), 4B4 anti-CD29 (1:50), or HB7 anti-CD38 (1:50) mAb for 2 h. Tumor cells were then labeled with ^{51}Cr (1 mCi/ml) in saline for 1 h, washed twice with HBSS (GIBCO BRL), and resuspended in RPMI 1640 medium containing 1% BSA (Sigma Diagnostics). ^{51}Cr-labeled tumor cells (0.05 × 10^6/well) were then incubated either on the LP101 BMSC monolayer or on FN-coated plates at 37°C for 30 min, followed by removal of nonadherent cells by inverted centrifugation at 90 g for 5 min. The cells remaining were lysed with 0.1% NP-40 (Sigma Diagnostics) in distilled water over 10 min and analyzed on a gamma counter (Cobra™ II Auto-Gamma; Packard Instrument Co., Meriden, CT).

Measurement of IL-6 secretion. An IL-6 ELISA detecting human IL-6 at 10 pg/ml was used to measure IL-6 levels in the culture super-

natants of 24-h cultures of MM cells with BMSCs, as previously described (29). Briefly LP101 BMSCs (0.02×10^6/well) were first cultured in 96-well tissue culture plates as a monolayer for 24 h. ARH-77 MM and MM1 cells were cultured with media or formalin-fixed NIH3T3/CD40LT cells for 16 h, and then cultured (0.05×10^6/well) on the LP101 BMSC monolayer either with PBS, 5E2 (20 μg/ml), 4B4 anti-CD29 (1:50), or HB7 anti-CD38 (1:50) mAb for 24 h. Serial dilutions (50 μl) of test supernatants and IL-6 standards were first added in duplicate to 96-well EIA/RIA plates (Costar Corp., Cambridge, MA) coated with IG61 anti–IL-6 mAb (murine IgG$_1$; TORAY, Shiga, Japan). Next, a biotinylated detector anti–IL-6 mAb (Genetics Institute, Cambridge, MA) was added, developed with hrp-conjugated streptavidin (Amersham Life Science), and detected at 450 nm excitation wavelength on a microplate reader (model 3550; Bio-Rad, Hercules, CA).

Isolation, peptide digestion, and sequencing of 5E2 mAb target Ag. ARH-77 MM cells in log-phase of growth were harvested, washed three times with ice-cold PBS, lysed on ice over 30 min in lysis buffer (50 mM Tris [GIBCO BRL] pH 8.0; 150 mM NaCl [Fisher Scientific, Fair Lawn, NJ]; 0.1% NP-40, 1.0 mM EDTA, 50 mM NaF, 1.0 mM sodium orthovanadate, and 5.0 μg/ml PMSF [all from Sigma Diagnostics]; 2.0 μg/ml aprotinin; and 2.0 μg/ml leupeptin [both from Boehringer Mannheim, Corp., Indianapolis, IN]), and centrifuged at 6,000 g at 4°C for 15 min. The protein concentration of the supernatant was assayed by Bradford's microtiter plate method (Bio-Rad).

2 ml of total cell lysate of ARH-77 MM cells (1.0 mg/ml) was immunoprecipitated with 5 μg of 5E2 mAb, separated on an 8% SDS-PAGE gel, and transferred to a polyvinylidine difluoride (PVDF) membrane (Immobilon™ P; Millipore Corp., Bedford, MA). The target Ag was identified by ponceau-S (Sigma Diagnostics) staining, and the band was carefully excised, diced into 1 mm × 1 mm pieces, and digested in 50 μl of digestion buffer (1% hydrogenated Triton X-100 [RTX-100], 10% acetonitrile [both Sigma Diagnostics], 100 mM Tris, pH 8.0, and 0.5 μg of sequencing grade modified trypsin [Promega Corp., Madison, WI]) at 37°C over 22–24 h (30). The membranes were next washed and sonicated for 5 min in 50 μl of digestion buffer (without trypsin), followed by 100 μl of 0.1% trifluoroacetic acid (Sigma Diagnostics). Supernatants were pooled (200 μl), immediately separated by HPLC (HP 1090 liquid chromatograph; Hewlett-Packard, Santa Clara, CA), and detected at 214 nm excitation wavelength. The peptide peak of interest was identified and analyzed (ABI 477 protein sequencer; Perkin-Elmer, Applied Biosystems Division, Foster City, CA).

Preparation of nuclear, cytoplasmic, and membrane cell lysate fractions. ARH-77 MM cells in log-phase growth were harvested, washed three times in ice-cold PBS, lysed on ice with 0.4 ml of TEM buffer (20 mM Tris HCl [GIBCO BRL], 0.5 mM EDTA, 0.5 mM EGTA [both Sigma Diagnostics], 10 mM 2-ME, 25 μg/ml aprotinin, and 25 μg/ml leupeptin), and sonicated twice for 10 s. Nuclei were then pelleted by centrifugation at 1,000 g at 4°C for 5 min, and lysed with 0.2 ml of lysis buffer. The cloudy supernatant was then ultracentrifuged at 100,000 g at 4°C for 30 min, and the cytoplasmic fraction was obtained from the supernatant. The pellet containing cell membranes was rinsed once with TEM buffer, resuspended in TEM buffer
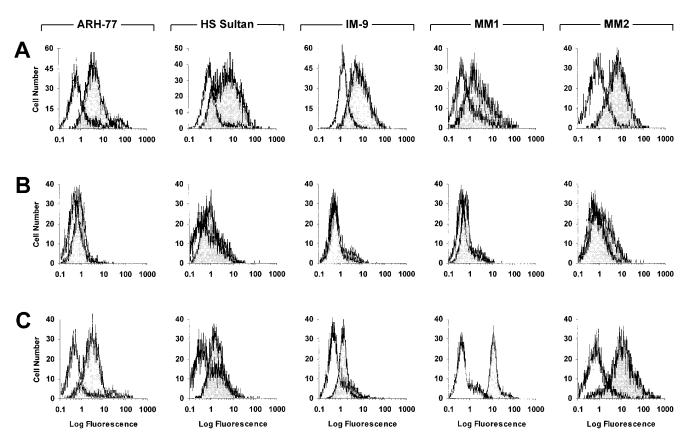

Figure 1. 5E2 mAb target Ag expression induced by CD40L on ARH-77, HS Sultan, and IM-9 MM cell lines, as well as patient MM1 and MM2 cells. ARH-77, HS Sultan, and IM-9 MM cell lines, as well as patient MM1 and MM2 cells, were cultured in media (*A* and *B*) or with formalin-fixed NIH3T3/CD40LT cells (*C*). Cells (0.5×10^6) were stained with either 10 μl of FITC-conjugated anti–human CD40 mAb (*A*) or 10 μl of 5E2 mAb (*B* and *C*), followed by 1 μl of FITC-conjugated goat (Fab′)$_2$ anti–mouse IgG mAb. The cell surface expression of CD40 (*A*) and 5E2 mAb target Ag (*B* and *C*) were analyzed by indirect immunofluorescence flow cytometry.

containing 0.5% Triton X-100, sonicated twice for 10 s, and incubated on ice for 1 h. The membrane fraction was obtained after centrifugation at 16,000 g at 4°C for 10 min from the supernatant.

Immunoprecipitation, immunoblotting, and Western blotting. For immunoprecipitation, 10 μg of either 5E2 mAb, C-20 anti–Ku-86 pAb, C-19 anti–Ku-70 pAb, anti-CD3 mAb, or C-15-G anti-pRb pAb was added to 1 ml of total cell lysate (1 mg/ml) from ARH-77 MM cells prepared as described above and incubated at 4°C overnight. 100 μl of 10% protein A–Sepharose CL-4B (Pharmacia Biotech AB, Uppsala, Sweden) was added next, incubated at 4°C for 1 h, pelleted by centrifugation at 10,000 g for 30 s, washed three times in ice-cold lysis buffer, and resuspended in 20 μl of modified Laemmli's sample buffer (2% SDS and 20% glycerol [GIBCO BRL]; 60 mM Tris, pH 6.8; 10% 2-ME, and 0.001% bromophenol blue [Sigma Diagnostics]). For direct Western blotting, 10 μl of total cell lysate (1 mg/ml) was mixed with an equal volume of 2× modified Laemmli's sample buffer. The samples were resolved on 8% SDS-PAGE, transferred to PVDF membranes, and blocked for 1 h in blotto A (5% skimmed milk [Mix and Drink®; Saco Foods, Inc., Middleton, WI), 0.2% Tween 20, and 0.02% sodium azide [both from Sigma Diagnostics]).

For immunoblotting, 1 μg of either 5E2 mAb, C-20 anti–Ku-86 pAb, C-19 anti–Ku-70 pAb, or AB-1 antiactin mAb was diluted in 10 ml of TBST (Tris-buffered saline with 0.2% Tween 20) and incubated at 4°C overnight. The membranes were then washed in TBST and incubated at room temperature for 1 h with the appropriate hrp-conjugated secondary Ab, either sheep anti–mouse Ig mAb (1:2,000) or donkey anti–goat Ig mAb (1:4,000). The membranes were next washed in TBST followed by hrp chemiluminescence detection (ECL™; Amersham Life Science) and exposure to photographic film (Biomax™ MR; Eastman Kodak, Rochester, NY). When required, the membranes were stripped in 25 ml of stripping buffer (2% SDS, 62.5 mM Tris, pH 6.8, and 0.7% 2-ME) at 56°C for 30 min and immunoblotted again as described above. Relative intensity of expression was assessed (model GS-700 imaging densitometer; Bio-Rad).

Results

Changes in cell surface phenotype induced by CD40L on ARH-77, HS Sultan, and IM-9 MM cell lines, as well as patient MM1 and MM2 cells. We first examined the expression of CD40 on ARH-77, HS Sultan, and IM-9 MM cell lines, as well as patient MM1 and MM2 cells, by indirect immunofluores-cence flow cytometry. As can be seen in Fig. 1 *A*, all of these MM cells highly express CD40. mAbs (5E2) were produced as described previously (26, 27) and selected for reactivity with CD40L-triggered ARH-77 MM cells (Fig. 1 *C*), but lack of reactivity on non–CD40L-treated tumor cells (Fig. 1 *B*). As can be seen in Fig. 1 *C*, 5E2 targets an Ag that is induced on the cell surface by CD40L on both MM cell lines and MM patient cells.

Homotypic aggregation of ARH-77 MM cells is blocked by 5E2 mAb. To screen for functionally important Ags identified by the mAbs, we next determined whether these mAbs could abrogate homotypic aggregation of CD40L-treated MM cells. As can be seen in Fig. 2 *A*, CD40L-treated ARH-77 MM cells spontaneously formed aggregates (++++) after 24 h of culture, and treatment with a novel (5E2) mAb significantly inhibited tumor cell aggregation (+) (Fig. 2 *B*). NKH-1 anti-CD56 mAb (15) also blocked formation of CD40L-treated tumor cell aggregates (+) (Fig. 2 *C*), whereas neither HB7 anti-CD38 (Fig. 2 *D*) nor anti-CD3 mAbs (Fig. 2 *E*) had similar effects. Non–CD40L-treated ARH-77 MM cells spontaneously produce smaller aggregates (++) (Fig. 2 *G*) than CD40L-treated tumor cells. Although NKH-1 anti-CD56 mAb blocked aggregation of non–CD40L-stimulated cells (+) (Fig. 2 *I*), the 5E2, HB7 anti-CD38, and anti-CD3 mAbs (Fig. 2, *H*, *J*, and *K*, respectively) had no effect.

Heterotypic adhesion between ARH-77 MM or MM1 cells to LP101 BMSCs and to human FN is abrogated by 5E2 mAb. To further define the potential functional significance of Ags identified by 5E2 mAb, we next examined whether this novel mAb could inhibit heterotypic adhesion of MM cells to BMSCs or FN, as in our prior studies (10). As can be seen in Fig. 3 *A*, treatment of ARH-77 MM cells or MM1 cells with CD40L increased tumor cell adhesion to LP101 BMSCs by 30–40% relative to adherence of non–CD40L-treated tumor cells to BMSCs. 4B4 anti-CD29 mAb, but not HB7 anti-CD38 mAb, blocked MM cell to BMSC adherence of both non–CD40L-stimulated tumor cells (50–60% inhibition) and CD40L-treated tumor cells (50–70% inhibition). Of great interest, although 5E2 mAb did not significantly block the binding of non–CD40L-treated ARH-77 MM cells or MM1 cells to
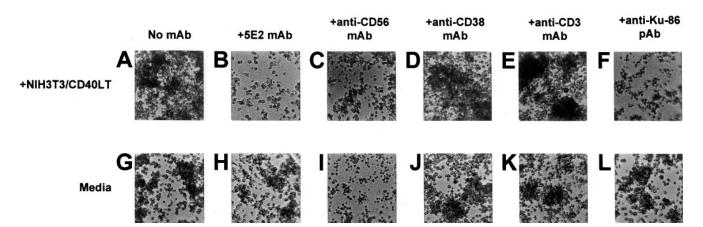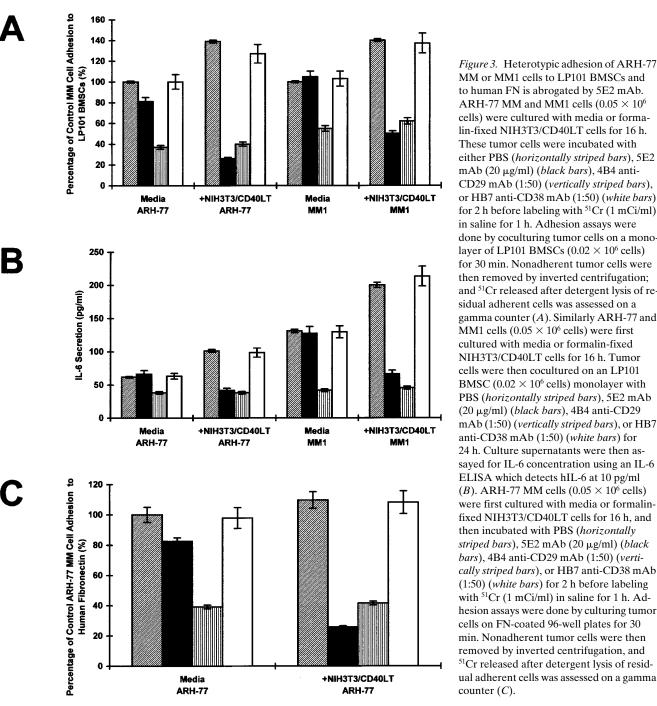

	No mAb	+5E2 mAb	+anti-CD56 mAb	+anti-CD38 mAb	+anti-CD3 mAb	+anti-Ku-86 pAb
+NIH3T3/CD40LT	A	B	C	D	E	F
Media	G	H	I	J	K	L

Figure 2. Homotypic aggregation of ARH-77 MM cells is blocked by 5E2 mAb. ARH-77 MM cells (0.2 × 10^6 cells) were cultured with formalin-fixed NIH3T3/CD40LT cells (*A–F*), or media (*G–L*) for 16 h. Blocking Abs (20 μg/ml) were next added: 5E2 mAb (*B* and *H*); NKH-1 anti-CD56 mAb (*C* and *I*); HB7 anti-CD38 mAb (*D* and *J*); anti-CD3 mAb (*E* and *K*); and C-20 anti–Ku-86 pAb (*F* and *L*). Homotypic aggregation of tumor cells was assessed using phase-contrast microscopy at 24 h.
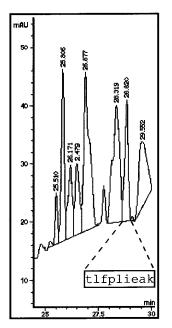
Figure 3. Heterotypic adhesion of ARH-77 MM or MM1 cells to LP101 BMSCs and to human FN is abrogated by 5E2 mAb. ARH-77 MM and MM1 cells (0.05×10^6 cells) were cultured with media or formalin-fixed NIH3T3/CD40LT cells for 16 h. These tumor cells were incubated with either PBS (*horizontally striped bars*), 5E2 mAb (20 µg/ml) (*black bars*), 4B4 anti-CD29 mAb (1:50) (*vertically striped bars*), or HB7 anti-CD38 mAb (1:50) (*white bars*) for 2 h before labeling with ^{51}Cr (1 mCi/ml) in saline for 1 h. Adhesion assays were done by coculturing tumor cells on a monolayer of LP101 BMSCs (0.02×10^6 cells) for 30 min. Nonadherent tumor cells were then removed by inverted centrifugation; and ^{51}Cr released after detergent lysis of residual adherent cells was assessed on a gamma counter (*A*). Similarly ARH-77 and MM1 cells (0.05×10^6 cells) were first cultured with media or formalin-fixed NIH3T3/CD40LT cells for 16 h. Tumor cells were then cocultured on an LP101 BMSC (0.02×10^6 cells) monolayer with PBS (*horizontally striped bars*), 5E2 mAb (20 µg/ml) (*black bars*), 4B4 anti-CD29 mAb (1:50) (*vertically striped bars*), or HB7 anti-CD38 mAb (1:50) (*white bars*) for 24 h. Culture supernatants were then assayed for IL-6 concentration using an IL-6 ELISA which detects hIL-6 at 10 pg/ml (*B*). ARH-77 MM cells (0.05×10^6 cells) were first cultured with media or formalin-fixed NIH3T3/CD40LT cells for 16 h, and then incubated with PBS (*horizontally striped bars*), 5E2 mAb (20 µg/ml) (*black bars*), 4B4 anti-CD29 mAb (1:50) (*vertically striped bars*), or HB7 anti-CD38 mAb (1:50) (*white bars*) for 2 h before labeling with ^{51}Cr (1 mCi/ml) in saline for 1 h. Adhesion assays were done by culturing tumor cells on FN-coated 96-well plates for 30 min. Nonadherent tumor cells were then removed by inverted centrifugation, and ^{51}Cr released after detergent lysis of residual adherent cells was assessed on a gamma counter (*C*).

BMSCs, it did significantly inhibit (60–80% decrease) heterotypic adhesion of CD40L-treated tumor cells to BMSCs.

To confirm the observed effect of 5E2 mAb on heterotypic adhesion of tumor cells to BMSCs, we next examined its impact on the IL-6 secretion induced by the adherence of tumor cells to BMSCs. As can be seen in Fig. 3 *B*, 4B4 anti-CD29 mAb, but not 5E2 or HB7 anti-CD38 mAbs, abrogated IL-6 secretion induced by adherence of non–CD40L-treated tumor cells to BMSCs. In contrast, both 4B4 anti-CD29 and 5E2 mAbs, but not HB7 anti-CD38 mAb, markedly diminished IL-6 secretion triggered by binding of CD40L-treated tumor cells to BMSCs.

Finally, CD40L stimulation of ARH-77 MM cells increases adhesion to human FN by 5–10% as compared with unstimulated cells (Fig. 3 *C*). 4B4 anti-CD29 mAb effectively blocks adhesion of both unstimulated and CD40L-treated ARH-77 MM cells to FN by 60–70%, and HB7 anti-CD38 mAb does not significantly alter the adhesion profile. In contrast, 5E2 mAb inhibits adhesion of CD40L-treated MM cells to FN (70–80% decrease) but does not abrogate binding of non–CD40L-treated tumor cells to FN.

Identification of 5E2 mAb target Ag in CD40-treated ARH-77 MM cells. To identify the target Ag of 5E2 mAb, we next performed direct protein sequencing. As can be seen in Fig. 4,

Human 86 kD subunit Ku AutoAg

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  1 mvrsgnkaav vlcmdvgftm snsipgiesp feqakkvitm fvqrqvfaen
 51 kdeialvlfg tdgtdnplsg gdqyqnitvh rhlmlpdfdl ledieskiqp
101 gsqqadflda livsmdviqh etigkkfekr hieiftdlss rfsksqldii
151 ihslkkcdis lqfflpfslg kedgsgdrgd gpfrlgghgp sfplkgiteq
201 qkegleivkm vmislegedg ldeiysfses lrklcvfkki erhsihwpcr
251 ltigsnlsir iaayksilqe rvkktwtvvd aktlkkediq ketvyclndd
301 detevlkedi iqgfrygsdi vpfskvdeeq mkyksegkcf svlgfckssq
351 vqrrffmgnq vlkvfaardd eaaavalssl ihalddldmv aivryaydkr
401 anpqvgvafp hikhnyeclv yvqlpfmedl rqymfsslkn skkyapteaq
451 lnavdalids mslakkdekt dtledlfptt kipnprfqrl fqcllhralh
501 preplppiqq hiwnmlnppa evttksqipl skik tlfpli eak kkdqvta
551 qeifqdnhed gptakklkte qggahfsvss laegsvtsvg svnpaenfrv
601 lvkqkkasfe easnqlinhi eqfldtnetp yfmksidcir afreeaikfs
651 eeqrfnnflk alqekveidq lnhfweivvq dgitlitkee asgssvtaee
701 akkflapkdk psgdtaavfe eggdvddlld mi
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Figure 4. Identification of 5E2 mAb target Ag in CD40L-treated ARH-77 MM cells. Immunoprecipitation of total cell lysates from CD40L-treated ARH-77 MM cells with 5E2 mAb was used to detect the 5E2 mAb target Ag. After separation on an 8% SDS-PAGE gel and transfer onto PVDF, the target Ag was identified by ponceau-S staining and careful excision of the membrane. The target protein was extracted from PVDF by incubating overnight in 1% RTX-100 buffer, digested with trypsin, and separated and detected by HPLC. The peptide of interest was thus isolated, sequenced, and compared with other reported protein sequences in the National Center for Biotechnology Information BLAST database.

a peptide peak at 28.820 min was identified on HPLC, with sequence "tlfplieak." The Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, Bethesda, MD) search demonstrated 100% sequence homology of the 5E2 mAb target Ag (amino acids 535–543) to the 86-kD subunit of human Ku autoantigen (Ku-86).

We further confirmed the target Ag of 5E2 mAb to be the 86-kD subunit of Ku by coimmunoprecipitation with C-20 anti–Ku-86 pAb. As shown in Fig. 5 *A*, the 5E2 mAb target Ag has a molecular mass of 86 kD (*second lane*) and coimmunoprecipitates with Ku-86 (*third lane*). Since Ku exists as a dimer of 70-kD (Ku-70) and 86-kD subunits (31, 32), Ku-70 also coimmunoprecipitates with 5E2 mAb target Ag (*fourth lane*). However, 5E2 mAb does not react specifically with Ku-70 protein (*first lane*).

The membranes were stripped and then immunoblotted simultaneously with both C-20 anti–Ku-86 and C-19 anti–Ku-70 pAbs (Fig. 5 *B*). All three immunoprecipitated samples (*second*, *third*, and *fourth lanes*) demonstrate coimmunoprecipitation with both anti-Ku pAbs. Furthermore, the 86-kD Ku-86

bands were completely superimposable on the 5E2 mAb bands of Fig. 5 *A*, confirming comigration of the 5E2 mAb target Ag with Ku-86. Finally the isotype controls, anti-CD3 mAb for mouse IgG$_1$ (*fifth lane*) and C-15-G anti-pRb pAb for goat pAb (*sixth lane*), do not demonstrate immunoreactivity with target Ags of 5E2 mAb, C-20 anti–Ku-86 pAb, and C-19 anti–Ku-70 pAb.

Depletion of Ku-86 protein also removes 5E2 mAb immunoreactivity. To confirm the specificity of 5E2 mAb for Ku-86, Ku-86 protein was depleted from ARH-77 MM cells by six repeated immunoprecipitations with either C-20 anti–Ku-86 pAb (Fig. 6 *A*) or 5E2 mAb (Fig. 6 *B*) followed by immunoblotting with 5E2 mAb, C-20 anti–Ku-86, and C19 anti–Ku-70 pAbs. Depletion using C-20 anti–Ku-86 pAb removed 5E2 mAb reactivity and vice versa. Immunoblotting with AB-1 antiactin mAb confirmed equal protein loading and specificity of Ku-86 removal.

To further confirm that 5E2 mAb identifies the Ku-86 protein, we examined whether this Ab inhibited homotypic aggregation of ARH-77 MM cells in a pattern similar to that for
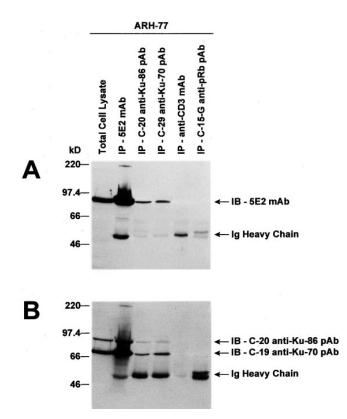
Figure 5. Confirmation that 5E2 mAb target Ag is the 86-kD subunit of the Ku autoantigen on ARH-77 MM cells. Total cell lysates of ARH-77 MM cells were immunoprecipitated with 5E2 mAb (*second lane*), C-20 anti–Ku-86 pAb (*third lane*), C-19 anti–Ku-70 pAb (*fourth lane*), anti-CD3 mAb (*fifth lane*), or C-15-G anti-pRB pAb (*sixth lane*); and then immunoblotted with 5E2 mAb (*A*). The membrane was then stripped and immunoblotted with C-20 anti–Ku-86 pAb and C-19 anti–Ku-70 pAb (*B*). Total cell lysate served as a positive control (*first lane*).

C-20 anti–Ku-86 pAb. As can be seen in Fig. 2, *B* and *F*, homotypic adhesion of these tumor cells in the presence of 5E2 mAb or C-20 anti–Ku-86 pAb was decreased (+) relative to that noted in the media culture (++++) (Fig. 2 *A*). Furthermore, blocking of homotypic adhesion by either of these Abs

was evident for CD40L-treated MM cells (Fig. 2, *B* and *F*), but not for unstimulated tumor cells (Fig. 2, *H* and *L*).

CD40L stimulation of ARH-77 MM cells increases cell surface membrane expression of Ku-86 Ag. Finally, we examined total cell lysates, as well as membrane, cytoplasmic, and nuclear fractions, of unstimulated and CD40L-treated MM cells for immunoreactivity with 5E2 mAb. As can be seen in Fig. 7 *A*, the total cellular expression of the 5E2 mAb target Ag (Ku-86) was equivalent in ARH-77 MM cells cultured with media, NIH3T3/CD40LT, NIH3T3/vt, or NIH3T3/wt cells. CD40L induced increased cell surface membrane expression of Ku (Fig. 7 *B*), associated with decreased expression of this protein in the cytoplasmic fraction (Fig. 7 *C*). Ku within the nuclear fraction remained unchanged (Fig. 7 *D*). These results suggest that CD40L induces a shift of Ku-86 from the cytoplasm to the cell surface: densitometric imaging revealed a threefold increase in membrane-associated Ku and a 30% decrease in cytoplasmic Ku on CD40L-treated tumor cells. Immunoblotting using AB-1 antiactin mAb confirmed equal protein loading.

Discussion

In this study, we extended our prior studies of adhesion molecules on MM cells. Specifically, we set out to identify novel Ags on MM cells which were upregulated when MM cells were activated via ligation of their cell surface CD40. Our earlier work had demonstrated upregulation of a variety of molecules, i.e., β1 and β2 integrins, induced by CD40L, suggesting the existence of other such Ags (8). Here we demonstrate that treatment with CD40L can induce proliferation of MM cells, formation of large, dense MM cell aggregates, and adherence of tumor cells to BMSCs and FN. Our study indicates that the 5E2 mAb recognizes the 86-kD subunit of Ku and that CD40 stimulation facilitates the migration of Ku to the cell surface. Moreover, 5E2 mAb inhibits homotypic cell aggregation of CD40L-activated MM cells and abrogates adhesion of CD40L-treated MM cells to BMSCs and FN, as well as related IL-6 secretion in BMSCs. This is the first report of CD40L-induced migration to the cell surface of Ku Ag, a predominantly nuclear protein. Moreover, we demonstrate a new function for this molecule, since Ku expressed on the cell surface can mediate both homotypic adhesion of tumor cells, as well as heterotypic adhesion of MM cells to BMSCs and FN.
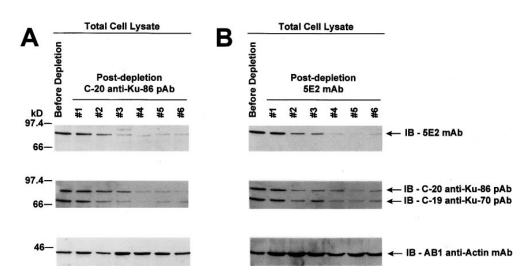

Figure 6. Depletion of Ku-86 protein also removes 5E2 mAb immunoreactivity. Total cell lysates (100 μg) of ARH-77 MM cells were repeatedly immunoprecipitated (six times) with 1 μg of either C-20 anti–Ku-86 pAb (*A*) or 5E2 mAb (*B*). Cell lysates after sequential depletion of Ku-86 protein or the 5E2 mAb target Ag were immunoblotted with 5E2 mAb, C-20 anti–Ku-86 pAb, or C-19 anti–Ku-70 pAb. Immunoblotting with AB-1 antiactin mAb confirmed equal protein loading and specificity of Ku-86 removal.

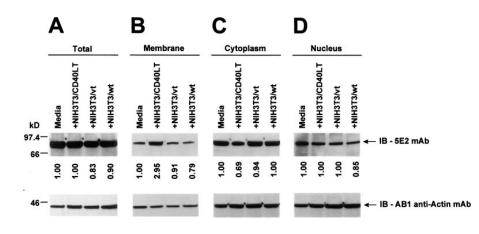|	A				B				C				D			
	Total				Membrane				Cytoplasm				Nucleus			

Figure 7. CD40L stimulation of ARH-77 MM cells increases cell surface membrane expression of Ku-86 Ag. ARH-77 MM cells were cultured with media, formalin-fixed NIH3T3/CD40LT, NIH3T3/vt, or NIH3T3/wt cells for 16 h. Total cell lysates (*A*), as well as membrane (*B*), cytoplasmic (*C*), and nuclear (*D*) fractions, were immunoblotted with 5E2 mAb. Intensity of expression relative to media was assessed by imaging densitometry. Immunoblotting with AB-1 antiactin mAb confirmed equal protein loading.

About a third of Japanese patients with polymyositis–scleroderma overlap syndrome possess Abs to an abundant acidic nuclear protein named Ku (19). This protein is composed of 70- and 86-kD subunits (31, 32), each of which has phosphoserine residues (33) and DNA binding regions (34–36). There is no sequence homology between the 70- and 86-kD subunits (34–36), nor is there conservation of Ag determinants between species (37). Ku is known to recruit the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) to DNA and is required for DNA-PKcs catalytic activity (38); DNA-PKcs in turn phosphorylates Ku (39). DNA-PKcs-Ku holoenzyme mediates nuclear processes including double-stranded DNA strand break repair and V(D)J recombination (20–24), DNA replication (40), DNA transcription (41–45), DNA unwinding (46), stimulation of the elongation property of RNA polymerase II (47), binding to HIV-1 TAR RNA (48), maintenance of normal telomere length (49), and regulation of the TCR β chain gene (50). Using purified anti-Ku Abs from patients, a reticular nuclear staining with sparing of nucleoli was observed previously on indirect immunofluorescence of human liver cells (19).

Although Ku has these multiple roles within the nucleus, its biological function in the cytoplasm or on the cell surface is at present undefined. Flow cytometric analyses (51), indirect immunofluorescence microscopy (52), hydrophobicity assays demonstrating a possible transmembrane region on Ku-70 (53), and structural concordance of Ku with somatostatin transmembrane receptors (54, 55) all suggest that Ku may also be membrane associated and function as a cell surface adhesion molecule. The current study demonstrates that Ku, as we have shown for other adhesion molecules (8), is upregulated on MM cells by CD40 ligation. This increased cell surface Ku expression is related to a shift from the cytoplasm. Most importantly, cell surface Ku can mediate both homotypic and heterotypic adhesion. In addition to these functions as an adhesion molecule, the known serine/threonine kinase activity of DNA-PKcs-Ku (56), coupled with our studies demonstrating a shift of Ku to the cell surface triggered via CD40L, suggest that DNA-PKcs-Ku may mediate CD40 signal transduction.

Our interest in focusing on the CD40L-activated MM cell is multifaceted. First, most current evidence suggests that the MM precursor cell is a late stage pre-Ig switch B cell (57), and CD40 ligation of MM cells triggers expansion of a CD19+ monoclonal B cell which may correspond to this late stage MM precursor (8). In addition, Zelazowski et al. reported recently that nuclear expression of Ku proteins could be upregulated when normal murine B splenocytes were triggered by a combination of CD40L and IL-4 (58), suggesting that CD40 and Ku molecules were actively involved in isotype switching. Moreover, mice deficient in either Ku-70 or Ku-86 genes have been shown to be profoundly lacking in normal B cell development (59, 60), further suggesting that Ku proteins play an essential role in the development of normal, as well as malignant, B cells. Second, it has been reported that CD19+ monoclonal B cells may represent a reservoir of drug-resistant tumor cells (61). Third, the induction of IL-6 production and proliferation upon CD40 ligation of MM cells suggests that this subset of tumor cells may represent those cells which are dependent on IL-6 for their self-renewal (7–9). Fourth, the demonstration of increased homotypic and heterotypic adhesion of CD40L-treated MM cells, and the mechanism of this binding at least in part being related to Ku, offers insights into disease pathogenesis. In particular, future studies will investigate the role of Ku in regulation of IL-6–dependent and –independent tumor cell growth and survival. Most importantly, this and our prior studies (8) suggest that CD40L treatment of MM cells markedly upregulates not only Ku, but also a variety of adhesion and accessory molecules including HLA classes I and II as well as CD11a, CD11b, CD11c, CD18, and CD80, on CD19+ neoplastic MM cells; this alteration in cell surface phenotype may enhance immunogenicity. Previous reports also demonstrate that 89% of patients with Ku-positive autoimmune disease express HLA class II Ag DQw1, suggesting that Ku protein may be MHC class II–restricted (62). To date our studies suggest that MM cells generate reactivity as stimulators in an allogeneic mixed lymphocyte reaction, and that CD40L treatment of tumor cells can markedly upregulate this response. Even more importantly, little if any reactivity is observed when MM cells are used as stimulators in an autologous MLR. However, CD40L-treated MM cells stimulate a marked proliferation of autologous T cells. Therefore, ongoing studies are characterizing the role of Ku and other cell surface molecules as target Ags for this response, as well as the resultant clonal T cell expansion. These observations support the view that immunization of patients with their own CD40L-activated MM cells may generate immunity to MM cells, and that CD40L-activated tumor cells may allow expansion of T cells ex vivo for novel adoptive immunotherapy approaches.

Acknowledgments

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