Multiple Myeloma Cell Adhesion-Induced Interleukin-6 Expression in Bone Marrow Stromal Cells Involves Activation of NF-κB

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Adhesion of multiple myeloma (MM) cells to bone marrow stromal cells (BMSCs) not only localizes MM cells in the marrow microenvironment, but also triggers interleukin-6 (IL-6) secretion by BMSCs and related MM cell proliferation. In the present study, we characterized the regulation of IL-6 gene expression in BMSCs during MM cell adhesion. Adhesion of ARH-77, HS-Sultan, IM-9, and U266 MM cells lines to BMSCs and BMSC lines (LP 101 and AA 101) triggered 5- through 15-fold and 2- through 4-fold increases in IL-6 secretion, respectively. IL-6 mRNA transcripts were undetectable by Northern blotting in IM-9 MM cells or LP 101 BMSCs cultured alone; however, adherence of IM-9 cells to LP 101 cells induced a transient increase in IL-6 transcripts at 6 hours, followed by peak IL-6 secretion at 24 hours. To confirm increased IL-6 transcription and characterize its regulation, LP101 BMSCs were transiently transfected with full length and deletion fragments of the IL-6 promoter linked to the chloramphenicol acetyltransferase (CAT) reporter gene. Transient transfection of LP101 BMSCs with plasmid containing an intact NF-κB site showed a 6.8 ± 0.4-fold increase in CAT activity triggered by IM-9 MM cell adhesion (n = 3, P < .05). Transfection of LP 101 cells with plasmid containing a single base pair deletion from the NF-κB binding motif abolished the MM adhesion-induced increase in CAT activity, whereas transfection with plasmid containing three copies of synthetic NF-κB sequence resulted in an 8.1 ± 0.7-fold increase in CAT activity related to MM adhesion (n = 3, P < .05). These data suggest that the NF-κB site is one of the essential regulatory elements for MM cell adhesion-induced IL-6 transcription in BMSCs. Electrophoretic mobility shift assays confirmed the involvement of NF-κB activation in regulating MM adhesion-induced IL-6 transcription in BMSCs. Further characterization of the upstream events in the signaling cascade regulating IL-6 may not only delineate mechanisms of IL-6 regulation during paracrine MM cell growth, but also provide new therapeutic strategies based on interruption of IL-6 mediated tumor cell growth.

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increases in IL-6 secretion by BMSCs. Paraformaldehyde fixation of BMSCs before MM adhesion abrogated this response, suggesting that BMSCs are the major source of IL-6. Adherence of IM-9 MM cells to LP 101 or AA 101 BMSCs triggered a transient increase in IL-6 transcripts that preceded maximal IL-6 secretion. Full-length or deletion fragments of the IL-6 promoter linked to the chloramphenicol acetyltransferase (CAT) reporter gene were transfected into BMSCs before MM cell adhesion; these experiments indicated involvement of the NF-κB binding site in the induction of IL-6 gene transcription. Finally, electrophoretic mobility shift assays (EMSAs) showed increased NF-κB binding activity in BMSCs after MM cell adhesion, and antibodies against members of the rel/NF-κB family revealed the presence of p50, c-rel, and smaller quantities of p65 in the complex induced by IM-9 MM cell adherence to LP101 BMSCs. These studies confirm a role for NF-κB activation in regulation of IL-6 transcription triggered in BMSCs after MM cell adhesion.

MATERIALS AND METHODS

Cells and cell culture. The IM-9, ARH-77, HS Sultan, and U-266 human MM-derived cell lines were obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI-1640 containing 10% to 15% fetal bovine serum (FBS), 100 U/mL penicillin (pen), and 100 μg/mL streptomycin (strept) (GIBCO, Grand Island, NY). The normal human BMSC lines LP101 and AA101 were kindly provided by Dr Shin Ajizawa (Tokyo Medical College) and cultured in Iscove's Modified Dulbecco Medium (IMDM), containing 10% FBS, and pen/strep. Assays of adhesion of MM cell lines to BMSCs were done as previously described. Briefly, BMSCs were cultured (1 x 10^5 cells/mL) for 24 hours to obtain a confluent monolayer. After BMSCs formed a confluent adherent layer, remaining nonadherent cells were washed three times with Hanks' Buffered Saline Solution. Myeloma cells (5 x 10^7 cells/mL) were then added directly to BMSCs. After incubation at 37°C for 24 hours, the supernatants were collected, and remaining bound cells were procured for either Northern blot analysis or CAT assays.

Measurement of IL-6 secretion. IL-6 levels in the supernatants of BMSCs cultured in media or with adherent MM cell lines were measured in a bioassay using the B9 IL-6 dependent hybridoma cells, as previously described. Test samples were heat-inactivated (56°C for 30 minutes) and sterile filtered before use. Triplicate test samples were serially diluted in 200 μL microtitre plates (100 μL/well) and 100 μL of B9 cells (5 x 10^5 cells/well) suspended in IMDM containing 5% FBS, 5 x 10^-5 mercaptoethanol, and pen/strep. Dilutions of recombinant IL-6 were included as a standard. B9 cells were then cultured for 72 hours, with the addition of tritiated thymidine (7.4 kBq/well) for the last 4 hours of culture. Thymidine uptake by the B9 cells was determined as described; the lower limit of detection of IL-6 was 0.5 pg/mL.

IL-6 was also measured using an enzyme-linked immunosorbent assay (ELISA; Endogen, Cambridge, MA), as previously described. Serial dilutions (100 μL) of test sample supernatants were added in duplicate to 96 well plates (Costar, Cambridge, MA) coated with IgG1 anti-IL-6 antibody (murine IgG1; TORAY, Kanagawa, Japan). Biotinylated detector anti-IL-6 monoclonal antibody (MoAb) (TORAY) was next added and developed with streptavidin (Amersham, Arlington Heights, IL). IL-6 levels in each supernatant were determined by comparison with a standard curve. The level of detection of IL-6 was 1 ng/mL.

Measurement of IL-1 and IL-8 secretion. The IL-1 and IL-8 levels in culture supernatants were measured using ELISA kits (Endogen). The minimal detection limits were 50 pg/mL and 1 pg/mL for IL-1 and IL-8, respectively.

RNA isolation and Northern blotting. Total cellular RNA was isolated by a modification of the guanidine-isothiocyanate technique, as previously described. Total cellular RNA (20 μg/lane) was subjected to electrophoresis in a 1% agarose/2.2 mol/L formaldehyde gel, transferred to nitrocellulose paper, and hybridized to one of the following 32P-labeled DNA probes: (1) the pA1 plasmid containing a 2.0-kb Pst I insert of the chicken β-actin gene; and (2) the BamH I-Taq I fragment containing nucleotides 215 to 657 of a full-length human IL-6 cDNA. The hybridizations were performed for 16 to 24 hours at 42°C in 50% (vol/vol) formamide, 2 x SSC (SSC: 0.15 mol/L sodium chloride, 0.015 mol/L sodium citrate), 1 x Denhardt's solution, 0.1% (wt/vol) sodium dodecyl sulfate, and 200 μg/mL salmon sperm DNA. Filters were washed and exposed to Kodak X-Omat XAR film (Eastman Kodak, Rochester, NY) using an intensifying screen. The autoradiograms were scanned using an LKB produker (Bromma, Sweden) Ultrascan XL laser densitometer and analysed with the Gelscan XL software package (LKB). Signal intensity was determined in a linear range and normalized to that for actin.

CAT assays. The various IL-6 CAT constructs were prepared as previously described. Briefly, the parental BAMBHII/Xol fragment of IL-6 promoter was subjected to restriction enzyme digestion with Mva I (–630) and Hho I (–225). 5' deletional constructs were prepared from the pIL-6 CAT 225. The 5' deletions were constructed by BAL 31 resection of pIL-6 CAT 225 from the Xba I site and addition of BamHI linkers. The resulting IL-6 BambHI-Xho I fragments were ligated to pIL-6 CAT 3 digested with BamHI and Xho I to generate 5' deletions (see Fig 3). For cloning, complementary oligonucleotides were treated with kinase, annealed, and inserted at the BamHI site of the plasmid pIL-6 CAT 59, creating pIL-6 CAT 59/NF-κB x 3.

The indicated plasmids (30 μg) (see Fig 3) were transfected into LP101 or AA101 BMSCs using electroporation, as previously described. Briefly, 1.5 x 10^5 BMSCs were procured and washed with phosphate-buffered saline. The cells were resuspended in 800 μL of media without serum, and incubated on ice for 10 minutes. The cells were pulsed with 240 V, 960 mF (Biorad Gene Pulser, Richmond, CA) using the standard vendor's protocol; incubated further on ice for 10 minutes; resuspended in complete media; and then incubated at 37°C. Twenty-four hours after transfection, IM-9 MM cells were added directly to the transfected confluent LP101 or AA101 BMSCs. The transfected BMSCs were also cultured alone as a control. After 24 hours, either BMSCs alone or BMSCs and IM-9 MM cells were procured and lysed by three cycles of freezing and thawing in 0.25 mol/L Tris-HCl (pH 7.8) and 1 mmol/L phenylmethylsulfonyl fluoride. To assay for CAT activity, equal amounts of the cell extracts were incubated with 0.025 μCi [3H]chloramphenicol (Amersham; 57 Ci/μmol), 0.15 mol/L Tris-HCl (pH 7.8), and 0.4 mmol/L acetyl-CoA (Sigma, St Louis, MO) for 4 hours at 37°C. The enzyme assay was terminated by the addition of ethyl acetate. The organic layer containing the acetylated [3H]chloramphenicol was separated by thin layer chromatography using chloroform:methanol (95%/5%; vol/vol). Following autoradiography, both acetylated and unacetylated forms of [3H]chloramphenicol were cut from the plates, and the conversion of chloramphenicol to the acetylated form was calculated by measurement of radioactivity in a β-scintillation counter, as previously described.

The IM-9 MM cells were also transiently transfected with pIL-6CAT 630 and pIL-6CAT 59/NF-κB x 3 constructs. After 24 hours of culture, the transfected IM-9 MM cells were adhered to LP101 BMSCs, and the extracts analysed for CAT activity.

Cotransfections were also performed with a luciferase gene reporter construct (pGL2) as an internal control plasmid, as in previous
studies. In particular, the CAT assays were performed with equal amounts of luciferase activity to normalize for any variation in transfection efficiency. Luciferase enzymatic activity was measured using “luciferin” as a substrate in the ‘Luciferase Assay System,’ per manufacturer’s directions (Promega, Madison, WI). Luminescence was measured with a luminometer (Model TD-20e luminometer; Turner, Sunnyvale, CA). The results showed similar transfection efficiencies for all pIL-6 constructs shown (see Fig 3).

Nuclear extracts and EMSAs. LP101 BMSCs were incubated in the presence or absence of IM9 MM cells for 1 hour, and nuclear extracts were prepared according to Dignam et al. The following double-stranded oligonucleotide was used as a probe in EMSAs:  

\[ \text{IL-6 NF-κB oligonucleotide:} \]
\[ \text{tgacATGTGGGATTTTCCCCATGAc} \]
\[ \text{gTACCCCTAAAGGGTACTgagct} \]

DNA binding reactions and EMSAs were performed as described. The 5′ ends of the oligonucleotides were 32P-labeled with T4 DNA polynucleotide kinase to a specific activity of approximately 5 × 108 cpm/μg and purified. Samples of 20 μL containing 2 μg nuclear extract were incubated with 10,000 cpm labeled oligonucleotides, 1 μg poly(dI-dC) (Pharmacia, Piscataway, NJ), 2 μL of buffer D, 2 μg bovine serum albumin (New England Biolabs, Beverly, MA), in 10 mmol/L Tris pH 7.5, 50 mmol/L NaCl, 1 mmol/L DTT, 1 mmol/L EDTA, 5% glycerol. The samples were incubated for 15 minutes at room temperature, and run on 4% polyacrylamide gels in 1 × TAE (6.7 mmol/L Tris-HCl pH 7.5, 3.3 mmol/L sodium acetate, 1 mmol/L EDTA) at 150 V. In supershift assays, nuclear extracts were preincubated for 10 minutes in the presence of specific antisera (anti-Rel B, anti-c Rel, anti-p65, anti-p50 and anti-p52) (Santa Cruz Biotechnology, Santa Cruz, CA), before addition of probe and incubation for 15 minutes at room temperature.

RESULTS

Adhesion of IM-9 MM cells to LP101 BMSCs triggers IL-6 secretion. In the present study, four MM lines (ARH-77, IM-9, HS-Sultan [HSS] and U266), as well as the myelomonocytic cell line U937 were adhered to normal BMSCs. As in prior studies, MM cell lines showed 40% to 50% specific adherence to BMSCs (data not shown). Peak IL-6 secretion in culture supernatants, measured by ELISA, was observed at 24 hours (Fig 1). IL-6 secretion by normal BMSCs in media alone was 2.1 ± 0.1 ng/mL; in contrast, IL-6 secretion by ARH-77, HSS, IM-9, U266 and U937 cells in media alone was undetectable. Binding of the ARH-77 and U266 cells to BMSCs increased IL-6 secretion by 5.0 ± 0.5-fold and 5.4 ± 0.3-fold, respectively (n = 3, P < .05). Adherence of HS Sultan and IM-9 MM cells triggered 9.9 ± 0.6-fold and 15.8 ± 1.4-fold increases, respectively, in IL-6 secretion by BMSCs (n = 3, P < .05). IL-6 secretion by BMSCs was also increased 6.2 ± 1.8-fold by adhesion of the U937 myelomonocytic leukemic cell line, suggesting that this effect is not specific for MM cells. To define the source of MM cell adhesion-induced IL-6 secretion, BMSCs were fixed with 1% paraformaldehyde before adherence of MM cells. Although MM cell lines ARH-77, HS-Sultan, IM-9, and U266 adhered to the fixed BMSCs, no significant increase in IL-6 secretion was noted (data not shown). These data suggest, as in our previous studies, that the predominant source of adhesion-related IL-6 secretion is rather than tumor cells.

BMSCs obtained from either MM patients or normal donors could not be used to characterize the molecular mechanism regulating adhesion-induced IL-6 secretion because of their low cell numbers and low transfection efficiency. Because our previous studies had showed that adherence of IM-9 MM cells to LP101 BMSCs resulted in increased IL-6 secretion similar to that observed after IM-9 cell adherence to BMSCs (Fig 1), we chose to use these cells as a model system for molecular studies. To assure that observed changes were not unique to LP 101 BMSCs, we also characterized IM-9 MM cell related induction of IL-6 secretion in another BMSC line, AA 101. IL-6 secretion by LP 101 and AA 101 BMSCs in media alone was 387 ± 82 pg/mL and 423 ± 84 pg/mL, respectively (Fig 2A). Adherence of MM cell lines to LP101 BMSCs for 24 hours triggered significant increases in IL-6 secretion: 2.8 ± 0.3 fold for ARH-77 (n = 3, P < .01), 2.4 ± 0.3-fold for HSS (n = 3, P < .01), 3.7 ± 0.7-fold for IM-9 cells (n = 3, P < .01), and 2.2 ± 0.3-fold for U-266 cells (n = 3, P < .02). Similarly, binding of MM cell lines to AA 101 BMSCs stimulated increased IL-6 secretion: 2.0 ± 0.3-fold for ARH-77 cells (n = 3, P < .02), 2.0 ± 0.2-fold for HSS cells (n = 3, P < .05), 2.8 ± 0.7-fold for IM-9 cells, and 1.7 ± 0.1-fold for U-266 cells (n = 3, P < .04).

Adhesion of IM-9 MM cells to LP101 and AA 101 BMSCs induces IL-6 mRNA expression. Northern blotting was used to probe for IL-6 mRNA expression during IM-9 MM cell adhesion to LP 101 and AA 101 BMSCs. Undetectable levels of IL-6 transcript were observed in either IM-9 MM cells or LP101 BMSCs cultured alone (Fig 2B). In contrast, adherence of MM cells to BMSCs for 6 hours induced maximal IL-6 mRNA expression (10.3 ± 0.7-fold increments, as determined by densitometry); longer periods of adherence (24 hours) resulted in downregulation of these transcripts. This transient increase in IL-6 mRNA level occurred in the absence of any changes in β-actin mRNA. A similar transient
The IL-6 promoter NF-κB site is required for MM cell adhesion-induced IL-6 gene transcription in BMSCs. To delineate MM cell adhesion-induced signalling pathways in BMSCs that induce IL-6 gene transcription, we next examined (1) whether MM cell adhesion to BMSCs induces the IL-6 promoter, and (2) which regulatory regions within the IL-6 promoter might be activated by adhesion. Cotransfections were performed by using the luciferase construct pGL2 as an internal control, with each of the various pIL-6 CAT constructs. The results showed similar transfection efficiencies for the pIL-6 CAT constructs shown in Fig 3. Low to undetectable constitutive CAT activity was observed on transient transfection of LP101 BMSCs with these constructs. CAT activity was induced by 4.8- to 6.6-fold in all promoter constructs up to position -59. Specifically, a 630-bp fragment of the IL-6 promoter, as well as different 5' deletion mutants thereof, were linked to the CAT reporter gene (Fig 3). The IL-6 promoter CAT construct (pIL-6CAT 630) was transiently transfected into LP101 BMSCs and IM-9 MM cells were added to a confluent layer of transfected LP101 BMSCs. The results showed an increase of 5.6 ± 0.6-fold in CAT activity in cell lysates from cocultures of pIL-6CAT 630 transfected LP101 cells and IM-9 cells, compared with cell lysates from pIL-6CAT 630 transfected LP101 cells alone (n = 3, P < .05), suggesting an induction of promoter activity by IM-9 cell adhesion.

The 630-bp IL-6 promoter construct contains an AP-1 binding site (-283 to -277); a cyclic-AMP responsive element (CRE) (-163 to -158); NF-IL-6 (-169 to -135); direct repeats (-128 to -101); an NF-κB binding site (-73 to -63); and TATA box element (-32 to -25). Transient transfections were performed with deletion constructs of the IL-6 promoter that eliminates some of these regulatory elements. Elimination of the AP-1 site, CRE, NF-IL-6, and direct repeats using pIL-6CAT225, pIL-6CAT138, pIL-6CAT126, and pIL-6CAT112 constructs, respectively, did not affect the inducibility of the IL-6 promoter (Fig 3). These data suggested that the inducibility of IL-6 promoter activity in BMSCs in response to MM cell adhesion is not influenced by deletion of the binding site for transcription factor AP-1 (-283 to -277), which has been shown to be induced by DNA damaging agents, e.g., DNA synthesis inhibitor β-D-arabinofuranosylcytosine or UV light; and deletion of the binding site for the transcription factor NF-IL-6 (-169 to -135), known to be induced by IL-1.

In contrast, deletion of the NF-κB binding site resulted in abrogation of transcriptional activation of the IL-6 promoter in response to MM cell adhesion to BMSCs. Transient transfection of LP101 BMSCs with the deletion plasmid pIL-6CAT72, which contains an intact NF-κB site, showed a 6.6 ± 0.4-fold increase in CAT activity in response to IM-9 MM cell adhesion (n = 3, P < .05) (Fig 3). However, a single base pair deletion from the NF-κB binding motif in the pIL-6CAT71 plasmid, which eliminates the NF-κB site, abolished the increase in CAT activity in transfected LP101 BMSCs on adhesion with IM-9 MM cells. These data suggest a potential role of the NF-κB element (-73 to -63) in regulating MM cell adhesion-induced IL-6 gene transcription in BMSCs.

To confirm our findings, we also used the plasmid pIL-6 CAT 59/NF-κB × 3, which contains three copies of synthetic NF-κB sequence (Fig 3). The CAT assay results showed an increase of 8.1 ± 0.7-fold in CAT activity in cell lysates from cocultures of pIL-6CAT 59/NF-κB × 3 transfected LP101 BMSCs and IM-9 MM cells, compared with pIL-6CAT 59/NF-κB × 3 transfected LP101 cells alone (n = 3, P < .05). Taken together, these findings show that the NF-κB site is an essential regulatory element in IM-9 MM cell adhesion-induced IL-6 transcription in LP101 BMSCs.
To address the possibility that a portion of the MM cell adhesion-induced increase in IL-6 transcription or NF-κB activity may be in MM cells rather than BMSCs, additional experiments were performed transiently transfecting the IL-6 CAT 630 or pIL-6CAT 59/NF-κB × 3 constructs into IM-9 MM cells. The transfected IM-9 MM cells were adhered to LP101 BMSCs, and the cellular extracts analyzed for CAT activity. No CAT activity was observed in either MM cells transfected with IL-6 CAT constructs (pIL-6CAT 630 or pIL-6CAT 59/NF-κB × 3) cultured in media alone or in the cultures of transfected IM-9 MM cells adherent to LP101 BMSCs. These data suggest that BMSCs are the predominant source of IL-6 transcription and NF-κB activity in these adhesion assays.

**IM-9 MM cell adhesion to AA101 BMSCs induces IL-6 gene expression.** The present findings, taken together with our and other prior studies,29-31,35 suggest that MM cell adhesion to either freshly isolated BMSCs, long-term BMSC cultures, or BMSC line LP101 stimulates IL-6 transcription and secretion in BMSCs. To test whether other BMSC lines are similarly responsive to adhesion of IM-9 MM cells, we determined whether adhesion of IM-9 MM cells to AA101, another normal human BMSC derived line, also induced IL-6 gene transcription. AA101 cells were transiently transfected with plasmids pIL-6CAT 630, pIL-6CAT72, or pIL-6CAT71 (Fig 3) before IM-9 MM cell adhesion, and cell lysates were analyzed for CAT activity as described above. A 4.8 ± 0.4-fold increase in CAT activity in cell lysates was observed after adherence of IM-9 cells to pIL-6CAT 630 transfected AA101 cells, compared with lysates from pIL-6CAT630 transfected AA101 cells alone (n = 3, P < .05) (Fig 4). Adherence of IM-9 cells to AA101 cells that were transiently transfected with the plasmid pIL-6CAT72 (containing an intact NF-κB element but lacking binding sites for transcription factors AP-1, CRE, and NF-IL-6) resulted in a 6.1 ± 0.6-fold increase in IL-6 promoter activity compared with pIL-6CAT72 transfected AA101 cells alone (n = 3, P < .05). In contrast, IM-9 MM cell adhesion to AA101 cells transfected with plasmid pIL-6CAT71, which has a single base pair deletion from the consensus NF-κB sequence, resulted in no increase in IL-6 promoter activity. These results suggested that NF-κB is potentially involved in the transcriptional activation of the IL-6 gene in AA101 BMSCs in response to MM cell adhesion, as was noted in LP101 BMSCs.

**IM-9 MM cell adhesion to LP101 BMSCs induces NF-κB binding activity.** To determine whether adhesion-induced transcriptional activation of the IL-6 promoter is associated with enhanced binding of nuclear proteins to the NF-κB site, we performed EMSAs using the IL-6 NF-κB site as a probe. Nuclear extracts were prepared from LP101 cells alone, IM-9 MM cells alone, and from IM-9 MM cells adherent to LP101 BMSCs. As positive controls for the activation of NF-κB we used nuclear extracts from unstimulated and CAMP-stimulated Pu5-1.8 monocyctic cells, which we have previously demonstrated to contain inducible NF-κB.36 EMSA analysis showed only low levels of NF-κB in nuclear extracts from either IM-9 MM cells or LP101 BMSCs cultured alone (Fig 5A). In contrast, in nuclear extracts prepared from IM-9 MM cells adherent to LP101 BMSCs for 1 hour (Fig 5A) a strong protein-DNA complex was formed that comigrated with CAMP-inducible NF-κB in Pu5-1.8 nuclear extracts. This protein-DNA complex appears to represent two different complexes that migrate relatively close to each other.

To gain further insight into which members of the rel/NF-κB family activated in the cocultured cells might interact with the IL-6 NF-κB site, we performed supershift assays using specific antiserum to c-Rel, p50, p65, p52, and Rel B. The electrophoretic mobility of the complexes was decreased maximally by p50 antiserum, and to a lesser extent by p65

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Fig 3. IM-9 MM cell-adhesion-induced IL-6 gene transcription in LP101 BMSCs is conferred by NF-κB. LP101 BMSCs were transfected with the indicated IL-6 CAT constructs by electroporation and maintained in media for 24 hours. IM-9 MM cells were added to the confluent layer of adherent IM-9 MM cells and increase over the control. Data shown are the average of three independent experiments.
has been shown to induce the growth of murine plasmacytoma IM-9 MM cells to LPlOl BMSCs does not upregulate either IL-1 or IL-8 expression. These results suggest that adhesion of IM-9 MM cells or BMSCs cultured alone or IM-9 MM cells adherent to LPlOl BMSCs decreases IL-1. Adhesion of IM-9 MM cells to LPlOl BMSCs decreases IL-1 secretion to 43.2 ± 2.7 pg/mL (n = 3, P < .05). IL-8 was not detectable in the supernatants from either MM cells or BMSCs cultured alone or IM-9 MM cells adherent to BMSCs. These results suggest that adhesion of IM-9 MM cells to LPlOl BMSCs does not upregulate either IL-1 or IL-8 expression.

DISCUSSION

IL-6 triggers the terminal differentiation of normal B cells, has been shown to induce the growth of murine plasmacytomas and hybridomas, and has also been proposed to be either an autocrine or paracrine growth factor in human MM. In vitro evidence exists for both autocrine and paracrine mechanisms of IL-6 stimulation of MM cell line growth. Moreover, high serum IL-6 levels observed in patients with advanced MM and plasma cell leukemia, coupled with transient inhibition of MM cell growth using anti-IL-6 antibodies in vivo suggest a role for IL-6 in the pathophysiology of MM. These studies support the view that IL-6 plays a role in growth of secretory B-cell neoplasms, even though other studies find the highest serum IL-6 levels to be present in the setting of quiescent MM.

Although some MM cells secrete IL-6, the major source of IL-6 in MM is BMSCs. Therefore, our studies to date have specifically characterized the adhesion of MM-derived cell lines to BMSCs from both normal volunteers and MM patients, and focused on determining the effect of adhesion on IL-6 secretion by BMSCs and related tumor cell proliferation. In chromium binding assays, the U266, ARH-77, and IM-9 MM cell lines showed specific adherence to normal and MM BMSCs. This binding of MM cells to BMSCs was partially blocked with anti-β1 integrin MoAb, anti-β2 integrin MoAb, and excess RGD peptide, suggesting multiple mechanisms for adhesion of MM cell lines to BMSCs. Importantly, significant increases in IL-6 secretion were evident in MM cell line adherent normal and MM BMSC cultures; paraformaldehyde fixation of BMSCs prior to adhesion abrogated IL-6 secretion, suggesting that IL-6 secretion was triggered primarily in BMSCs rather than MM cells. These studies support the view that adhesion to BMSCs may not only localize tumor cells in marrow, but also permits cell-to-cell contact that is essential to trigger IL-6 secretion by BMSCs. Moreover, adherance of IL-6-dependent B9 cells increased their proliferation, suggesting that adherence may trigger IL-6-mediated tumor cell proliferation in a paracrine growth mechanism. The current studies extend these observations and show that MM cell line adherence to BMSC lines also triggers IL-6 secretion, as is true for MM and normal BMSCs. Moreover, they show that MM cell adhesion to BMSCs stimulates transient induction of IL-6 transcription, which precedes peak IL-6 secretion.

To understand the molecular mechanisms regulating IL-6 gene expression in BMSCs triggered by tumor cell adherence, we have begun to systematically analyze the transcriptional control mechanisms induced by adherence of MM cells to BMSCs. Although the specific ligand receptor interaction between MM cells and BMSCs triggering IL-6 remains undefined, these studies nonetheless likely reflect the interaction between tumor and BMSCs that occurs in the marrow microenvironment in vivo. We have found that induction of IL-6 secretion in BMSCs by MM cells is indeed at least partially because of an increase in the transcription rate of the IL-6 gene, suggesting the activation and/or inactivation of specific transcription factors that can bind to the regulatory regions of the IL-6 promoter. Several transcriptional control elements have been described on the IL-6 promoter: an AP-1 site, the multiple regulatory element site which contains a CRE, the NF-IL6 site, and the NF-κB site. Other potential regulatory elements include a glu-

![Fig 4. IM-9 MM cell adherence to AA 101 BMSCs induces IL-6 promoter activity. BMSC line AA101 was transfected with the plasmids pIL-6 CAT630, pIL-6 CAT71, or pIL-6 CAT72. Cells were cultured for 24 hours in media with or without adherent IM-9 MM cells, and conversion of chloramphenicol to its acetylated form was then determined. The results (mean ± SD of three independent experiments) are expressed as the fold increase in percentage conversion of chloramphenicol to its acetylated form for cultures of IM-9 MM cells adherent to BMSCs, relative to control cultures of transfected BMSCs cultured alone.](image-url)
Fig 5. IM-9 MM cell adhesion to LP101 BMSCs induces NF-κB binding activity. (A) Nuclear proteins were isolated from the IM-9 MM cells alone, LP101 BMSCs alone, and from IM-9 MM cells adherent to LP101 BMSCs. Nuclear extracts were then examined for NF-κB binding activity with end-labeled probe containing the NF-κB sequence present within the IL-6 promoter. (B) Nuclear extracts from IM-9 MM cells adherent to LP101 BMSCs were analyzed for the specificity and composition of the NF-κB complexes in supershift EMSAs, by probing with specific antiserum indicated.

corticoid responsive element and a region highly analogous to the retinoblastoma control element. In the present studies we show, using promoter deletion analysis, that the NF-κB site appears to be at least one of the crucial regulatory elements for induction of the IL-6 promoter by MM cell adherence to BMSCs. This is correlated with an increase in nuclear NF-κB binding activity. Involvement of NF-κB motifs in IL-6 regulation has been extensively characterized in other cell systems, and our future studies will be directed at defining those signaling cascades acting through NF-κB to upregulate IL-6 in BMSCs to delineate and eventually interfere with IL-6-mediated paracrine growth mechanisms in MM.

Deletion analysis of the promoter has the disadvantage of interrupting potential cooperative interactions among different regulatory elements and, therefore, will only give partial answers. This is especially important because a cooperative interaction between NF-IL-6 and NF-κB has been postulated; for example, transcription factors NF-IL-6 and NF-κB synergistically activate transcription of the inflammatory cytokines IL-6 and IL-8. We have shown in this study that adherence of MM cells to BMSCs leads to a transient activation of IL-6 gene transcription, as shown by a correlation of an increase in IL-6 mRNA with increased IL-6 promoter activity. Enhanced IL-6 promoter activity appears to be associated with activation of NF-κB and is abolished on deletion of the IL-6 NF-κB site. These studies suggest that NF-κB is involved in IL-6 gene induction by MM cell adher-
ence to BMSCs and is essential for this process, but that other transcription factors might also be induced, which together with NF-kB, lead to induction of IL-6 gene expression. More detailed analysis of other regulatory elements is currently underway using site-directed mutations of individual regulatory elements within the context of the full-length IL-6 promoter. These studies of the mechanisms regulating expression of IL-6 may eventually suggest innovative therapies that interfere with IL-6 production in MM.

REFERENCES


upregulate IL-6 but downregulate osteocalcin production by osteoblastic cells through cell-cell-contact. Blood 86:3151, 1995


47. Ishihiki H, Akira S, Tanabe O, Nakajima T, Shimamoto T, Hirano T, Kishimoto T: Constitutive and IL-1 inducible factors interact with the IL-1 responsive element in the IL-6 gene. Mol Cell Biol 10:2577, 1990


60. Ballester OF, Moscinski LC, Lyman GH, Chaney JV, Saba HI, Spiers ASD, Klein B: High levels of interleukin-6 are associated with low tumor burden and low growth fraction in multiple myeloma. Blood 83:1903, 1994


Multiple myeloma cell adhesion-induced interleukin-6 expression in bone marrow stromal cells involves activation of NF-kappa B

D Chauhan, H Uchiyama, Y Akbarali, M Urashima, K Yamamoto, TA Libermann and KC Anderson