

The Role of IL-1 in Myeloma Cell Proliferation

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SPECIFIC AIMS:

Multiple myeloma is a cancer of the plasma cells of the blood. In this cancer, a B cell divides uncontrollably and develops into an abnormal plasma cell, called a myeloma cell. Interleukin-1 (IL-1) is a cytokine that has been shown to play an important role in the proliferation of cancerous plasma cells. IL-1 is not expressed by plasma cells from healthy individuals but is expressed by myeloma cells in virtually all patients with multiple myeloma. This differential expression of IL-1 suggests that IL-1 may be important in the pathogenesis of multiple myeloma. The IL-1 promoter contains a functional binding site for CBF1, a transcriptional repressor in mammalian cells that represses IL-1 expression in normal B cells. When CBF1 binding to the IL-1 promoter in B cells is dysregulated, IL-1 expression increases. This leads to the hypothesis that increased expression of IL-1 in myeloma cells may be due to disruption of CBF1 binding to the IL-1 promoter.

(1) To identify the defect in the transcriptional regulation of IL-1 in myeloma cells.

Hypothesis: Defects in CBF1 repression of IL-1 transcription lead to myeloma cell proliferation.

Bone marrow stromal cells are fibroblast-like cells that support myeloma cell proliferation through the release of cytokines such as IL-6. The production of IL-1 by myeloma cells may be responsible for stimulating the generation of IL-6 by bone marrow stromal cells. In several examples of IL-1-induced IL-6 release, IL-1 signals through the p38 mitogen activated protein kinase (MAPK) cascade. This leads to the hypothesis that in myeloma cells, IL-1 signals through the p38 MAPK pathway to stimulate release of IL-6 from bone marrow stromal cells.

(2) To delineate the signaling pathways invoked by IL-1 that stimulate the release of IL-6 from bone marrow stromal cells in multiple myeloma.

Hypothesis: IL-1 signals through the p38 MAPK pathway in order to stimulate release of IL-6 from bone marrow stromal cells in multiple myeloma.

The results of the proposed work will provide a better understanding of the molecular basis of myeloma cell progression and could be used to develop possible therapies for treating multiple myeloma.

BACKGROUND:

Multiple myeloma is a plasma cell neoplasm that accounts for 10% of all hematologic cancers (1). Each year approximately 14,000 new cases of multiple myeloma are diagnosed and an additional 9,100 patients die from the disease. Median survival rates from the date of diagnosis are three years, (2) and only approximately 5% of patients will live 10 years after diagnosis (3). Understanding the molecular mechanisms by which multiple myeloma progresses could lead to therapies to treat this serious disease.

The plasma cells of the blood are responsible for the production of antibodies (immunoglobulins) that are released in response to antigens. Plasma cells are terminally-differentiated B-cells. When stimulated by antigen, mature B cells proliferate and differentiate into memory B cells and plasmablasts. The plasmablasts further differentiate into either short-lived plasma cells that survive for 3 days or into long-lived plasma cells that survive for 30 days. The plasmablasts that precede the long-lived plasma cells migrate to the bone marrow, where they

interact with stromal cells and undergo differentiation into long-lived plasma cells (4). The cytokine interleukin-6 (IL-6) causes a late B cell to terminally differentiate into a plasma cell. Normal B-cells produce immunoglobulin in response to IL-6 but do not proliferate uncontrollably (4).

Multiple myeloma affects the long-lived plasma cells that are in close association with the stromal cells in the bone marrow. In the bone marrow, the myeloma cells and the stromal cells both release cytokines and interact through adhesion molecules, which activate the stromal cells that further support proliferation of the myeloma cells (10,11). Multiple myeloma proliferation is dependent upon these growth factors the same way that plasmablasts are dependent upon the same growth factors to proliferate and differentiate into mature plasma cells.

The Role of Cytokines in Myeloma Cell Proliferation

Two cytokines that have been shown to have an important role in myeloma cell proliferation are interleukin-1 (IL-1) and IL-6 (12-15). IL-1 induces bone lesions characteristic of multiple myeloma through its action as an osteoclast-activating factor. An osteoclast is a large multinucleated cell that erodes bone matrix, tunneling deep into bone, forming cavities into which capillaries grow (20, 21). Additionally, production of IL-1 by myeloma cells is responsible for stimulating IL-6 release by the stromal cells (20, 21). IL-6 release by bone marrow stromal cells may in turn stimulate the further release of IL-1 by the myeloma cells (Fig. 1). Thus, the IL-6 and IL-1 regulate one another in a positive feedback loop.

FIGURE 1: Role of IL-1 in myeloma cell proliferation.

(Lust, 1993)



Interleukin-6

IL-6 has been shown to be one of the most important growth factors in myeloma cell survival *in vitro* and *in vivo* through several experimental and clinical findings. In one of these experiments, IL-6 was shown to induce *in vitro* growth of myeloma cells that were freshly isolated from patients (16). In another experiment, myeloma cells were shown to spontaneously produce IL-6 and express IL-6-receptor, which normally only bone marrow stromal cells produce (17). Still another experiment showed that anti-IL-6 antibodies inhibited the growth of multiple myeloma cells or cell lines *in vitro*, and a clinical trial showed that treatment of multiple myeloma patients with monoclonal antibodies to IL-6 showed some antitumor effect (18, 19). Stromal cells secrete IL-6 to keep myeloma cells proliferating, and some of the plasma cells acquire the ability to produce the cytokine themselves (4, 21). The signaling pathway through which IL-1 released from myeloma cells stimulates the release of IL-6 from stromal cells is unknown. In many examples of IL-1-induced IL-6 release, including in human fibroblast-like synoviocytes and in macrophages, IL-1 signals through the p38 MAP kinase cascade (48-51). Involvement of the p38 MAP kinase pathway in IL-1-induced IL-6 synthesis in human MRC-5 lung fibroblasts was demonstrated by the use of the specific p38 MAP kinase inhibitor

SB203580 (33). Delineating the signaling pathway through which IL-1 stimulates IL-6 release from bone marrow stromal cells would be an important development for designing therapies used in the treatment of multiple myeloma.

Interleukin-1

IL-1 mediates several physiological responses to infections and injuries, including stimulation of thymocyte proliferation, B lymphocyte maturation and proliferation, induction of acute-phase protein synthesis by hepatocytes, and induction of fever (34,35). Additionally, intracellular IL-1 acts as a specific endogenous competitive inhibitor of certain apoptosis-associated proteins such as FAS in human B lymphoblastoid cells, highlighting an important role for IL-1 in inhibiting apoptosis and therefore enhancing cell survival and possibly cell proliferation (36). IL-1 mRNA is expressed by plasma cells from virtually all patients investigated with multiple myeloma, while it was not detectable in healthy individuals (25, 26), suggesting that IL-1 may be important in the pathogenesis of multiple myeloma.

Production of IL-1 by myeloma cells may be responsible for stimulating generation of IL-6 by the stromal cells and several adhesion molecules important for myeloma cell adhesion to bone marrow stromal cells (20, 21). In addition, this cytokine is an important mediator in inflammation, hematopoiesis, and lymphocyte activation and is also an osteoclast activating factor (21). The IL-1 gene is highly inducible and its expression can be affected by many microbial and cellular products (22). It is normally under stringent transcriptional control both at the transcriptional level by cis- and trans-acting factors and at the posttranscriptional level through translation and the processing of the mature protein (23, 24). Transcriptionally, IL-1 may be regulated by CBF1. This protein is a transcriptional repressor in mammalian cells that functions by binding to DNA and inhibiting the transactivation of genes (38). Krauer *et al.* demonstrated that the IL-1 promoter in immortalized lymphoblastoid cell lines contains a functional binding site for CBF1 (37). This DNA binding protein binds to the DNA consensus sequence 5-GTGGGAAA (39), which has been identified in the promoter region of various cytokines and their receptors, including IL-1, interferon-, IL-2R-, IL-6R, and IL-2 (37, 40). Thus, CBF1 may control the expression of many cytokines.

CBF1 has been shown to repress IL-1 transcription in B cell lines (37). Krauer *et al.* studied the dysregulation of CBF1 repression of IL-1 transcription by Epstein-Barr virus latent proteins EBNA 3 and EBNA 6. When B cells are transformed by Epstein-Barr virus, EBNA 3 and EBNA 6 dysregulate CBF1 repression of the IL-1 promoter and IL-1 is expressed (29-31, 37). This model of modulation of expression of IL-1 by CBF1 in B cell lines leads to the hypothesis that this transcriptional repressor is dysregulated in myeloma cells, leading to cell proliferation.

SUMMARY:

Multiple myeloma is a plasma cell neoplasm that accounts for 10% of all hematologic cancers. In this disease, long-lived plasma cells divide uncontrollably. The cancerous myeloma cells interact with bone marrow stromal cells through the release of cytokines and adhesion molecules. Two cytokines that play an important role in myeloma cell proliferation are IL-1 and IL-6. IL-6 has been shown to be an important growth factor in myeloma cell survival, acting both as an autocrine growth factor and as an anti-apoptotic factor to myeloma cells. IL-1 is not secreted by normal plasma cells and may be important in the pathogenesis of multiple myeloma. IL-1 can induce paracrine expression of IL-6 and several adhesion molecules important in myeloma cell pathogenesis. Therefore,

understanding the molecular mechanisms that regulate IL-1 expression and IL-6 release could lead to therapies to prohibit multiple myeloma disease progression.

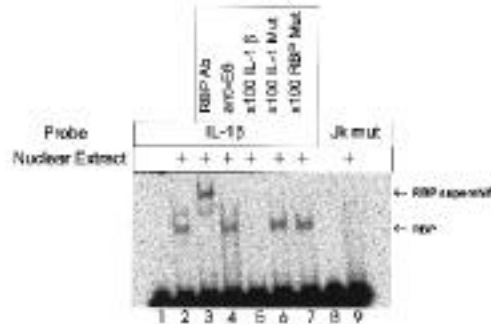
PRELIMINARY DATA:

Krauer et al. studied the regulation of IL-1 transcription by Epstein-Barr virus (EBV) latent proteins (EBNAs) in B cells (37). As in multiple myeloma, IL-1 is upregulated in B cells transformed by EBV. After EBV infection *in vitro*, B cells express the full set of EBV latent antigens that act to initiate and maintain B cell growth. Expression of these latent proteins, which include EBV nuclear proteins (EBNAs) 1–6 and the latent membrane proteins (LMP-1 and -2), results in the transformation of B cells and the generation of lymphoblastoid cell lines. There is evidence that IL-1 is synthesized by EBV-transformed B cells (45, 46) and that IL-1 may function as an autocrine growth factor of these cells (46, 47). The EBNA 2, 3, 4, and 6 gene products have been shown either by immunoprecipitation, the yeast two-hybrid system, or *in vitro* binding assays to interact with the DNA-binding protein CBF1 (38, 41-44). Interaction of these EBNA gene products with CBF1 prevents CBF1-mediated repression of IL-1 in EBV-transformed B cell lines. These data serve as a model for the molecular mechanisms that may cause IL-1 expression in myeloma cells and lead to the hypothesis that dysregulation of CBF1 repression of the IL-1 promoter in myeloma cells leads to cell proliferation.

The IL-1 promoter contains a CBF1 binding site.

Nuclear extracts prepared from dG75 EBV- minus Burkitt's lymphoma B cells were incubated with ³²P-labeled IL-1 probe, and binding complexes were analyzed on polyacrylamide gels (Fig. 2). These results show that two complexes were formed when dG75 nuclear extract was incubated with the IL-1 probe (Fig. 2, lane 2). The subsequent addition of anti-CBF1 monoclonal antibody led to the lower complex being super-shifted, demonstrating the presence of CBF1 in this complex (Fig. 2, lane 3). The addition of an irrelevant antibody (anti-E6) led to no change in the binding complex (Fig. 2, lane 4). To demonstrate the specificity of this lower complex for the CBF1 DNA consensus site, binding reactions were performed in the presence of unlabelled probe. The addition of excess unlabelled probe led to abolition of the binding of the CBF1 protein to the radio-labeled probe (Fig. 2, lane 5). Competition experiments also were performed using a probe with six of seven bases in the CBF1 DNA consensus site mutated (MUT). The CBF1 binding complex was not altered by addition of this mutated probe (Fig. 2, lane 6). As an additional control for CBF1 binding to the IL-1 promoter sequence, a probe was prepared in which the first two bases of the CBF1 site were mutated (CBF1 mut). The addition of nuclear extract to this probe did not result in binding of the lower complex CBF1 (Fig. 2, lane 9), indicating that interaction of CBF1 to its DNA consensus sequence requires the presence of the first two bases in the DNA site. Competition of the IL-1 probe with excess CBF1 mut probe resulted in no change in the CBF1 binding complex, whereas the weaker upper band was abolished (Fig. 2, lane 7). These results demonstrate that CBF1 is able to specifically interact with the CBF1 DNA consensus sequence present in the IL-1 promoter.

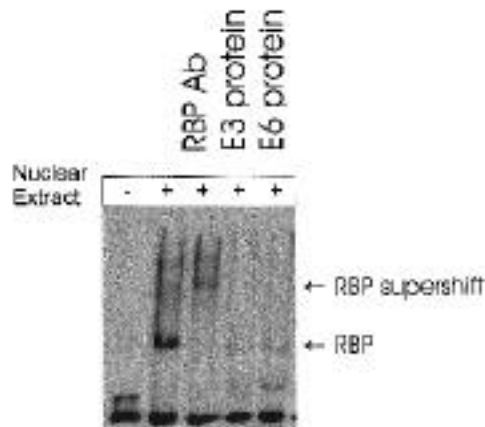
FIGURE 2. CBF1 specifically interacts with the CBF1 DNA consensus sequence present in the IL-1 promoter.



EBNAs 3 and 6 decrease CBF1 binding to its consensus binding sequence.

EBNAs 3 and 6 have previously been shown to bind CBF1 and prevent it from binding to its DNA recognition site in the IL-1 promoter (43). To determine whether this may be the mechanism by which EBNA 3 and 6 up-regulate IL-1, gel shift analyses were performed in the presence of baculovirus-purified EBNA 3 and 6 proteins (Fig. 3) (37). The addition of purified EBNA 3 (Fig. 3, lane 4) or EBNA 6 (Fig.3, lane 5) resulted in a decrease in the binding of CBF1 to the IL-1 probe (Fig. 3, lane 2). These results confirm the findings of Robertson et al. (43) that the EBNA 3 family proteins can disrupt the binding of CBF1 to its consensus binding site and suggest that EBNA 3 and 6 may up-regulate IL-1 via interaction with CBF1.

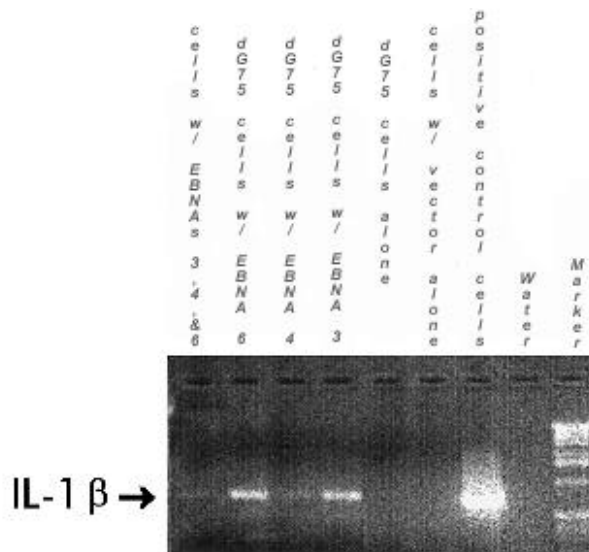
FIGURE 3. EMSA for CBF1 activity in dG75 nuclear extracts after addition of purified baculovirus EBNA 3 and 6 proteins using the IL-1 probe (5'-TCTTCTAACGTGGGAAAATCCAGT-3'). The positions of the CBF1 and CBF1 supershifted complexes are indicated by the position of the arrows. The lanes show the addition of nuclear extract (+ or -), CBF1 antibody (CBF1 Ab), and purified baculovirus EBNA 3 (E3 protein) and EBNA 6 (E6 protein).



The Epstein-Barr Virus EBNA 3 family of latent proteins upregulate IL-1 .

To identify which of the EBNA proteins may have been responsible for the up-regulation of IL-1 , Krauer *et al.* used RT-PCR to quantify IL-1 cDNA in a panel of cell lines stably transfected with individual EBNA genes. After 35 cycles of PCR, an 800-bp fragment corresponding to IL-1 was detected in dG75 cells transfected with either EBNA 3, 4, 6, or all three EBNAs, whereas dG75, dG75 EC3 (control vector transfected cells expressing EBNA 1), and the water control was negative (Fig. 4). Expression of EBNA 3 or 6 alone led to greater increases in IL-1 message than EBNA 4 or expression of all EBNA 3 family proteins.

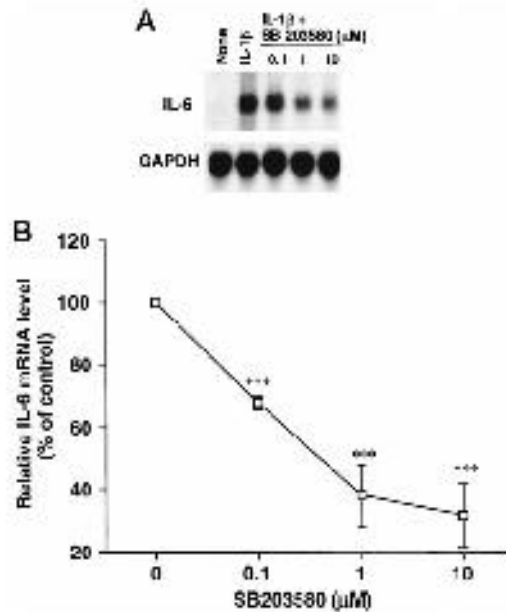
FIGURE 4. Expression of EBNA 3 or 6 alone leads to increases in IL-1 message.



P38 MAP kinase is activated in response to IL-1 in human fibroblast-like synoviocytes.

Miyazawa *et al.* (48) demonstrated that p38 MAP kinase is activated in response to IL-1 in human fibroblast-like synoviocytes (FLSs) and is involved in IL-6 synthesis by stabilizing IL-6 mRNA. In response to stimulation by IL-1 and other factors, FLSs secrete enzymes involved in the degradation of cartilage. They also secrete IL-6, which allows the FLSs to proliferate (48). When SB203580, a p38 Map kinase inhibitor was added to FLS cells, IL-1 -induced IL-6 mRNA was greatly reduced (48). SB203580 (0.01-10 μM) inhibited protein production of IL-6 in a concentration-dependent fashion, suggesting that SB203580 acts on human FLSs to suppress IL-6 synthesis. These results were obtained by stimulating human FLSs with Il-1 for 6 h and adding various concentrations of SB203580 at the start of the designated cultures. Total cellular RNA (20 μg) were then prepared, fractionated on a 1.2% (w/v) agarose gel, transferred onto Hybond N nylon membranes, and then hybridized to human IL-6 cDNA probes or GAPDH cDNA as a quantitative control. (Fig. 5) (48).

FIGURE 5. Dose-response study of SB203580 effect on IL-1 -induced IL-6 mRNA. Figure A is a Northern blot autoradiograph of the RNA and Figure B shows the relative levels of IL-6 mRNA expression.



EXPERIMENTAL DESIGN:

SPECIFIC AIM 1. To identify the defect in the transcriptional regulation of IL-1 in myeloma cells..

Hypothesis: Defects in CBF1 repression of IL-1 transcription lead to myeloma cell proliferation.

Rationale. It is known that IL-1 is not expressed in plasma cells from healthy individuals, however, IL-1 is expressed by myeloma cells in virtually all patients with multiple myeloma (25, 26). This production of IL-1 by myeloma cells may be responsible for stimulating the generation of IL-6 by bone marrow stromal cells and the generation of several adhesion molecules important for myeloma cell adhesion to the stromal cells (20, 21). This differential expression of IL-1 suggests that IL-1 may be important in the pathogenesis of multiple myeloma.

Krauer *et al* demonstrated that the IL-1 promoter contains a functional binding site for CBF1, a transcriptional repressor in mammalian cells that binds to the consensus sequence 5'-GTGGGAAA-3' (39). CBF1 represses IL-1 expression in normal B cells but that when CBF1 interacts with various EBV proteins such as EBNA 3 or EBNA 6, CBF1 repression is dysregulated and IL-1 expression increases (29-31, 37). These data lead to the hypothesis that increased expression of IL-1 in myeloma cells may be due to disruption of CBF1 binding to the IL-1 promoter.

This specific aim will test whether defects in the repression of IL-1 transcription due to dysregulation of CBF1 binding lead to myeloma cell proliferation. Through *in vivo* analyses we will determine if CBF1 is present in normal plasma cell extracts and contributes to IL-1 repression. Analyses of the IL-1 promoter region will also be performed *in vitro* to better understand the mechanism of transcriptional regulation of IL-1.

*Protocol.***1A. Comparison of CBF1 expression between normal plasma cells and myeloma cells.**

In order to compare CBF1 expression between normal plasma cells and myeloma cells, western blots will be performed. Cell extracts from primary normal plasma cells and from primary myeloma cells isolated from patients (54) will be electrophoresed on 7.5% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The cell lysates will be probed with α -CBF1 monoclonal antibody as described (56) to detect CBF1, and with α -IL-1 from Biosource (37) to detect IL-1. As a positive control for the presence of CBF1, we will use cell extracts of dG75 EBV-negative Burkitt's lymphoma B cells. As a negative control, we will use MUTU III clone 62 EBV-transformed Burkitt's lymphoma B cells. A recombinant CBF1 protein will be made in a yeast system and a known concentration will be input into one lane to serve as a standard (55). α 2 microglobulin will be used as a quantitative control to standardize loadings of the samples (37).

Data analysis and expected results.

If CBF1 is expressed in normal plasma cells, western analysis will detect this expression. The α -CBF1 antibody will bind to any expressed CBF1 protein. The α 2 microglobulin will be used as a quantitative control to standardize loadings of the samples and the recombinant CBF1 protein input at a known concentration will serve as a size standard to identify CBF1 in other lanes. The positive control dG75 cells express CBF1, and therefore a band corresponding to CBF1 will be detected on the blot corresponding to these cells. In the negative control MUTU III clone 62 cells, CBF1 is not expressed and therefore a band corresponding to CBF1 will not be detected. If CBF1 is present in plasma cells, it will be detected when probed with antibody. If CBF1 is dysregulated in myeloma cells, a band may not be detected. Since IL-1 is not expressed in normal plasma cells but is expressed in myeloma cells, this experiment will confirm previous data.

1B. Gel Shift Analysis for presence of CBF1.

IL-1 is not expressed in normal plasma cells, but is expressed in myeloma cells (25, 26). Gene expression can be negatively regulated by binding a transcriptional repressor. CBF1 is a transcriptional repressor that has been shown to bind to the IL-1 promoter (37). To determine if IL-1 is repressed in normal plasma cells due to CBF1 repression, we will ascertain if CBF1 binds to its consensus sequence through electromobility shift assays. A complementary synthetic 32 P-end-labeled double-stranded probe (5'-TCTTCTAACGTGGGAAAATCCAGT-3') corresponding to the CBF1 binding sequence will be prepared, annealed, and used in gel shift analyses. A similar labeled probe with the first two bases of the CBF1 DNA consensus site mutated (5'-TCTTCTAACACGGGGAAAATCCAGT-3') will be prepared and used in this analysis as well to test if interaction of CBF1 to its DNA consensus sequence requires the presence of the first two bases in its DNA binding site. Nuclear extracts prepared from primary normal plasma cells, primary myeloma cells, dG75 B cells as a positive control for CBF1 binding, and EBV-transformed MUTU III clone 62 cells as a negative control for dysregulated CBF1 binding will be prepared and incubated with these probes. Binding complexes will be analyzed on polyacrylamide gels. Subsequent addition of α -CBF1 monoclonal antibody will cause any complexes with CBF1 to supershift. Addition of irrelevant antibody will serve as a negative control. To demonstrate the specificity of the CBF1 complex for the CBF1 DNA consensus sequence, competition experiments will be performed by adding excess unlabeled probe.

Data analysis and expected results.

When nuclear extracts from normal plasma cells are incubated with the ^{32}P -end-labeled probe, a binding complex should occur. Subsequent addition of α -CBF1 should supershift this complex, indicating the presence of CBF1 in this complex. The addition of irrelevant antibody should show no change in the binding complex. When additional unlabeled probe is added, abolition of the binding of CBF1 to the ^{32}P -labeled is expected. When the mutated probe is incubated with cell extracts, binding complexes are not expected to form, indicating that interaction of CBF1 to its DNA consensus sequence requires the presence of the first two bases in the DNA site. The same results are expected for the positive control dG75 cells. When nuclear extracts from myeloma cells are incubated with the probes, binding complexes are not expected to form for any of the conditions, indicating that CBF1 is not expressed. The same results are expected with the negative control EBV-transformed MUTU III clone 62 cells.

1C. *In vitro* transcription.

The induction of IL-1 is mediated at the transcriptional level. An alternative approach to studying the transcriptional regulation of the IL-1 promoter by CBF1 will involve the use of an *in vitro* transcription system (57). In this assay, nuclear extracts prepared from primary normal plasma cells, primary myeloma cells, dG75 B cells as a positive control, and EBV-transformed MUTU III clone 62 cells as a negative control will be used to transcribe a G-free cassette reporter under the control of the IL-1 promoter. A second construct composed of the adenovirus major late promoter and a G-free cassette of 190 bp will be included in all reactions as an internal control of transcription levels (57). In each experiment, varying amounts of α -CBF1 antibody, including no antibody, will be preincubated with the nuclear extracts before the addition of the templates to dysregulate CBF1 binding to the IL-1 promoter. Another informative experiment would be to preincubate the nuclear extracts with varying amounts of the EBV-latent protein EBNA 3, which has been shown to dysregulate CBF1 binding to the IL-1 promoter in EBV-transformed B cells, using the same controls as the experiment proposed above. The transcription reaction mixture will include 10 μCi of [α - ^{32}P]UTP and 0.1 mM 3-*O*-methyl-GTP and the radiolabeled transcripts will be detected and quantified using a phosphoimager. The quantitation ratio of the IL-1 transcript to the internal control will be used as the measurement of each transcription reaction.

Data analysis and expected results.

RNA resulting from the *in vitro* transcription will be purified by phenol/chloroform extraction, ethanol precipitated, and then applied onto an 8 M urea, 6% polyacrylamide gel and visualized by autoradiography (57). When no α -CBF1 antibody or EBNA 3 is added to the reaction mixture, it is expected that in the presence of nuclear extracts from normal plasma cells, transcription will not occur, while in the presence of nuclear extracts from myeloma cells, transcription will occur. The positive control nuclear extracts from dG75 cells also are expected to inhibit transcription, and transcription is expected to occur in the negative control EBV-transformed MUTU III clone 62 nuclear extracts. Addition of α -CBF1 antibody in increasing amounts is expected to dysregulate CBF1 binding to the repressor, so as the concentration of antibody increases for the normal plasma cells and the dG75 cells, transcription is expected to increase but will stay the same for the myeloma cells and the MUTU III clone 62 cells since CBF1 is not expected to bind in these cells. The same results obtained from addition of α -CBF1 antibody are expected to be obtained with the addition of EBNA 3. The results of

these experiments will test if CBF1 is binding to the IL-1 promoter in normal plasma cells and is involved in transcriptional regulation.

1D. Chromatin Immunoprecipitation to demonstrate *in vivo* CBF1 binding in normal plasma cells.

To determine if CBF1 is bound to the IL-1 promoter in plasma cells, thereby repressing transcription of the IL-1 gene, we will immunoprecipitate chromatin from normal plasma cells and myeloma cells (58). Proteins will be crosslinked *in situ* followed by sonication to randomly fragment the DNA. DNA associated with CBF1 will be immunoprecipitated by α -CBF1 antibody. Controls included in this portion of the experiment will include a no antibody control and an unrelated antibody control. After harvesting, washing, and eluting the immunoprecipitated DNA, the crosslinks will be reversed and the DNA will be purified with a 25:24:1 phenol:chloroform:isoamyl alcohol solution followed by ethanol precipitation. The immunoprecipitated DNA will be analyzed using Southern analysis with a synthetic ^{32}P -end-labeled probe (5'-TCTTCTAACGTGGGAAAATCCAGT-3') corresponding to the CBF1 binding sequence. Chromatin from primary normal plasma cells, primary myeloma cells, dG75 B cells as a positive control, and EBV-transformed MUTU III clone 62 cells as a negative control will be immunoprecipitated and analyzed by Southern analysis.

Data analysis and expected results.

This experiment will provide additional evidence of CBF1 binding to the IL-1 promoter in normal plasma cells and dysregulation of CBF1 binding in myeloma cells. When normal plasma cell immunoprecipitates are analyzed by Southern analysis, a band corresponding to CBF1 complexed to its binding site should appear. Conversely, this band should not appear in the immunoprecipitates from the myeloma cells. These results would be compared to the immunoprecipitates from the positive control dG75 cells, where a band is expected to appear and the negative control MUTU III clone 62 cells, where no band is expected. For the immunoprecipitation, no immunoprecipitates should form when no antibody is added, and therefore no bands are expected when analyzed by Southern analysis. The addition of irrelevant antibody should not immunoprecipitate the DNA associated with CBF1 either, and therefore no bands are expected when analyzed by Southern analysis.

Alternative Approaches.

If the Western analysis reveals that CBF1 is not present in normal plasma cell extracts, a protein other than CBF1 in plasma cells may repress the IL-1 promoter. This could be investigated by fractionating cell extracts and identifying which proteins bind to the IL-1 promoter. These proteins could be purified and used in the *in vitro* transcription assay proposed above to determine if any of these proteins repress the reporter. Then the experiments proposed above could be modified to accommodate the new protein, and the factor responsible for repression of IL-1 transcription in plasma cells could be identified and studied.

SPECIFIC AIM 2. To delineate the signaling pathways invoked by IL-1 that stimulate the release of IL-6 from bone marrow stromal cells in multiple myeloma. **Hypothesis:** IL-1 signals through the p38 mitogen activated protein kinase (MAPK) pathway in order to stimulate release of IL-6 from bone marrow stromal cells in multiple myeloma.

Rationale. The cytokines IL-1 and IL-6 have been shown to have an important role in myeloma cell proliferation. Production of IL-1 by myeloma cells stimulates the fibroblast-like bone marrow stromal cells to generate IL-6, which in turn may stimulate further release of IL-1 from the myeloma cells. In most examples of IL-1-induced IL-6 release, IL-1 signals through the p38 MAP kinase cascade (48-51). This leads to the hypothesis that in myeloma cells, IL-1 signals through the p38 MAPK pathway to stimulate release of IL-6 from bone marrow stromal cells. This specific aim will test if IL-1 signals through the p38 MAP kinase pathway to induce the release of IL-6 from bone marrow stromal cells. This hypothesis will be tested through the use of enzyme inhibitors of p38 MAP kinase. To rule out the other major MAP kinase pathways, ERK and SAPK/JNK, inhibitors of these enzymes will also be employed. A complementary approach to testing this hypothesis will be to employ a dominant negative p38 MAP kinase construct.

2A. Effect of enzyme inhibitors on IL-6 production in bone marrow stromal cells.

One method of determining if IL-1 signals through the p38 MAP kinase pathway to stimulate the release of IL-6 from bone marrow stromal cells is to employ pharmacologic enzyme inhibitors. A highly specific inhibitor of p38 MAP kinase is SB203580 (48-53). HS-5 bone marrow stromal cells will be cultured in 24-well plates at 5×10^4 cells/well at 37°C for 24 hours. SB203580 will be added in varying concentrations (0.1-10 μ M) at the beginning of the cultures. After 24 hours the cells will be stimulated with IL-1 (1 ng/ml) for 24 hours. Then the cells will be lysed and the IL-6 concentration of the supernatant will be assayed by a specific sandwich-enzyme-linked immunosorbent assay (52). Briefly, supernatants or serial dilutions of recombinant IL-6 standards (Genzyme) will be incubated overnight at 4°C in 96-well microtiter plates, which will be precoated overnight at 4°C with an anti-human IL-6 monoclonal antibody (2 μ g/mL; R&D Systems). After the plates have been washed, a biotinylated anti-human IL-6 polyclonal antibody (2 μ g/mL) (R&D Systems) will be added and incubated for 4 hours at room temperature. After subsequent incubation for 2 hours at room temperature with horseradish peroxidase-conjugated avidin (Zymed Laboratories, Inc), 3,3',5,5'-tetramethylbenzidine (Dojindo Labs, Kumamoto, Japan) will be added to the wells, and the absorbance at 450 nm will be measured by a microplate reader (Bio-Rad Laboratories) (52). As a control, PD98059, a specific inhibitor of ERK MAP kinase (50,51) will be employed to determine if it has any effect on IL-6 expression by the HS-5 cells. There is no specific inhibitor of SAPK/JNK MAP kinase, but a dominant negative approach (59) could be used to investigate the involvement of this kinase in the IL-1-stimulated release of IL-6, as will be proposed below. For each assay, controls with no inhibitor or IL-1 and controls with no inhibitor but with stimulation by IL-1 will be run.

Data analysis and expected results.

This experiment will test if the p38 MAP kinase inhibitor SB203580 will inhibit IL-6 in a concentration-dependent manner, thereby indicating that SB203580 acts on the HS-5 cells to suppress IL-6 synthesis. If IL-1-induced IL-6 synthesis signals solely through the p38 MAP kinase pathway, the inhibitor of ERK MAP kinase (PD98059) will show no effect on the levels of IL-6 in the cells. The

control with no inhibitor and no IL-1 will show no IL-6 released from the cell since IL-1 is needed for this release. The control with no inhibitor but IL-1 stimulation will show high levels of IL-6.

2B. Effect of pharmacologic enzyme inhibitors on IL-6 mRNA production in bone marrow stromal cells.

Since MAPK is a transcription factor, regulation of IL-6 release by p38 would likely be at the transcriptional level. To determine if SB203580 affects the steady-state levels of IL-6 mRNA in bone marrow stromal cells, Northern analysis will be performed. HS-5 bone marrow stromal cells will be grown to confluence in 75 cm² culture flasks at 37°C, with various amounts of SB203580 added at the start of the designated cultures (0.1 – 10 µM). The cells will then be stimulated for 6 hours with IL-1 (1ng/ml). Total cellular RNA will then be prepared by use of a Qiagen *Rneasy* kit. Northern blotting will be performed as described previously (53). RNA will be loaded onto a 1.2% (w/v) agarose gel containing formaldehyde and, after electrophoresis, will be transferred onto Hybond N nylon membranes (Amersham Pharmacia Biotech) by use of a VacuGene XL vacuum blotting apparatus (Amersham Pharmacia Biotech). Specific mRNAs will be detected by hybridization with randomly primed ³²P-labeled cDNA probes. The probes used will be a 1.16 kb pair *EcoRI/EcoRI* fragment of human IL-6 cDNA, and a 0.8 kb pair *PstI/XbaI* fragment of human GAPDH cDNA to normalize the signal. Relative levels of IL-6 mRNA expression to GAPDH will be determined by densitometric scanning of the autoradiographic bands and normalized to the GAPDH signal. As a control, PD98059, a specific inhibitor of ERK MAP kinase will be employed to determine if it has any effect on IL-6 mRNA expression by the HS-5 cells. There is no specific inhibitor of SAPK/JNK MAP kinase, but the dominant negative approach proposed below can be used to investigate the involvement of this kinase in the IL-1 -stimulated release of IL-6 mRNA. For each assay, controls with no inhibitor and no IL-1 and controls with no inhibitor but stimulation by IL-1 will be run.

Data Analysis.

It is expected that SB203580 will inhibit IL-1 -induced IL-6 mRNA expression in a concentration-dependent manner. When the Northern blots are analyzed, the control with no IL-1 and no inhibitor will show no band corresponding to IL-6 mRNA. The control with IL-1 only, with no inhibitor will show the strongest band corresponding to IL-6 mRNA expression. As SB203580 is added in increasing amounts, the band corresponding with IL-6 mRNA will decrease in intensity. The GAPDH will be of equal intensity in all lanes. If IL-1 -induced IL-6 mRNA synthesis signals solely through the p38 MAP kinase pathway, the inhibitor of ERK MAP kinase (PD98059) will show no effect on the levels of IL-6 in the cells.

2C. A dominant-negative strategy.

A complementary approach to the use of enzyme inhibitors in investigating if IL-1 -induced expression of IL-6 signals through the p38 MAP kinase pathway is to employ a dominant negative (59) of MEKK1, an immediate upstream regulator of p38 (60). We will transiently transfect HS-5 human bone marrow stromal cells with either an empty pSR expression vector control, a wild type pSR -MEKK1, or a dominant-negative pSR -MEKK1 expression vector (61). HS-5 cells will be seeded into 100 mm dishes and transfected at 80% confluency with optimized levels of vector. After 24 hours, cells will be stimulated with IL-1 (1 ng/ml) for 6 hours, then lysed. The lysates will be

immunoprecipitated with anti-MEKK1 polyclonal antibody, separated on SDS-PAGE, and transferred onto nitrocellulose. The membrane will first be blotted with anti-MEKK1 to confirm equal precipitation of the wild type and dominant negative proteins, then the membrane will be blotted with anti-IL-6 to evaluate the levels of IL-6 (59). Controls using dominant-negative constructs for ERK MAP kinase and JNK/SAPK MAP kinase (61) will be employed to eliminate the possibility of IL-1 signaling through these pathways to stimulate release of IL-6. For ERK, the dominant negative used will be MEK1, an immediate upstream regulator of ERK. For SAPK, the dominant negative used will be SEK-AL, a dominant-negative mutant of SEK1, an immediate upstream regulator of JNK/SAPK. This protocol will also be performed using a control where IL-1 is not added to the cells.

Data Analysis.

It is expected that the MEKK1 dominant-negative will inhibit IL-1 -induced IL-6 mRNA expression. To test this, analysis of the Western blots of the empty pSR control and wild-type pSR -MEKK1 control would show a band corresponding to IL-6, since its expression would not be inhibited. In the case of the dominant-negative pSR -MEKK1, no band corresponding to IL-6 would appear because IL-6 expression should be inhibited. For all ERK MAP kinase and JNK/SAPK MAP kinase controls, a band corresponding to IL-6 would appear. For the controls when IL-1 is not added to the cells, IL-6 expression is not expected.

Alternative approaches.

The majority of studies on IL-1 -induced IL-6 expression have shown that IL-1 signals through a MAP kinase pathway (48-51). If IL-1 -induced IL-6 expression does not signal through the p38 MAP kinase pathway, it is likely to signal through one of the other MAP kinase pathways. If IL-1 signals through the ERK MAP kinase pathway or the JNK/SAPK MAP kinase pathway, these experiments will appropriately test this.

The proposed work will provide a better comprehension of the molecular basis of myeloma cell progression. The results of this proposal will contribute greatly to the understanding of multiple myeloma and to the design of therapies to treat this disease.

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