Advances in Brief

Prevention of Myeloma Cell Apoptosis by Ectopic *bcl-2* Expression or Interleukin 6-mediated Up-regulation of *bcl-x$_L$*[1]

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Abstract

Upon cytokine withdrawal, interleukin (IL) 6-dependent murine plasmacytoma/hybridoma (myeloma) cells die in a way characteristic of apoptosis. Although gene transfer-mediated elevation in Bcl-2 protein levels has been demonstrated to repress a number of apoptotic death programs, it has been reported that ectopic *bcl-2* expression is unable to prolong the survival of IL-6-deprived myeloma cells. In view of the recent identification of Bax as a protein that antagonizes the anti-apoptotic function of Bcl-2, we sought to determine whether the inability of transfected *bcl-2* to protect against myeloma cell apoptosis might simply be due to insufficient levels of Bcl-2 protein produced to counteract this inhibitor. We show here that high-level expression of an exogenous *bcl-2* gene, introduced into IL-6-dependent B9 myeloma cells via retroviral or bovine papilloma virus-based vectors, is indeed able to suppress apoptotic death following cytokine deprivation, with the extent of protection provided correlating with the amount of Bcl-2 protein synthesized in relation to the amount of endogenous Bax protein present in the cells. Of note, however, we found that IL-6-mediated suppression of B9 apoptosis does not involve induction of endogenous *bcl-2* expression but is associated instead with the up-regulation of cellular *bcl-x* mRNA and Bcl-x$_L$ protein. These results thus extend the apoptotic death mechanisms that are inhibitable by both *bcl-2* and *bcl-x$_L$* to include that operative in IL-6-dependent cells and suggest that apoptosis in other cell types using the gp130 subunit of the IL-6 receptor might also be *bcl-2* regulable or *bcl-x$_L$* dependent.

Introduction

IL[3]-6 is a pleiotropic cytokine that functions as a major growth factor for murine and human plasma cell tumors (myelomas; Ref. 1). Previous work from our laboratory showed that besides stimulating proliferation of myeloma cells, IL-6 promotes their survival by suppressing apoptosis (2). The *bcl-2* proto-oncogene, which was identified at the t(14;18) chromosomal breakpoint of follicular lymphoma (3), has been documented to prevent apoptosis in certain factor-dependent hematopoietic cell lines (4, 5) but, at least in one study (5), was found to be incapable of protecting either IL-2-dependent T cells or IL-6-dependent myeloma cells from apoptosis following cytokine removal. This result suggested that the signaling pathways triggered by IL-2 and IL-6 might suppress distinct apoptotic death programs. An alternative possibility raised by the recent discovery of proteins that interact with Bcl-2 and that can repress its apoptosis-blocking ability, if present in excess (reviewed in Ref. 6), is that the Bcl-2 protein levels attained by gene transfer were inadequate to counter the death facilitator activity of such a protein(s) (7). Consistent with this

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latter notion, Deng and Podack (8) determined that apoptosis in IL-2-dependent T cells induced by cytokine deprivation can in fact be inhibited by higher levels of ectopically expressed *bcl-2*. Therefore, to assess whether the apoptotic death pathway suppressed in myeloma cells by signaling through the IL-6 receptor might likewise be *bcl-2* regulable, we used the bcl-2 expression vector of Deng and Podack (8) as well as a recombinant bcl-2 retrovirus based on the MSCV retroviral vector (9) to overexpress the murine *bcl-2* cDNA in IL-6-dependent B9 myeloma cells. In contrast to an earlier report (5), we found that, irrespective of the mode of gene delivery, B9 cells stably expressing high levels of exogenous *bcl-2* exhibited significant resistance to apoptosis in the absence of IL-6. Nonetheless, endogenous *bcl-2* is not regulated by IL-6 in these cells; instead, IL-6 induces expression of the *bcl-2*-related gene, *bcl-x$_L$*, that has previously been demonstrated to prolong the viability of IL-3-dependent lymphoid progenitor cells deprived of factor (10). Since IL-6 is a member of a family of broadly acting cytokines whose cognate receptors share the common signal-transducing component gp130 (11), these data have important implications with regard to the regulation of apoptotic death mechanisms in a wide variety of cell types.

Materials and Methods

Cell Culture and *bcl-2* Gene Transduction. Maintenance and viability studies of IL-6-dependent B9 myeloma cells and genetically engineered derivatives were carried out as described previously (2). The BMGneo-bcl-2 vector, a gift from E. Podack (8), harbors the murine *bcl-2* cDNA in the bovine papilloma virus-based expression vector BMGneo, which contains the bacterial neomycin phosphotransferase (*neo*) gene conferring resistance to G418 (12). To construct MSCVpac-bcl-2, the 700-bp *XhoI* bcl-2-containing fragment was excised from BMGneo-bcl-2 and inserted into the *XhoI* site of the MSCVpac retroviral vector, which expresses the bacterial puromycin *N*-acetyl-transferase (*pac*) drug resistance gene (9). The vectors were introduced into B9 cells by electroporation (BMGneo-bcl-2) or retroviral infection (MSCVpac-bcl-2) as described (13). B9/BMGneo-bcl-2 cells and B9/MSCVpac-bcl-2 cells stably expressing exogenous *bcl-2* were maintained in medium containing 750 μg/ml G418 (Life Technologies, Gaithersburg, MD) and 4 μg/ml puromycin (ICN Biomedicals, Costa Mesa, CA), respectively.

Cell Cycle Analysis. Cytofluorometric analysis of cell cycle distribution and apoptosis was performed by propidium iodide staining of nuclei as reported previously (2), except that samples were analyzed on a FACScan equipped with Lysis II software (Becton Dickinson, San Jose, CA).

Western Blot Analysis. Western blotting was carried out according to previously published procedures (14). The membranes were probed with: hamster anti-mouse Bcl-2 mAb (PharMingen, San Diego, CA) using goat anti-hamster IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) as a secondary antibody; mouse anti-Bcl-x mAb (Transduction Laboratories, Lexington, KY) using anti-mouse IgG as a secondary antibody (Jackson ImmunoResearch); or rabbit anti-Bax polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) using anti-rabbit IgG (Jackson ImmunoResearch) as a secondary antibody. All of the secondary antibodies were peroxidase conjugated. The blots were developed using the ECL system (Amersham Life Science, Inc., Arlington Heights, IL).

Northern Blot Analysis. RNA samples (20 μg total RNA/lane) were electrophoresed through agarose gels and transferred to nylon membranes, and

the blots were hybridized with ^{32}P-labeled probes as described in detail (2). Probes were restriction enzyme fragments from plasmids carrying murine bcl-2 (8); murine bcl-x, a gift from C. Thompson (15); murine bax, a gift from C. Guidos (7); and rat glyceraldehyde-3-phosphate dehydrogenase (2) cDNAs.

Results and Discussion

Withdrawal of IL-6 from B9 cells activates a programmed death sequence which results in the formation of oligonucleosomal-sized DNA fragments that is the hallmark of apoptosis (2). In preliminary experiments, we determined that B9 cells express high levels of Bax. Since excessive Bax levels counter the death repressor ability of Bcl-2 in IL-3-dependent FL5.12 pro-B lymphoid cells (7), we were interested in determining whether expressing Bcl-2 at levels comparable to those of Bax would provide any protection against IL-6-deprived B9 apoptosis. Episomal (bovine papilloma virus-based BMGneo-bcl-2) or integrating (recombinant MSCVpac-bcl-2 retrovirus) bcl-2-con-
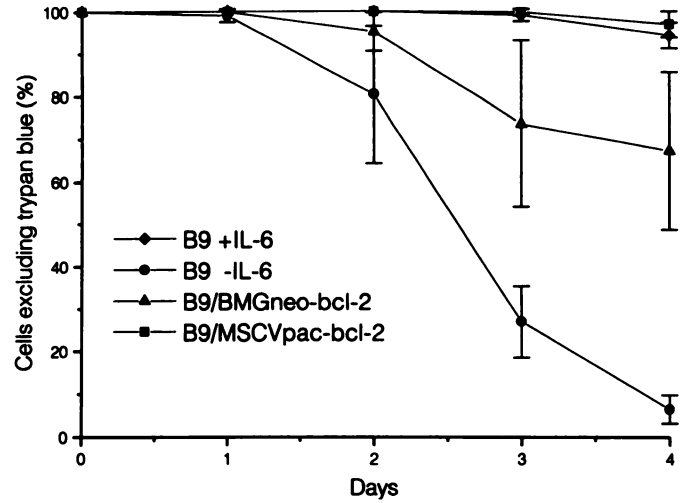

Fig. 2. Ectopic bcl-2 expression prolongs survival of IL-6-deprived B9 cells. Parental B9, B9/BMGneo-bcl-2, and B9/MSCVpac-bcl-2 cells were cultured in the absence of IL-6, and the percentage of viable cells was determined by trypan blue dye exclusion at the times indicated. Note that B9/MSCVpac-bcl-2 cells, which synthesize greater amounts of exogenous Bcl-2 than B9/BMGneo-bcl-2 cells, were found to be more resistant to apoptotic cell death induced by IL-6 withdrawal. B9 cells exponentially growing in medium containing IL-6 served as controls. Data are presented as the mean ± SD of values obtained in three separate experiments.
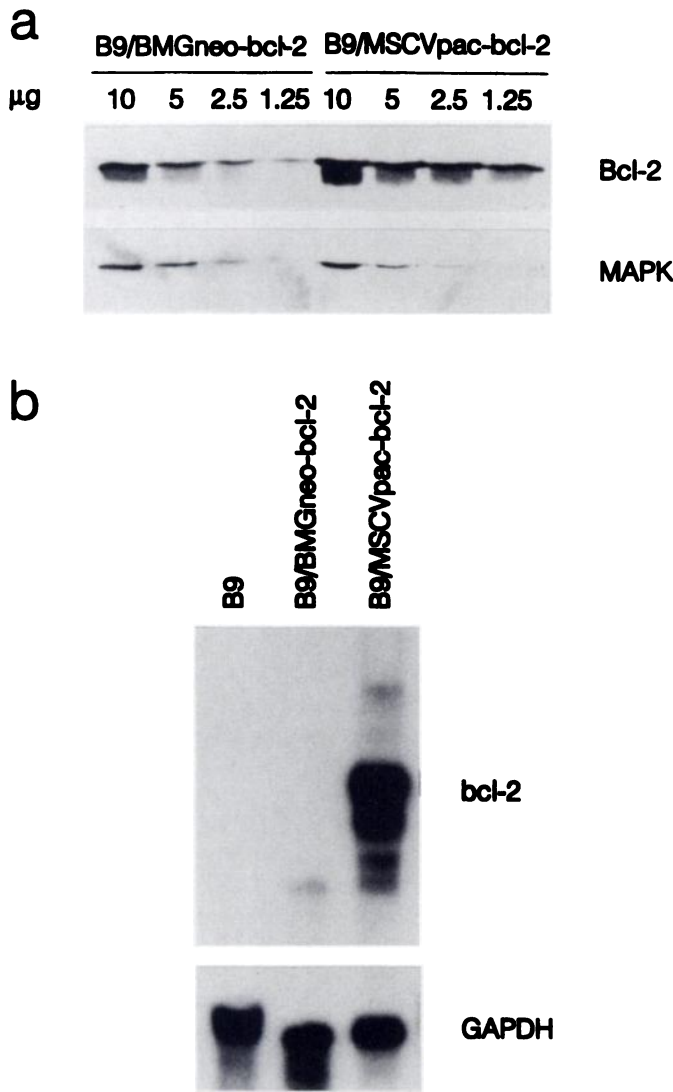

Fig. 1. Ectopic bcl-2 expression in genetically engineered B9 derivatives. a, Western blot analysis of serially diluted lysates prepared from exponentially growing B9/BMGneo-bcl-2 and B9/MSCVpac-bcl-2 cells probed with anti-Bcl-2 (26-kDa) antibody. The blots were stripped and reprobed with an antibody to mitogen-activated protein kinase (MAPK; 42 kDa) (anti-Erk; Transduction Laboratories) as a control for amount of protein loaded. b, Northern blot analysis of bcl-2 mRNA levels in B9 cells, B9/BMGneo-bcl-2 cells (1.5 kb), and B9/MSCVpac-bcl-2 cells (3.4 kb). Rehybridization of the blot with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (1.4 kb) indicated that similar amounts of RNA were loaded.

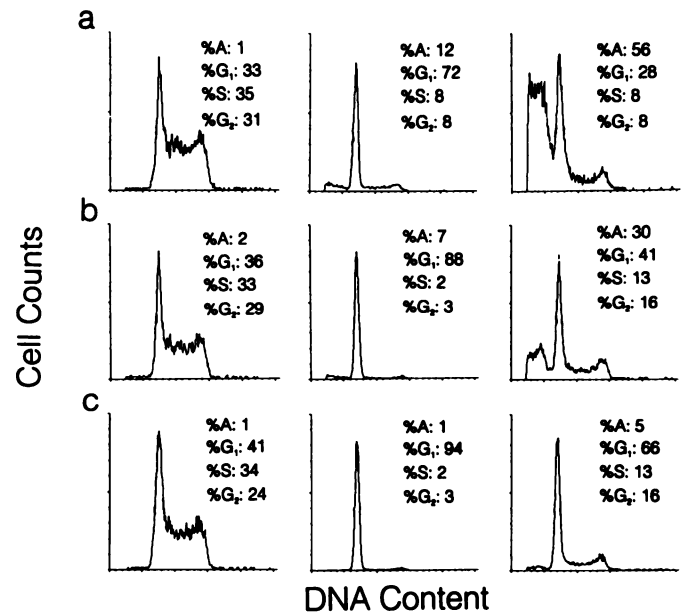

Fig. 3. Cell cycle distribution of parental and bcl-2-transduced B9 cells deprived of IL-6. Shown are the DNA frequency histograms, as determined by cytofluorometric analysis of propidium iodide-stained nuclei, 0 (left panels), 2 (middle panels), and 5 (right panels) days after IL-6 withdrawal. a, B9 cells; b, B9/BMGneo-bcl-2 cells; and c, B9/MSCVpac-bcl-2 cells. Note the diminished percentage of cells with low DNA stainability (A = sub-G$_1$), representative of apoptotic cells, in cultures of bcl-2-transduced versus parental B9 cells at days 2 and 5.

taining vectors were therefore introduced into B9 cells. Both BMGneo-bcl-2-transfected (B9/BMGneo-bcl-2) and MSCVpac-bcl-2-infected (B9/MSCVpac-bcl-2) B9 derivatives stably expressed exogenous bcl-2, as revealed by Western and Northern blot analyses (Fig. 1). Cultures of parental and bcl-2-transduced B9 cells were then depleted of IL-6, and the number of viable cells was monitored by trypan blue dye exclusion at 24-h intervals. As shown in Fig. 2, withdrawal of IL-6 from parental B9 cultures resulted in extensive loss of cell viability (>90%) over a 4-day period, as reported previously (2). Identical results were obtained in separate experiments with
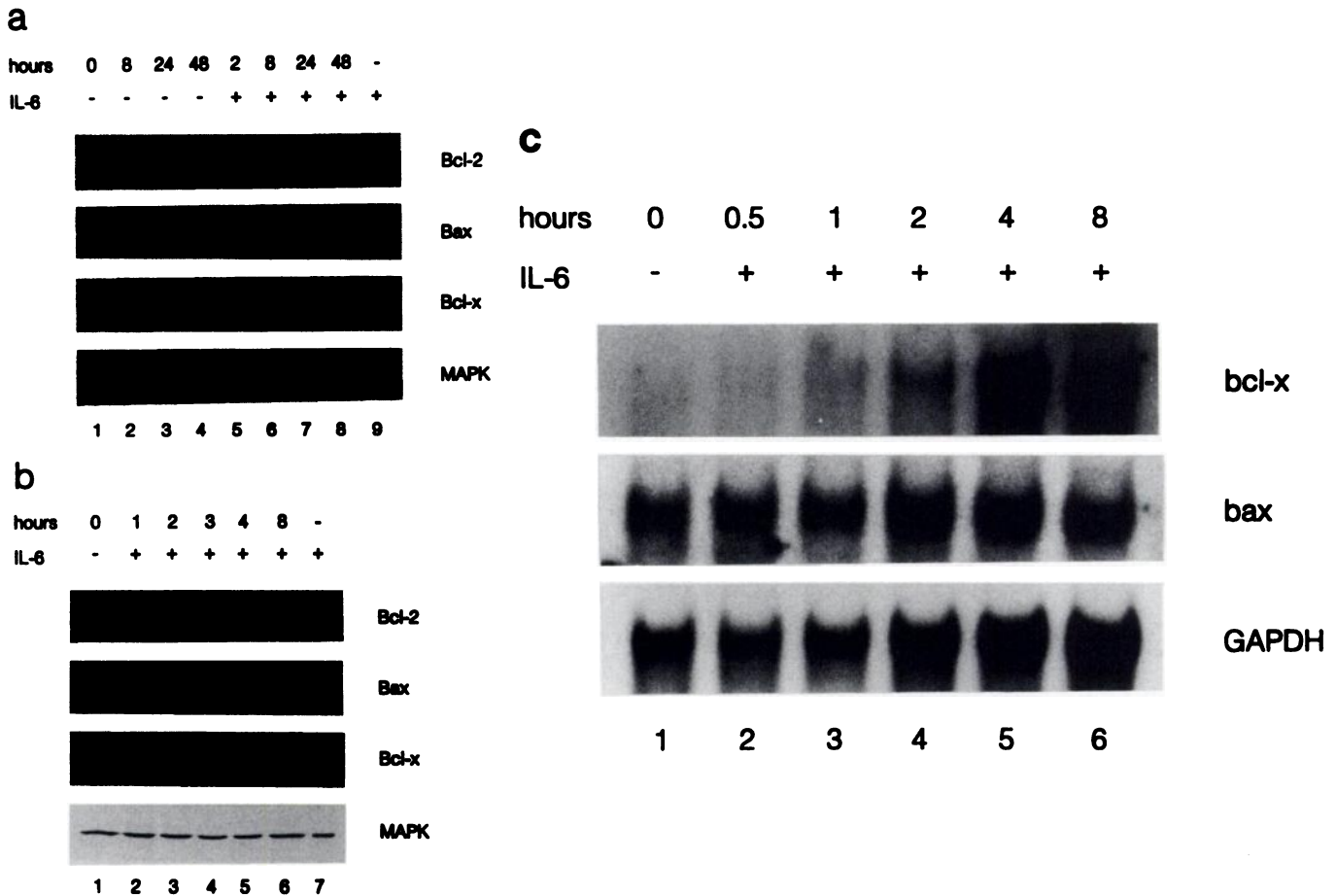
a

hours 0 8 24 48 2 8 24 48 -

IL-6   - - - - + + + + +

Bcl-2

Bax

Bcl-x

MAPK

1 2 3 4 5 6 7 8 9

b

hours 0 1 2 3 4 8 -

IL-6   - + + + + + +

Bcl-2

Bax

Bcl-x

MAPK

1 2 3 4 5 6 7

c

hours 0 0.5 1 2 4 8

IL-6   - + + + + +

bcl-x

bax

GAPDH

1 2 3 4 5 6

Fig. 4. IL-6 responsiveness and kinetics of bcl-x_L expression in B9 cells. a and b, Western blot analysis of cell lysates prepared at the times indicated after IL-6 withdrawal (Lanes 1–4 in a and Lane 1 in b) and following IL-6 addition (Lanes 5–8 in a and Lanes 2–6 in b). The blots were sequentially probed with antibodies specific for Bcl-2 (26 kDa), Bcl-x (28 kDa), and Bax (21 kDa). Lysate prepared from exponentially growing B9/MSCVpac-bcl-2 cells was included as a positive control for Bcl-2 (Lane 9 in a and Lane 7 in b). Reprobing of the blots with anti-MAPK (42-kDa) antibody demonstrated that approximately equal amounts of protein were loaded. c, Northern blot analysis of total RNA isolated from B9 cells after 48 h of IL-6 starvation (Lane 1) and following IL-6 restimulation for the times indicated (Lanes 2–6). The blot was sequentially hybridized with probes specific for bcl-x (3.0 kb) and bax (1.0 kb). A final hybridization of the blot with a probe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1.4 kb) confirmed that similar amounts of RNA were loaded.

B9 cells that had been transduced with control expression vectors carrying the neo or pac genes as selectable markers (16).[4] By comparison, there was much less cell death observed in IL-6-deficient cultures of bcl-2-transduced B9 cells over the same time period; there was only a 30% reduction in the number of surviving cells in B9/BMGneo-bcl-2 cultures whereas similarly-treated B9/MSCVpac-bcl-2 cultures exhibited less than 10% cell death. Although the stoichiometry between Bcl-2 and Bax was not investigated (but see, for example, Fig. 4a, Lane 9), the extent of resistance to IL-6-deprived apoptosis correlated with the levels of ectopic Bcl-2 (Fig. 1), consistent with a mechanism whereby cell survival depends on the ratio of Bcl-2:Bax (7, 17).

Concomitant with the death process, B9 cells arrest predominantly in G_1 upon IL-6 withdrawal (2). To assess whether bcl-2-enhanced survival was due to continued cell proliferation in the absence of IL-6, nuclei were stained with propidium idodide and subjected to cytofluorometric analysis. As can be seen in Fig. 3, growth arrest was not affected by bcl-2 overexpression, with ~95% of the bcl-2-transduced cells having intact DNA being in G_1 2 days following IL-6 removal. This result is in accord with previous reports that bcl-2 does not control cell proliferation, and that cell survival and proliferation are independently regulated by cytokine signaling (4, 5).

Although bax expression could be detected in B9 cells by Western and Northern blot analyses (Fig. 4), we were unable to detect expression of endogenous bcl-2 at either the protein (Fig. 4) or mRNA (Fig. 1b) levels. For this reason, we examined whether B9 cells expressed the endogenous bcl-x_L gene. Western blot analysis with anti-Bcl-x antibody showed that Bcl-x_L protein was present, that the levels decreased during a 48-h period in which the cells were deprived of IL-6, and then increased to steady-state levels within 8 h of IL-6 readdition to cytokine-depleted cultures (Fig. 4, a and b). No 19-kDa band corresponding to the Bcl-x_S isoform was observed (15). Upon Northern blot analysis (Fig. 4c), it was revealed that bcl-x_L expression was regulated in part at the mRNA level, with bcl-x transcripts being detected as early as 2 h after IL-6 restimulation of starved cells. By comparison, bax mRNA levels remained relatively unchanged throughout the deprivation and restimulation regimen.

We have shown here that IL-6 induces bcl-x_L expression in IL-6-dependent B9 cells having abundant Bax but no detectable Bcl-2. Our finding that overexpression of exogenous bcl-2 is capable of suppressing IL-6-deprived B9 apoptosis, on the one hand, and previous data showing that transfected bcl-x_L protects against IL-3-deprived apoptosis in a B-lineage cell line at least as well as bcl-2 (10), on the other, argues for a role of bcl-x_L in the IL-6-mediated regulation of apoptotic cell death in these murine myeloma cells. In this regard, it is noteworthy that synthesis of Bcl-x_L protein in normal human plasma cells

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[4] T. S. Hawley and R. G. Hawley, unpublished data.

has recently been described (18); it will be of obvious interest to determine whether *bcl-x$_L$* also contributes to the survival of some human myeloma cells (19).

Other investigators have demonstrated that IL-6 is capable of inhibiting p53-induced apoptosis in murine M1 myeloid leukemic cells (20). We have found that B9 cells express wild-type p53.[5] In addition, B9 cells constitutively express c-*myc* as a result of a chromosomal translocation involving the immunoglobulin heavy chain locus (2). Since Bcl-2 prevents c-*myc*- and p53-induced apoptosis (6), our future experiments will focus on these genes as well as the newly discovered *bad* gene (21) as potential mediators of the death process regulated by *bcl-x$_L$* in this system.

Acknowledgments

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[5] A. Z. C. Fong and R. G. Hawley, unpublished data.