Role for c-myc in Activation-Induced Apoptotic Cell Death in T Cell Hybridomas

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Immature T cells and some T cell hybridomas undergo apoptotic cell death when activated through the T cell receptor complex, a phenomenon that is probably related to antigen induced negative selection of developing T cells. This activation-induced apoptosis depends on active protein and RNA synthesis in the dying cells, although none of the genes required for this process have previously been identified. Antisense oligonucleotides corresponding to c-*myc* block the constitutive expression of c-Myc protein in T cell hybridomas and interfere with all aspects of activation-induced apoptosis without affecting lymphokine production in these cells. These data indicate that c-*myc* expression is a necessary component of activation-induced apoptosis.

Apoptosis, a form of cell death in which the cell actively participates in its demise, is characterized by chromatin condensation, DNA fragmentation, and cytoplasmic blebbing, whereas mitochondria and other cytoplasmic organelles remain intact during the early stages (*1*). The dying cells fragment into small, membrane-bound apoptotic bodies, which are rapidly taken up by resident phagocytic cells with no ensuing inflammation (*2*). Frequently, the process of apoptosis is dependent on RNA and protein synthesis in the dying cell (*1*, *3*), leading to the concept of "death genes" that are responsible for the phenomena.

Activation in mature and immature T cells involves a cascade of gene expression, including the early induction of several proto-oncogenes, including c-*myc* (*4*). T cell hybridomas undergo apoptosis on activation through the CD3-T cell receptor (TCR) complex (*5*), and this phenomenon is likely to be related to activation-induced apoptosis and negative selection in immature thymocytes (*6*). We therefore sought to determine whether c-*myc* was involved in the process of activation-induced apoptosis. Our approach was the use of antisense oligodeoxynucleotides to specifically control function of this gene.

Antisense oligodeoxynucleotides corresponding to the c-*myc* protooncogene inhibit c-Myc protein expression and affect a number of cell functions (*7*), including inhibition of mitogenesis in human T cells, inhibition of growth of transformed B cells, and induction of differentiation of the HL-60 cell line. To determine the role of c-*myc* in activation-induced apoptosis, antisense oligonucleotides were added to A1.1 cells at the same time that the cells were exposed to a monoclonal antibody to CD3 (CD3 MAb), which activates the cells through the TCR. Phosphorothioate-derivatized antisense oligonucleotides corresponding to c-*myc* dramatically blocked activation-induced cell death, whereas antisense oligonucleotides corresponding to c-*fos* did not (Fig. 1A). Nonsense oligonucleotides with the same base composition as that of the c-*myc* antisense also had no effect. The ability of c-*myc* antisense to prevent activation-induced apoptosis was observed in other T cell hybridomas as well (*8*). This effect was not dependent on the use of phosphorothioates; conventional oligonucleotides with the antisense c-*myc* sequence also inhibited cell death after activation (*8*). Other antisense oligodeoxynucleotides corresponding to the translation start regions of murine c-*myb*, c-*abl*, and *bcl-2* failed to inhibit the induction of cell death in this system (*8*), despite the observations that antisense to c-*myb* and *bcl-2* have effects in other systems (*9*).

Activation-induced apoptosis is a rapid

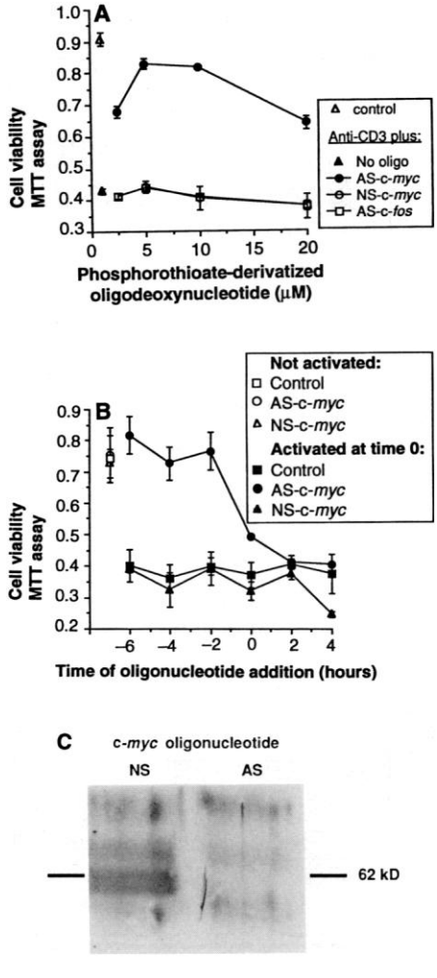

Fig. 1. Effects of c-*myc* antisense oligodeoxynucleotides on activation-induced cell death in a T cell hybridoma. Dose response (**A**) and kinetics (**B**) of inhibition of activation-induced cell death and effect on c-Myc protein expression (**C**) were determined for different oligodeoxynucleotides added to cultures of A1.1 T hybridoma cells. (**A**) Cells were cultured in 96-well plates that had previously been coated with CD3 MAb (145-2C11) as described (*5*). Phosphorothioate oligodeoxynucleotides (Regional DNA Synthesis, Calgary, Alberta) were added at the indicated concentrations at the start of culture. Sequences used were as follows: antisense c-*myc*, CACGTTGAGGGGCAT; antisense c-*fos*, ACCCGAGAACATCAT; and nonsense c-*myc*, AGTGGCGGAGACTCT. In many experiments, another nonsense sequence with the base composition of c-*myc* was also used (TGAATCAGCGGGCTG), and this also failed to have any significant effects (*8*). "Control" cells were cultured in the absence of CD3 MAb or oligonucleotides. No effects on survival of any of the oligonucleotides were seen in cultures without CD3 MAb (*8*). Relative survival of A1.1 cells after activation by plastic-bound CD3 MAb in the presence of the indicated antisense or nonsense oligodeoxynucleotides was determined after approximately 18 hours of culture by tetrazolium staining (MTT) and determining optical density at 595 nm (*5*). (**B**) Antisense c-*myc* oligonucleotide (5 μM) was added at various times relative to activation (exposure to CD3 MAb-coated plastic). Relative cell survival was determined by MTT approximately 18 hours after activation. (**C**) Effect of oligonucleotides on c-*myc* protein expression. A1.1 cells were cultured with antisense or nonsense c-*myc* oligonucleotides (10 μM) for approximately 18 hours, following which protein was extracted and prepared for Western blotting with a rabbit antiserum to a peptide of c-Myc (*26*). C-Myc resolved as a 62-kD band (*11*).

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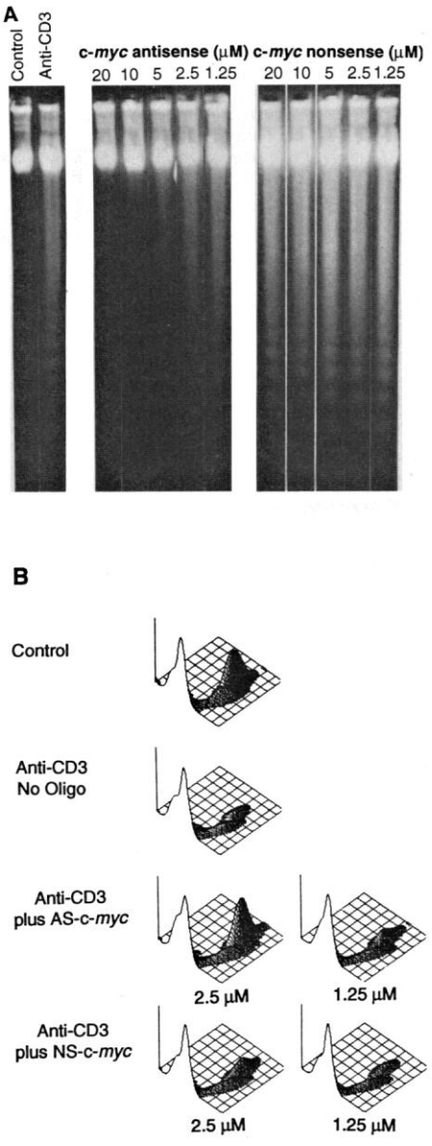

Fig. 2. Inhibition of activation-induced apoptosis by c-*myc* antisense oligodeoxynucleotides. (**A**) DNA fragmentation in CD3 MAb-activated A1.1 cells protected by antisense c-*myc*. A1.1 cells were cultured in 24-well plates that had been coated with CD3 MAb antibody as described (*5*) and phosphorothioate oligodeoxynucleotides (legend to Fig. 1) were added at the indicated concentrations at the start of culture. Cells were recovered after 10 hours of CD3 MAb activation, lysed, and centrifuged at 12,000*g* to remove debris and high molecular weight chromatin, and the DNA was extracted from supernatants with phenol-chloroform as described (*12*). DNA fragments were visualized after agarose gel electrophoresis by ethidium bromide staining. (**B**) Light scattering properties of CD3 MAb-activated A1. 1 cells protected by c-*myc* antisense oligodeoxynucleotides. A1.1 cells were cultured on CD3 MAb-coated 24-well plates in the presence or absence of either 2.5 μM or 1.25 μM antisense as indicated (Fig. 1). Controls included uncoated wells. After 16 hours in culture, the cells were harvested and analyzed with a FACScan (Becton-Dickenson, Mountain View, CA). Results shown are ungated data from forward angle light scatter (*x* axis) versus side scatter (*y* axis).

event in T cell hybridomas, with initial effects observable within about 4 to 6 hours after stimulation (5). Because c-Myc has a rapid turnover time of about 30 min (*10*), we postulated that the effects of the antisense would be facilitated by exposure of the cells before stimulation (Fig. lB). Al.l cells exposed to a suboptimal dose of c-*myc* antisense oligonucleotides for 2 to 6 hours before activation were completely resistant to activation-induced cell death, whereas exposure at the time of activation had a smaller effect. Addition of the oligonucleotides at 2 to 4 hours after activation failed to have any inhibitory effect on cell death.

The ability of c-*myc* antisense oligodeoxynucleotides to block cell death in Al.1 cells correlated with their effects on c-Myc protein expression. Immunoblot analysis with antiserum to a c-Myc peptide (anti-*c-myc*) revealed a loss of c-Myc from cells cultured with the c-*myc* antisense oligonucleotides (Fig. 1C) as described (*7, 11*).

Whereas the c-*fos* antisense oligonucleotide failed to block activation-induced cell death in A1.1, this oligonucleotide was as active as the c-*myc* antisense oligonucleotide in inhibiting growth of mouse L929 cells, whereas a *c-myc* nonsense sequence had no significant effect on cell growth, as observed by others (*11*). Thus, 2 x 10^5 cells grown for 3 days in the absence of oligonucleotides increased in number by a factor of 2.79 ± 0.02, and those grown in the presence of the nonsense oligonucleotide increased 2.44 ± 0.13 times. Cells grown with the c-*myc* or c-*fos* antisense oligonucleotides increased by factors of only 1.32 ± 0.12 and 1.47 ± 0.11, respectively (8).

The fragmentation of DNA into an oligonucleosomal "ladder" is a hallmark of apoptosis and can be readily demonstrated in T cell hybridomas undergoing activation-induced death (5). DNA extracted from cells undergoing activation-induced apoptosis showed the characteristic fragmentation pattern, whereas this fragmentation was dramatically reduced when cells were cultured in the presence of the c-*myc* antisense phosphorothioate (Fig. 2A). Similar results were obtained with other T cell hybridomas as well (8). In cells labeled with ^{125}I-deoxyuridine, DNA fragmentation after activation was quantitated (*3, 5*) as 68.8% with CD3 MAb alone versus 24.5% fragmentation with CD3 MAb plus c-*myc* antisense (8).

Cells undergoing apoptosis display changes in morphology that can be detected by comparing forward and side scatter profile from a fluorescence-activated cell sorter (FACS) (*12*). These morphologic changes were also inhibited by c-*myc* antisense oligonucleotides but not by nonsense controls (Fig. 2B). The peak present in the control group but not in the CD3 MAb-treated cells represents cells with normal forward and side scatter. As cells undergo apoptosis, the increased granularity is reflected as an increase in side scatter, and the cellular shrinkage and fragmentation into apoptotic bodies results in a decrease in forward angle light scatter (12). The normal cell peak was also present in cells treated with CD3 MAb in the presence of 2.5 μM phosphorothioate antisense. [Essentially identical results were obtained when oligonucleotides were added at 5, 10, 20, and 40 μM (8)]. These data demonstrate that the cells protected by the c-*myc* antisense display the morphologic characteristics of normal cells. Visually, we confirmed that the cytoplasmic and membrane blebbing associated with activation-induced apoptosis in T cell hybrido-

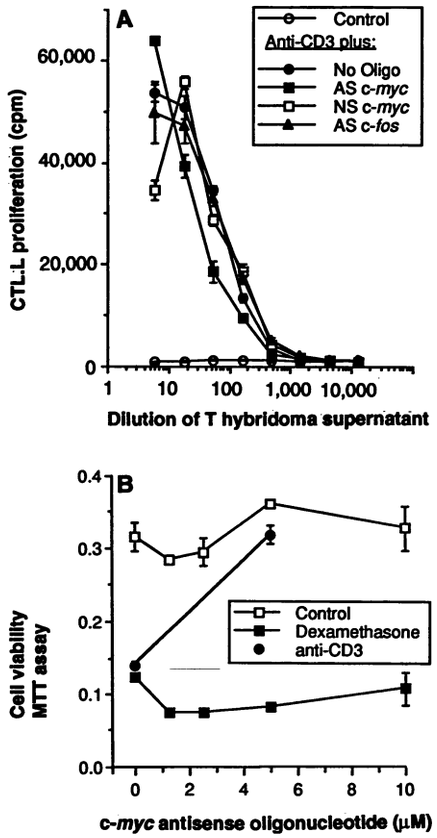

Fig. 3. C-*myc* antisense oligodeoxynucleotides do not inhibit activation-induced lymphokine production or corticosteroid-induced apoptosis. (**A**) Cell supernatants from the 10 μM oligonucleotide cultures shown in Fig. 1A were collected ~18 hours after activation. Lymphokine in the supernatants was determined by proliferation of the IL-2–responsive cell line CTLL (*10*). (**B**) A1.1 cells were cultured with dexamethasone (10^{-6} M) or on CD3 MA–coated plastic with the indicated concentration of c-*myc* antisense oligonucleotide added at the start of culture. Cell viability was assessed by MTT after ~18 hours of culture and expressed as optical density at 595 nm.

mas (5) did not occur in the presence of the c-*myc* antisense but did occur in the presence of nonsense or c-*fos* antisense oligonucleotides (8).

T cell hybridomas release lymphokines such as interleukin-2 (IL-2) after activation through the CD3-TCR complex, as do A1.1 cells in response to specific antigen plus antigen-presenting cells (*13*). The CD3 MAb-induced apoptosis was inhibited by the addition of c-*myc* antisense oligonucleotides, but neither these nor control oligonucleotides affected the amount of lymphokine released after activation by CD3 MAb, as assessed with lymphokine-responsive CTL.L cells (*12*). Supernatants of CD3 MAb-activated A1.1 cells induced equivalent proliferation in the assay cells, regardless of whether activation of the A1.1 cells occurred in the presence or in the absence of 10 μM c-*myc* antisense or nonsense phosphorothioate oligonucleotides (Fig. 3A). Virtually identical results were obtained with 20 M oligonucleotides (8). Thus, c-*myc* antisense inhibits activation-induced apoptosis in the T cell hybridoma, without inhibiting another outcome of activation: the production of lymphokines. This is reminiscent of observations that c-*myc* antisense oligonucleotides inhibit entry into S phase of activated mature T cells but do not inhibit blast transformation or the expression of the IL-2 receptor in these cells, although both follow c-*myc* expression during T cell activation (*7*).

Although c-*myc* antisense inhibits activation-induced cell death in T cell hybridomas, it has no effect on the induction of apoptosis in the same cell line by dexamethasone (Fig. 3B). Glucocorticoid-induced and activation-induced apoptosis is known to proceed through different pathways, as each mode of induction mutually inhibits the apoptosis induced by the other (*14*). Dexamethasone inhibit *c-myc* expression (*15*), and our results suggest that this inhibition may account for the ability of dexamethasone to inhibit activation-induced apoptosis.

The apparent role for c-*myc* in apoptotic cell death was surprising because *myc* is usually implicated in cell cycle progression and cell transformation (16). Nevertheless, the idea that c-*myc* may play a role in cell death has precedents. Chinese hamster ovary cells transfected with c-*myc* under the control of a heat shock promoter undergo cell death on heatshock (*17*). A similar relation between c-*myc* expression and apoptosis was described by Wyllie and colleagues (*18*), who observed an increased rate of apoptotic death in fibroblasts that were transformed with the *myc* oncogene. More recently, it has been observed that c-*myc* expression induces apoptosis in cells deprived of growth factors (*19*).

Apoptosis is blocked in some cases by the inhibition of RNA or protein synthesis. Examples include apoptosis induced in thymocytes by glucocorticoids or calcium ionophore, apoptosis induced by growth factor withdrawal, and activation-induced apoptosis in T cell hybridomas (*1, 3, 5*). Such observations led to the idea that apoptosis involves the activation of "death genes" required for the process. Candidate "death genes" include transglutaminase, SGP-2/TRMP-2, and others (*20*). An alternative view was proposed by McConkey and colleagues (*21*), who observed that brief exposure to RNA or protein synthesis inhibitors led to rapid loss of an endogenous endonuclease activity believed to be required for apoptosis. Thus, constitutively expressed gene products with a high turnover rate might play roles in the apoptotic process, and inhibition of the synthesis of such proteins would therefore prevent apoptosis. We have observed that c-*myc* mRNA (and protein) are constitutively expressed in T cell hybridomas, with no effects observed up to 4 hours after activation with CD3 MAb (8). Thus, c-*myc* expression is necessary but not sufficient for activation-induced apoptosis.

How c-*myc* contributes to activation-induced and other forms of apoptosis is not known. The c-Myc protein has two distinct properties: (i) Myc specifically binds to nucleotide sequences in DNA (*22*) and as a heterodimer with other proteins (23) regulates gene transcription (24); and (ii) Myc specifically binds to the retinoblastoma protein (25), which in turn regulates cell cycle events. It will be interesting to determine whether one or both of these properties are responsible for the role of c-*myc* in cell death.

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26. A conserved c-*myc* peptide, APSEDIWKKFELL, provided by B. Singh (University of Alberta Edmonton), was coupled to keyhole limpet hemacyanin and used to immunize rabbits. The resulting anti–c-*myc* antiserum was tested by Western Immunoblotting of protein extracted from heat-shocked Chinese hamster ovary (CHO) cells expressing c-*myc* under the control of a heat shock promoter (*17*). Western immunoblotting of extracts of the T cell hybridoma was performed as described (*11*). Single letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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