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Fas-independent apoptosis in T-cell tumours induced by the CD2-*myc* transgene

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Abstract

Depending on the cellular context, the Myc oncoprotein is capable of promoting cell proliferation or death by apoptosis. These observations suggest that apoptosis in response to deregulated gene expression may represent a natural brake to tumour development. The pathways by which Myc induces apoptosis are as yet poorly characterised although recent observations on rat fibroblasts over-expressing Myc have demonstrated a requirement for the Fas pathway. To investigate the role of Fas in Myc-induced lymphomagenesis we backcrossed CD2-*myc* mice onto an *lpr* background. Rates of tumour development and phenotypic properties, including levels of apoptosis were indistinguishable from CD2-*myc* controls. Further, tumour cell lines derived from mice expressing a regulatable form of Myc showed inducible apoptosis at similar rates regardless of their *lpr* genotype. These results show that activation of c-*myc* and loss of Fas do not collaborate in T lymphoma development and that Myc-induced apoptosis in T-cells occurs by Fas-independent pathways. *Cell Death and Differentiation* (2000) **7,** 80–88.

Keywords: Fas; lpr; Myc; T-cell; thymus; lymphoma

Abbreviations: lpr, lymphoproliferation; ER^{TM}, mutated oestrogen receptor; TcR, T-cell receptor; TUNEL, TdT-mediated dUTP-biotin nick end labelling; MuLV, Murine Leukaemia Virus; 4-OHT, 4-hydroxytamoxifen

Introduction

Apoptosis is a genetically controlled form of cell death characterised by distinct biochemical and morphological changes.[1] Genetic lesions that result in the disruption of apoptotic pathways or in the inappropriate expression of survival signals represent key events in tumourigenesis.[2–8] Events which block apoptosis may take on a special significance when combined with the activation of specific oncogenes such as c-*myc*. A number of studies have shown that under certain growth limiting conditions c-*myc* can induce apoptosis, a property that may act to limit the oncogenic potential of *myc* following deregulation.[9–11]

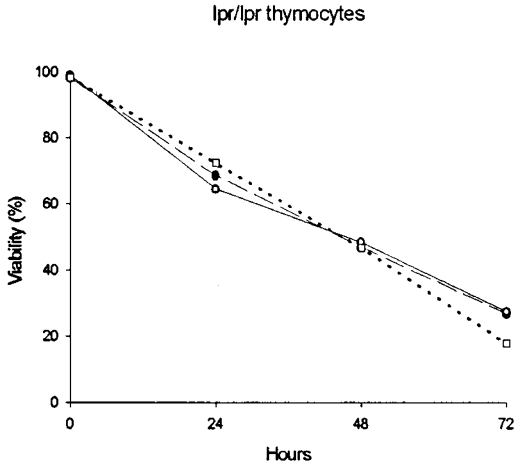
Fas (CD95/APO-1) is a cell surface receptor and a member of the tumour necrosis factor (TNF) receptor family. Crosslinking Fas with antibody or its natural ligand, expressed on the surface of activated T-cells, induces cell death. Recent studies have shown that c-*myc* induced apoptosis in fibroblasts is mediated by, and dependent on, cell surface interaction of Fas and its ligand.[12] However, the role of the Fas pathway in tumour development is not yet clear. Both haematopoetic and non-haematopoetic tumours have been shown to express Fas,[13–17] despite this not all of these tumours are sensitive to anti-Fas induced apoptosis suggesting that selection events have occurred to disrupt this pathway.[13–15,17] Further, transplantation studies involving Fas positive tumour cell lines have shown that the growth of such tumours can be inhibited using anti-Fas antibodies.[18] Although the lymphadenopathy observed in *lpr* and *gld* mice results from a failure of T cell homeostasis these cells do not appear to be prone to transformation.[19] However, a significantly higher incidence of late onset plasmacytomas has been observed in *gld* mice[20] and T-cell deficient mice show an increased incidence of B-cell tumours when the Fas pathway is absent.[21] In addition, Fas appears to restrict myeloid tumour development in transgenic animals overexpressing *bcl-2*.[22] It is not clear from these studies whether a defect in the Fas pathway creates an environment that permits tumour development or whether cells that lack Fas are intrinsically more sensitive to transformation.

CD2-*myc*[23] and CD2-*myc*ER^{TM} transgenic mice carry c-*myc* under the control of the CD2 dominant control region and show a moderate incidence of thymic lymphoma. To investigate if a defect in the Fas pathway could synergise with the c-*myc* oncogene in tumour development by abrogating c-*myc* induced apoptosis, we crossed these transgenic mice onto an *lpr* background. CD2-*myc* thymic lymphoma cells show increased cell death following anti-Fas treatment *in vitro* indicating that this pathway is still functional in transformed thymocytes. However, this pathway does not act to restrict tumour development as *myc* mediated transformation is not enhanced when Fas is absent and *myc* induced apoptosis in transformed thymocytes does not require the presence of Fas.

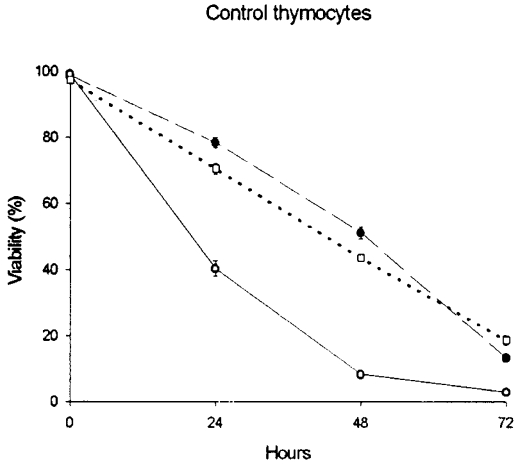
Results

Fas can mediate apoptosis of transformed thymocytes *in vitro*

CD2-*myc* mice develop thymic lymphomas with a moderate incidence of 30 to 70% by 12 months of age depending on the genetic background.[23] To investigate the integrity of the Fas



(a)



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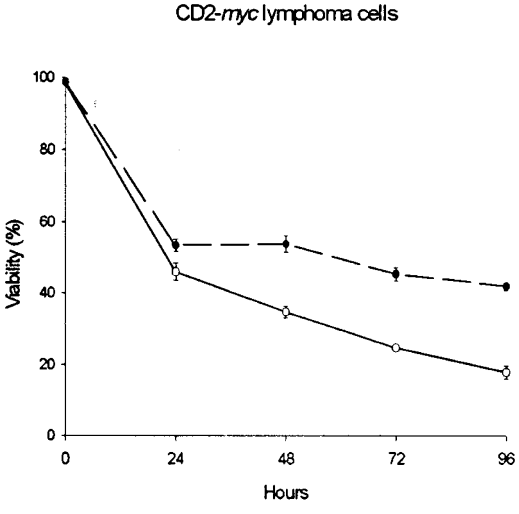

(c)

pathway in CD2-*myc* tumours, transformed cells from CD2-*myc* tumours were explanted and incubated in the presence of anti-Fas antibody (Jo2). Despite the high background rate of cell death typical of explanted tumour cells, treatment with Jo2 antibody resulted in significantly increased cell death in these short term cultures (Students *t*-test: $P<0.001$). These data are shown in Figure 1, similar results were obtained with five additional tumours. The Fas antibody also had a cytotoxic effect in normal untransformed thymocytes from C57Bl6 mice but did not significantly increase cell death in thymocytes from *lpr/lpr* mice. These findings indicate that, at least in the tumours tested, the Fas pathway was intact and there had been no apparent selection for events that abrogated Fas induced death.

Myc induced lymphomagenesis is not accelerated in *lpr* mice

To investigate the possibility that Fas mediated apoptosis could restrict tumour development and/or growth, CD2-*myc* mice were backcrossed on to *lpr/lpr*-MRL mice. CD2-*myc* transgenic mice, homozygous for the *lpr* mutation (CD2-*myc/lpr*), developed either thymic lymphoma or lymphoproliferative disease whereas animals heterozygous for *lpr* (hereafter referred to as CD2-*myc*) only developed lymphomas. Tumours were diagnosed on the basis of gross pathology, clonality as defined by TcR rearrangements, and transgene expression (results not shown). As preneoplastic tissues from CD2-*myc* mice do not express the transgene, but emerging tumours invariably show high level expression, it is possible to identify neoplastic tissues by this criteria.[24]

There was no difference in overall survival between CD2-*myc/lpr* and CD2-*myc* mice (Figure 2). Further, the incidence of thymic lymphoma was not increased in CD2-*myc/lpr* animals (14/48) compared to CD2-*myc* mice (25/38). Indeed the incidence appears to be low in the CD2-*myc/lpr* group. The apparent discrepancy between the two groups is due to the large number of animals (28/48) removed from this cohort due to the ongoing development of *lpr* associated lymphadenopathy and autoimmune disease. Tumour-free survival was analyzed using the Generalised Wilcoxon Test, a statistical approach that takes account of tumour-free animals leaving the cohort during the study period. There was no significant difference between the two groups in terms of tumour-free survival. These results suggest that apoptosis mediated through the Fas receptor does not act to inhibit tumour initiation and/or progression.

Figure 1 Viability of explanted CD2-*myc* lymphoma cells following anti-Fas treatment. *In vitro* culture of (**a**) *lpr/lpr* thymocytes, (**b**) C57Bl6 thymocytes and (**c**) CD2-*myc* tumour cells are shown. Quadruplicate cultures were assayed and viability was calculated by comparing the average number of live cells against the average total. Cells were treated with either anti-Fas antibody, Jo2 (solid line, open circle) or with isotype control (dashed line, filled circle). Untreated cells (dotted line, open square) are also shown in (**a**) and (**b**)

The phenotype of CD2-*myc* thymic lymphomas was not altered by the *lpr* background

CD2-*myc/lpr* tumours were analyzed by flow cytometry to ascertain the phenotype of these cells. The majority (9/10) of CD2-*myc/lpr* tumours were of the $CD4^+/CD8^+$ phenotype. Similarly, 9/9 of the CD2-*myc* cohort were also $CD4^+/CD8^+$. These results agree with previous, more extensive, analyses of CD2-*myc* tumours in which 70% of tumours belong to the $CD4^+/CD8^+$ phenotype[25] and demonstrate that the *lpr* background did not alter the tumour phenotype.

Lymph nodes from the CD2-*myc/lpr* mice with thymic lymphomas were also examined in detail. Of these nodes 9/10 revealed evidence of *lpr* lymphadenopathy on the basis of an expanded $CD4^-/CD8^-$ population, a recognised diagnostic feature of *lpr* induced disease.[19,26] One lymph node appeared to be enlarged as a result of tumour metastasis, having an identical population of $CD4^+/CD8^+$ cells to the thymic lymphoma and high levels of transgene expression, this is in keeping with CD2-*myc* pathology where occasional metastasis to the lymph nodes is observed. These results suggest that most of the CD2-*myc/lpr* mice succumbing to thymic lymphoma also had well developed changes associated with the *lpr* phenotype.

Six out of six of the CD2-*myc/lpr* mice presenting with *lpr* disease and 4/4 of *lpr* control mice displayed a marked increase in the population of $CD4^-/CD8^-$ and $CD4^+$ thymocytes in keeping with previous reports.[19,26]

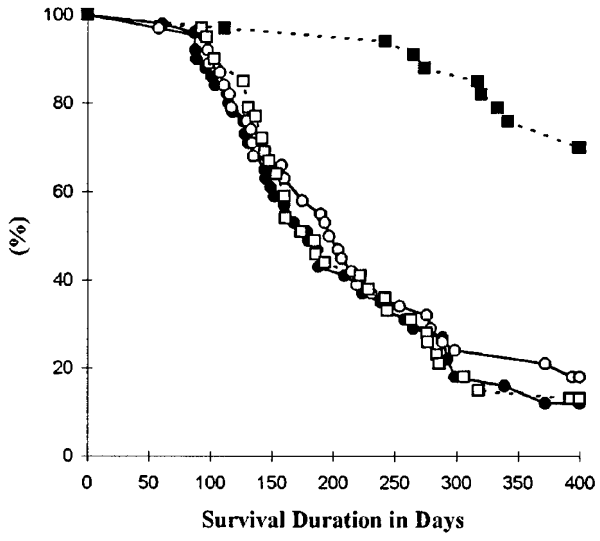

Figure 2 Disease free survival curve of CD2-*myc/lpr* mice. Survival curves for CD2-*myc/lpr* (solid line, filled circles, *n*=48), CD2-*myc* (solid line, open circles, *n*=38), *lpr* (dashed line, open square, *n*=39) and control mice (dashed line, filled square, *n*=33). These data represent overall survival. Deaths in the CD2-*myc/lpr* group were due to either thymic lymphoma development or severe autoimmune disease associated with the *lpr* phenotype. Statistical analysis was carried out using the Generalised Wilcoxon test (SAS statistical package, SAS Institute, Cary, NC, USA)

Myc induced tumours show high levels of apoptosis independent of *lpr* status

Hueber *et al*,[12] have shown that *myc* induced apoptosis is abrogated in fibroblasts harbouring a defect in the Fas pathway. CD2-*myc* thymic lymphomas are characterised by high levels of apoptosis, to investigate if Fas signalling is involved in this process we performed TUNEL on lymphomas from CD2-*myc/lpr* mice. There was no significant difference in the levels of apoptosis in thymic lymphomas from CD2-*myc/lpr* and CD2-*myc* animals (Figure 3, Table 1) suggesting that loss of Fas did not permit increased survival of tumour cells. In general, tumours displayed high levels of apoptosis and extensive clumping of stained cells indicating active macrophage activity, a feature absent in control sections. In contrast to the neoplastic tissues only moderate levels of apoptosis were observed in normal thymocytes and positively stained cells were scant in thymic sections from *lpr* mice suffering from lymphoproliferative disease.

Fas independent *myc* induced apoptosis occurs in CD2-*myc*ER^{TM} lymphoma cells

To confirm that deregulated *myc* and loss of Fas do not collaborate in the formation of T-cell tumours we crossed CD2-*myc*ER^{TM} transgenic mice on to an *lpr* background. These mice express a hybrid protein that brings c-*myc* under the control of a modified oestrogen receptor.[27] This construct can be regulated by the oestrogen agonist/antagonist 4-hydroxytamoxifen (4-OHT) but is insensitive to actions of oestradiol. Although *myc* activity can be successfully modulated in CD2-*myc*ER^{TM} mice, animals not treated with 4-OHT show a background tumour incidence, presumably due to residual activity of the

Table 1 Apoptotic index of thymic lymphomas and *LPR* affected thymi

		Apoptotic cells		
		Average	**S.D.**	**(Range)**
Thymic lymphomas	CD2-*myc/lpr* group	30.1	12.0	(21–44)
	CD2-*myc* group	35.0	8.1	(23–51)
Thymus-*Lpr* disease	CD2-*myc/lpr* group	2.8	1.2	(2–6)
	lpr group	2.4	1.5	(0–5)
Thymus-control tissue	Control group	4.9	1.7	(2–8)

Apoptotic cell average was based on the examination of at least eight mice in each group. The apoptotic index for each sample was based on the examination of at least four fields using the TUNEL technique. Statistical analysis was carried out using the Students *t*-test

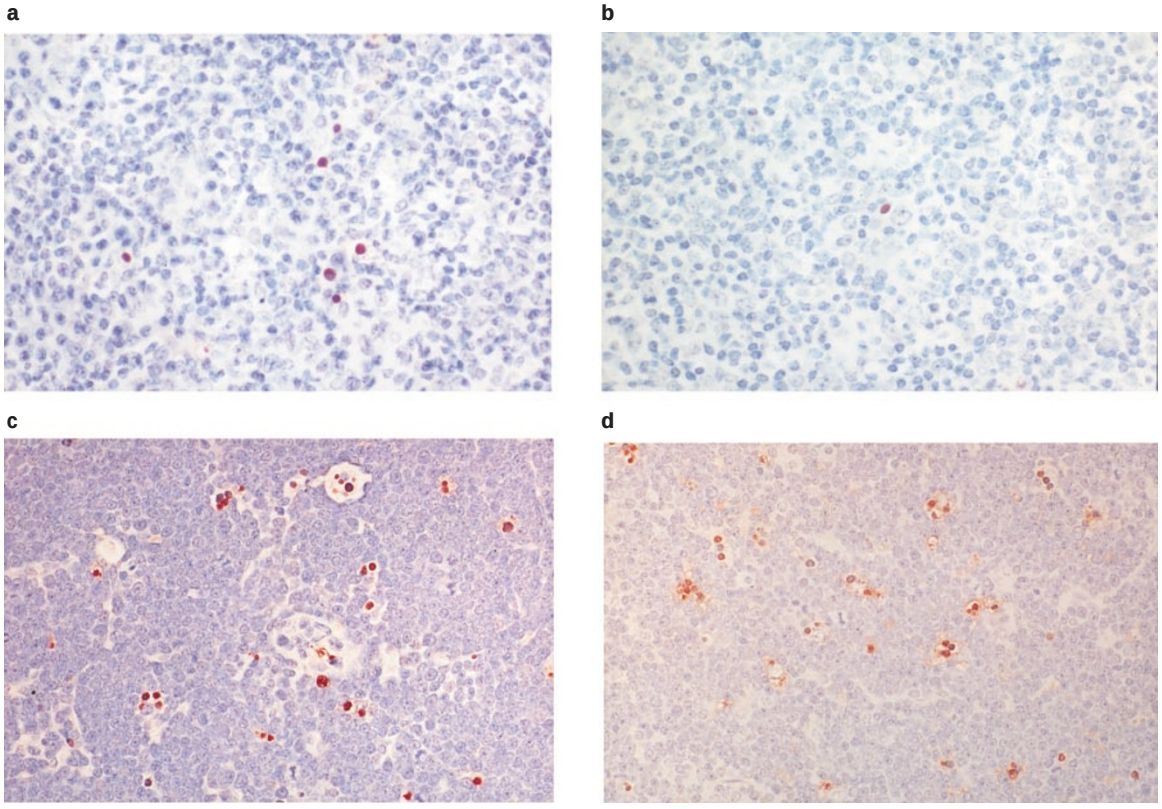

Figure 3 Apoptosis in CD2-*myc*/*lpr* tumours. Photomicrographs of TUNEL stained sections of thymic tissue (×400). (**a**) Control thymus, (**b**) thymus from *lpr* mouse with lymphadenopathy, (**c**) thymic tumour (CD2-*myc*) and (**d**) thymic tumour on an *lpr* background (CD2-*myc*/*lpr*)

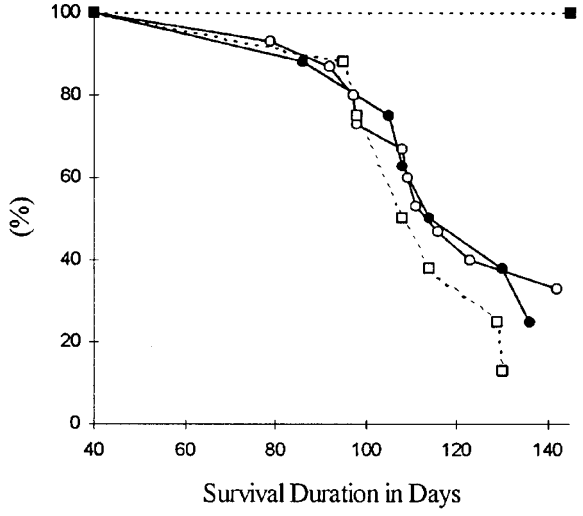

Figure 4 Disease free survival curve of CD2-*myc*ER^{TM}/*lpr* mice. Survival curves for CD2-*myc*ER^{TM}/*lpr* (solid line, filled circles, *n*=8) CD2-*myc*ER^{TM} (solid line, open circles, *n*=15), *lpr* (dashed line, open square, *n*= 8) and control mice (dashed line, filled square, *n*=8). These data represent overall survival. Deaths in the CD2-*myc*ER^{TM}/*lpr* group were due to either thymic lymphoma development or severe autoimmune disease associated with the *lpr* phenotype. Statistical analysis was carried out using the Rank Sum test

Myc-ER fusion protein (Blyth *et al,* submitted). Tumour incidence and latency were compared in CD2-*myc*ER^{TM} mice homozygous for the *lpr* mutation and in heterozygote controls. As shown in Figure 4 survival rates were unaffected by *lpr* status in the CD2-*myc*ER^{TM} mice.

An interesting feature of CD2-*myc*ER^{TM} tumours is that despite their transformed status *myc* activity can still be modulated. Analysis of a series of CD2-*myc*ER^{TM} lymphomas has shown that explanted tumour cells show increased cell death following 4-OHT treatment (Figure 5a). This is seen in both primary tumour cells and established lines and is due to the effect of 4-OHT on transgene activity as treatment doesn't influence cell survival in lymphoma cells not carrying the CD2-*myc*ER^{TM} construct (Figure 5b). These data indicate that *myc*'s apoptotic function is not abolished during transformation. CD2-*myc*ER^{TM} lymphoma cells homozygous for *lpr* were placed in culture and treated with 4-OHT. *Myc* induced apoptosis was not blocked in *lpr/lpr* cells indicating that, at least in T cell lymphoma cells, Fas was not required for *myc* induced apoptosis *in vitro*. Figure 5c shows a representative survival curve for 4-OHT treated primary CD2-*myc*ER^{TM}/*lpr* lymphoma cells. *Lpr* mice are known to express very low levels of Fas, to exclude the possibility that increased levels of Myc could sensitise *lpr* cells to Fas induced apoptosis, an established CD2-*myc*ER^{TM}/*lpr* lymphoma cell line was treated with 4-OHT and anti-Fas antibody (Jo2). Jo2 had no effect on the viability of CD2-*myc*ER^{TM}/*lpr* lymphoma cells when Myc activity was upregulated (Figure 5d).

Proviral insertions at c-*myc* do not occur preferentially in MuLV infected *lpr/lpr* mice

Insertional mutagenesis of c-*myc* is frequently observed in Murine Leukaemia virus (MuLV) induced lymphomas.[28] If *myc* deregulation and loss of Fas signalling represent synergistic events in T cell lymphomagenesis the frequency of proviral insertions at c-*myc* would be expected to be significantly increased in infected *lpr/lpr* mice. To confirm the apparent lack of synergy observed in the transgenic studies, *lpr/lpr*-MRL and MRL control mice were infected with MuLV. As shown in Figure 6 the latency of tumour development was not significantly affected by the *lpr* status of the infected mice. These results are consistent with those previously reported by Zornig *et al*.[29] However the number of tumours containing insertions at c-*myc* in *lpr/lpr*-MRL mice (10/53) was not significantly greater than in the MRL controls (15/42) indicating that c-*myc* was not a preferential target in *lpr*

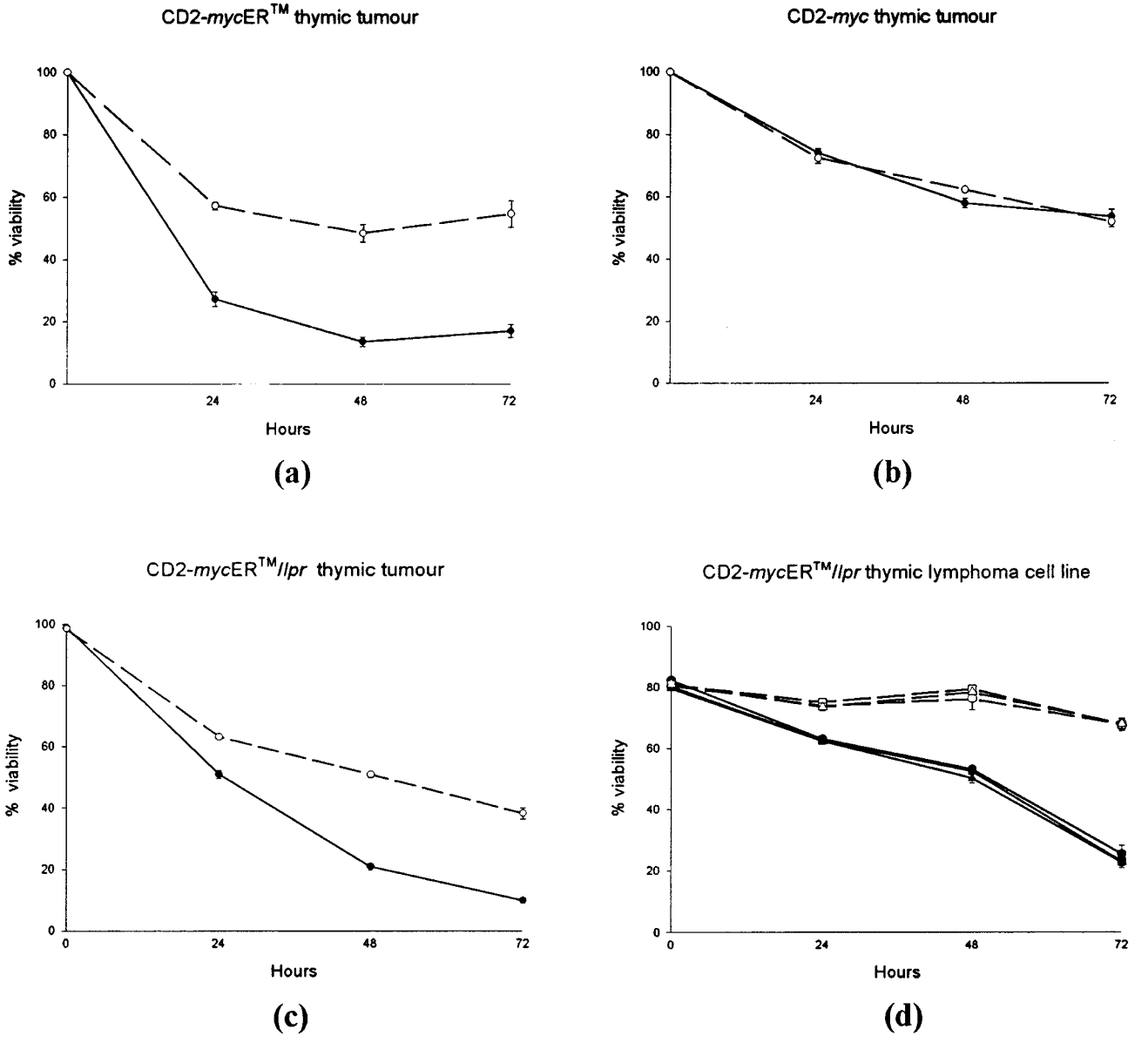

Figure 5 Viability of explanted CD2-*myc*ER^{TM} lymphoma cells treated with 4-OHT. Cell survival is shown during short-term *in vitro* culture with (solid line, filled circle) and without (dashed line, open circle) 4-OHT treatment. Analysis of viability is based on trypan blue exclusion and results are expressed as percentage viable over total, each result is based on quadruplicate counts. (**a**) CD2-*myc*ER^{TM} lymphoma cells, results were similar for another 12 tumours. (**b**) CD2-*myc* lymphoma cells (i.e. those that do not carry the regulatable form of the transgene), similar results were obtained for another three tumours. (**c**) CD2-*myc*ER^{TM}/*lpr* lymphoma cells, results were similar for another four tumours. (**d**) CD2-*myc*ER^{TM}/*lpr* lymphoma cell line: 4-OHT (solid line, filled circle); no treatment (dashed line, open circle); 4-OHT and Jo2 anti-Fas antibody (solid line, filled square); Jo2 anti-Fas antibody (dashed line, open square); 4-OHT and Ig isotype control antibody (solid line, filled triangle) and Ig isotype control antibody (dashed line, open triangle)

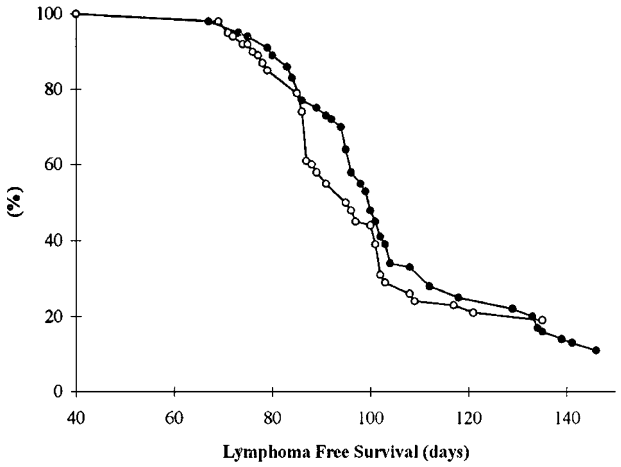

Figure 6 Tumour free survival of MuLV infected *lpr/lpr* mice. Survival curves for MuLV infected *lpr/lpr*-MRL (filled circles, *n*=60) and MRL control mice (open circles, *n*=49). Statistical analysis was carried out using the Rank Sum test

tumours. Further, there was no correlation between tumour latency and insertions at c-*myc*. In the *lpr* cohort the average latency of *myc* negative tumours and *myc* positive tumours was each 103 days. Similarly in the MRL mice the latency of tumours with and without *myc* insertions was 90 and 95 days respectively.

Discussion

In addition to proliferation, *myc* has the ability to induce apoptosis under certain circumstances. The capacity of *myc* to induce diverse outcomes has led to the hypothesis that its apoptotic role may represent an important restraint to tumour formation and coexisting survival signals or anti-apoptotic lesions are required to allow *myc* to realise its full oncogenic potential (reviewed in[30]). The dramatic acceleration of tumour incidence in transgenics expressing both *myc* and anti-apoptotic genes such as *bcl*-2 is consistent with this theory. Fas represents an important death pathway in T lymphocytes and has been shown to be involved in activation induced cell death, thymocyte negative selection, elimination of autoreactive cells in the periphery,[31–38] as well as being implicated as a tumour suppressor.[20–22]

We have investigated the putative tumour restricting properties of Fas in *myc* mediated tumourigenesis and here present evidence that loss of Fas signalling and deregulated *myc* expression do not synergise in T cell lymphomagenesis. Survival data from two similar but distinct CD2-*myc* transgenic models has shown that c-*myc* mediated tumour development is not increased on an *lpr* background and that tumour phenotype is not altered by the absence of a functional Fas pathway. These results are at variance with those of Zornig *et al,*[29] who reported an increased rate of tumour formation in Eμ-L-*myc* mice on an *lpr* background. The reason for the discrepancy between the data obtained from the two models used here and that of Zornig *et al*,[29] is not clear.

The lack of collaboration observed between *myc* and Fas loss in CD2-*myc* transgenic mice was confirmed by infecting *lpr*-MRL and MRL control mice. Survival was not reduced in the *lpr* cohort and, perhaps more importantly, *myc* did not represent a preferential target for proviral insertional mutagenesis. If *myc*'s oncogenic role in T cells was capable of being restricted by a functional Fas pathway then a high proportion of MuLV induced tumours in *lpr* mice might be expected to contain insertions that deregulate *myc*. Such a pattern is frequently observed in MuLV infected transgenic animals bearing a known *myc* collaborating oncogene. For example, all tumours arising in Eμ-*pim*-1 transgenics infected with MuLV have insertions at either c-*myc* or N-*myc*.[39]

Previously it has been shown in fibroblasts that *myc* induced apoptosis was dependent on the presence of a functional Fas pathway and that c-*myc* can sensitise cells to Fas mediated death signalling.[12] To investigate the functional interaction between Fas signalling and *myc* induced apoptosis in transformed T cells, and by extension the relevance of *myc* induced apoptosis to tumour suppression, we made use of cell lines arising from CD2-*myc*ER^{TM} transgenic mice. CD2-*myc*ER^{TM} tumour cells die at increased rates when *myc* activity is upregulated indicating that, despite their transformed status, these cells retain their sensitivity to *myc* induced apoptosis. This apoptotic response is not blocked by the absence of Fas as tumour cells arising from CD2-*myc*ER^{TM} mice on an *lpr* background are equally susceptible. These results are consistent with the *in vivo* data obtained using TUNEL that show that CD2-*myc/lpr* tumours do not display a reduced apoptotic index compared to CD2-*myc* tumours on a wild-type background.

It is clear therefore that *myc* mediated apoptosis can occur in a Fas independent manner, at least in transformed T cells. These results are in contrast to those of Hueber *et al,*[12] and indicate that *myc* may be able to utilise alternative death pathways in different cell types. Recent data has suggested that interactions between *myc* and Fas are involved in activation induced death. Using T cell hybridomas Wang *et al*,[40] have shown that CD3 cross-linking of the T cell receptor induces *myc* expression that in turn serves to upregulate FasL cell surface expression. It would appear, therefore, that *myc* can induce apoptosis in T cells by both Fas dependent and Fas independent mechanisms and that unlike fibroblasts functional Fas pathways are not essential for *myc* to realise its apoptotic function. Additional differences exist between *myc* induced apoptosis in T lymphoma cells and fibroblasts. In fibroblasts *myc* induced apoptosis is only observed when serum levels are suboptimal but in all CD2-*myc*ER^{TM} lymphoma cell lines tested, upregulation of *myc* activity induces apoptosis at normal serum concentrations. This may reflect the different *in vitro* requirements of these cell types as fibroblasts can be easily cultured *in vitro* whereas mouse thymocytes and the majority of thymic tumours do not establish. Further, specific growth factors such as IGF-1 have been shown to block *myc* induced apoptosis in fibroblasts[41] as does over expression of eukaryotic translation initiation factor 4E (eIF4E) which may be involved in mediating growth factor survival signalling.[42]

Fas may have an important role in restricting tumour formation within a specific cell lineage. Due to the extended

survival of B lineage cells, Eμ-Bcl-2 transgenic mice develop lymphoid hyperplasia that can progress to high grade lymphomas.[5] Although lymphoproliferative disease is markedly accelerated, these mice do not show an increased tumour incidence on an *lpr* background.[43] Similar results were obtained with *pim*-1 transgenic mice[44] and in mice null for p53 and Fas (ER Cameron *et al* 1999, unpublished results), both of which display accelerated lymphoproliferative disease but no increase in tumour incidence. By contrast, deregulated expression of Bcl-2 in myeloid cells results in a progressive monocytosis and aberrant myeloid differentiation that in the absence of Fas signalling can progress to a condition resembling acute myeloblastic leukaemia.[22]

The physiological consequences of Fas ligation can also be context dependent. Although usually associated with induction of apoptosis, Fas has been shown to be capable of transducing a proliferative signal in human fibroblasts.[45] Further, different cell types can utilise different pathways downstream of Fas. Type I cells are characterised by rapid activation of caspase-8 and caspase-3 with strong induction of the death-inducing signalling complex (DISC). By contrast, in type II cells caspase activation occurs following loss of mitochondrial transmembrane potential. Overexpression of *bcl*-2 can block activation of caspases-8 and -3 in type II but not in type I cells.[46]

Our results are reminiscent to those describing the role of p53 in *myc* induced apoptosis with some studies showing this process being dependent on the presence of functional p53 but others identifying p53 independent pathways.[47–49] Here we have demonstrated the existence of a Fas independent pathway that can mediate *myc*'s apoptotic function in T cell lymphomas. These results suggest that *myc* induced death can be transduced by alternative pathways and emphasise the complexity of events that can influence the balance between proliferation and cell death within an individual tumour.

Materials and Methods

Mouse experiments

Mice carrying CD2-*myc* or CD2-*myc*ER^{TM} transgenes, on a mixed C57B16 and CBA/Ca background,[23] were crossed with *lpr/lpr* MRL homozygous mice. Transgenic offspring were backcrossed onto *lpr/lpr* homozygous mice. Animals were sacrificed when obvious signs of malaise were present. In CD2-*myc/lpr* mice a diagnosis of thymic lymphoma was made when the lesion was (oligo)clonal with respect to TcR rearrangements and CD2-*myc* expression was present, previous work having established that the transgene is only expressed in transformed cells.[23]

DNA and RNA hybridisation analysis

Extraction of high-molecular weight DNA, digestion with restriction enzymes and Southern blotting was carried out as previously described.[23,24] Rearrangement of the T-cell receptor β-chain gene was determined by hybridisation to a radiolabelled 497 bp *Eco*RI fragment containing TCR Cβ sequence from the 86T5 clone.[50] *Myc* transgene sequences were detected using a human c-*myc* exon 3 probe (1.38 Kb *Cla*I/*Eco*RI fragment). Analysis of c-*myc* RNA expression was carried out by Northern blotting using the 1.38 Kb *Cla*I/*Eco*RI fragment.[23]

Polymerase chain reaction (PCR)

PCR was used to determine the genotype of *lpr/lpr* backcrossed mice. Three primers were designed as follows; A: sense 5′-CAC TTT ACT CAT TGA CTT AT-3′; B: antisense 5′-CAG CCG GTG CAG CCA GAA GC-3′; C: antisense 5′-CGT TGC TCC GAT GTC CGA TA-3′. Amplification of genomic DNA was carried out in a 50 μl reaction mix which consisted of 2 μg DNA, 2 units Taq polymerase (Perkin Elmer), 200 μM of each deoxynucleoside in 10 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$ buffer and either primers A and B or primers A and C to a final concentration of 0.5 μM. The conditions used were denaturation at 94°C for 5 min followed by 35 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 1 min ending with a final extension of 72°C for 7 min and 4°C soak indefinitely. Primers A and B detect wild-type allele (296 bp). Primers A and C detect the *lpr* mutation of the allele (547 bp).

Flow cytometry

Mouse lymphocytes were disaggregated in RPMI 1640 medium (Gibco BRL) and isolated on a Ficoll-Paque (Pharmacia) density gradient. Viable cells (1×10^6/ml) were resuspended in phosphate buffered saline containing 0.1% sodium azide and 2% FCS and directly labelled for 30 min at 4°C using a combination of the following antibodies: Quantum red conjugated rat anti-mouse CD3, Fluorescein Isothiocyanate (FITC) conjugated rat anti-mouse CD8, RPE conjugated rat anti-mouse CD4, FITC conjugated rat anti-mouse CD45R (Sigma). RPE conjugated mouse IgG2a (Serotec), FITC and Quantum red conjugated mouse IgG2a (Sigma) antibodies were used as isotype controls. Flow cytometric analysis was performed using a Coulter Epics Elite.

Cell culture

Thymocytes were cultured in RPMI 1640 (Gibco BRL) containing β mercaptoethanol (BDH), 10% foetal calf serum, 2% Penicillin and Streptomycin and 1% Glutamine (Gibco BRL). Cells were cultured at 2×10^6/ml in 24-well plates and viable cell numbers were counted at 24 h intervals by dye exclusion using 0.4% Trypan Blue solution (Sigma). Jo2 monoclonal antibody (Pharmingen) was used at a concentration of 2 μg/ml. Hamster IgG1 kappa monoclonal antibody (Sigma) was used as an isotype control at a concentration of 2 μg/ml. CD2-*myc*ER^{TM} cells were treated with 4-hydroxytamoxifen (4-OHT, Sigma) at a final concentration of 250 nM. All cultures were performed in quadruplicate and viability curves were based on the average number of live cells expressed as a percentage of the average total. Comparisons at specific time points were carried out using the Students *t*-test.

In situ terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL)

TUNEL was performed on 10% neutral buffered formalin fixed, paraffin embedded tissue sections and is based on the procedure described by Gavrieli *et al*.[51] Sections were dewaxed prior to nick end labelling. All incubations were carried out using 50–100 μl of solution in humidified chambers at room temperature unless otherwise stated. Sections were incubated with 20 μg/ml proteinase K (Sigma) for 15 min, rinsed in PBS then endogenous peroxidase activity was blocked using hydrogen peroxide (3% in methanol) for 5 min. The sections were then rinsed in PBS and immersed in TDT buffer (30 mM Trizma base pH 7.2, 140 mM

sodium cacodylate, I mM cobalt chloride) for 2 min, followed by 60 min, 37°C incubation in 0.15–0.25 U/μl TdT (Terminal deoxynucleotidyl Transferase) and 1 : 50–1 : 100 biotin-16-dUTP in TdT buffer. Following two 5 min rinses in TB buffer (300 mM sodium chloride, 30 mM sodium citrate) and four PBS rinses the sections were incubated for 60 min in 1 : 200 peroxidase conjugated streptavidin. Staining was visualised using the chromogen AEC (Vector Laboratories) prior to counter-staining with Mayers haematoxylin. At least five different microscopic fields were examined for each tissue section.

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