Abstract. Background: Increased glucose uptake is characteristic of many tumours and transformed cells and, consequently, glucose deprivation would be expected to differentially affect cancer cell survival and growth. In this study we investigated whether specific oncogenes sensitise cells to apoptosis induced by glucose deprivation. Materials and Methods: Oncogene-transformed 32D cells were deprived of glucose with or without IL-3 and $[^3H]2$-deoxyglucose uptake measured. Apoptosis was determined by AnnexinV/propidium iodide staining, cell cycle distribution was analysed and MYC expression determined by Western blotting. Results: v-Hras and to some extent v-src and v-abl enhanced apoptosis induced by glucose deprivation in the presence of IL-3 but attenuated apoptosis in its absence. In contrast, bcr-abl was highly protective against glucose deprivation-induced apoptosis for 72 hours in the presence and absence of IL-3, while bcl-2 was mildly protective in the presence of IL-3. With strongly transforming oncogenes, c-MYC expression correlated with cell sensitivity to apoptosis induced by glucose deprivation. Conclusion: Oncogenes vary markedly in their ability to protect cells from apoptosis following glucose deprivation and, in some situations, promote apoptosis.

Cells must maintain a constant supply of energy to avoid apoptosis. Should intracellular ATP levels drop to 10-15% of those found in normal cells, a pre-existing program for apoptosis will be activated (1,2). However, maintenance of energy balance is not the sole regulatory point governing cell survival, since glucose, ATP and other metabolites cannot reverse apoptosis induced in various ways in haemopoietic cells. Survival of cells is a highly coordinated process whereby signal transduction pathways integrate both external and internal signals with metabolic pathways to ensure that energy levels meet cellular requirements. A common feature of growth factor/cytokine signalling is the ability to promote glucose uptake across the plasma membrane (3,4) and to stimulate cellular metabolism. Because cell transformation often overrides growth factor dependence, it is not surprising that cancer is often associated with increased glucose uptake. Increased glucose uptake has been attributed to increased glucose transporter expression (5), translocation (6) and intrinsic activation of glucose transporters (3,7,8). For example, the expression of ras (5), abl (9) or fps (10) has been found to stimulate glucose uptake in fibroblasts and haemopoietic cell lines.

Increased glucose uptake has been demonstrated in many different types of cancers, with advanced and aggressive tumours consuming greater quantities of glucose than early or benign tumours (1,11-13). The observation that transformed cells utilise aerobic glycolysis to metabolise glucose was made almost 80 years ago by Warburg (14). This form of metabolism produces only 2 molecules of ATP compared with 36 during oxidative phosphorylation and is usually associated with high rates of glucose uptake. Increased glucose uptake by cancer cells is commonly employed to locate and assess metabolic activity using $[^{18}F]$-deoxyglucose and positron emission tomography. Transformed cells often maintain high levels of glucose uptake by over-expressing glucose transporter subtypes. For example, elevated levels of Glut-1 have been reported in a wide range of human malignancies including bladder, lung, ovary, testis, skin and breast (15-19), elevated Glut-2 in hepatic, pancreatic and breast carcinomas, and Glut-3 in high-grade gliomas (20). In addition, atypical expression of glucose transporter subtypes 4 and 5 has been observed in breast cancers (21).

The metabolic differences between cancer cells and normal cells remain attractive targets for anti-cancer
therapies, although to date this approach has not been fully exploited. A few studies have shown promising results, e.g. regional administration of the non-metabolisable glucose analog 2-deoxyglucose, or “armed” glucose analogs, such as those conjugated to nitric oxide releasing agents, are novel approaches to target cancers that have high glucose consumption (22-24). In addition, suppression of Glut-1 mRNA expression using anti-sense has been demonstrated to suppress tumour growth (25). As well as targeted approaches, indirect evidence suggests that the overall reduction in glucose, i.e. caloric restriction, reduces tumour incidence and growth (26,27), while hypoglycaemic shock has been shown to preferentially kill cancer cells due to their increased dependence on glucose (28).

While previous studies have found a correlation between the introduction of an active oncogene and increased glucose uptake, the effects of specific oncogenes on cell sensitivity to apoptosis induced by glucose deprivation has not been determined. In this study we investigated whether transformation of 32D cells with v-Hras, v-src, v-abl, bcr-abl or bcl-2 sensitises these cells to apoptosis induced by glucose deprivation and whether IL-3 alters this response. We show that these oncogenes differ widely in their ability to protect cells from glucose deprivation-induced apoptosis in the absence of IL-3 and that v-Hras, and to some extent v-src and v-abl, promote apoptosis in the presence of growth factor.

**Materials and Methods**

Cells. 32Dc3 and c32 cells, originally derived by Greenberger (29), have been used widely to study transforming oncogenes. 32Dc3 cells transformed with v-src, v-abl or v-Hras (JCS2) constructs are kindly provided by Dr S.M. Anderson (Department of Pathology, University of Colorado Health Science Centre, Colorado, USA). Cells were cultured at 37°C in a humidified incubator maintained at 5% CO2. Exponentially growing 32Dc3 cells were transfected with the pC3CMVcmyc or pC3CMVbcr-abl construct (Dr S.M. Anderson) or the pC3CMVbcr-abl construct, PEF Bcl-2-2pGKpurap (Dr A. Strasser and Eliza Hall Institute, Melbourne, Australia) by electroporation using a Cell-Porator (GIBCO-BRL, Grand Island, NY, USA). For stable transfection, cells were cultured in RPMI 1640 containing 5% FBS for 48 h, and then selected by the addition of 1 mg/ml G418 (GIBCO-BRL) for 7 days. Cells were maintained as polyclonal populations to avoid introduction of clonal artefacts. All oncogene-transfected cells were maintained in RPMI 1640 supplemented with 10% FBS. Bcl-2-transfected cells were supplemented with 10% Wehi-3-conditioned medium to provide IL-3 as bcl-2 did not confer growth factor independence. Morphologically, v-myc-, v-src-, or v-abl-transformed cells were similar to parental cells which were non-adherent and rounded in appearance in the phase contrast microscope. In contrast, bcr/abl-transformed cells were non-adherent and elongated with small dendrite-like processes (results not shown).

**Glucose deprivation.** 32D cells were washed three times in glucose-free RPMI, then cultured in the same medium supplemented with 10% dialysed FBS and 1 mM sodium pyruvate. FBS (40 ml), was dialysed (M r 10 KDa cut-off) at 4°C against two 1 L changes of PBS pH 7.4. Dialysed FBS was calculated to contain less than 1% glucose and was capable of supporting the long-term growth of 32D cells when supplemented with glucose and IL-3.

**Glucose uptake.** Glucose uptake was measured by the zero-trans method. Cells (10 6) were suspended in 250 µl glucose-free RPMI 1640 (GF-RPMI) without serum and preincubated for 3 min at 37°C. [3H] 2-deoxy-D-glucose (0.5 µCi) (Amersham, Little Chalfont, England) and 2-DOG were added at a final concentration of 100 µM. Uptake was determined at 37°C for 3 min under conditions where glucose uptake was linear. Uptake was terminated by adding 250 µl ice-cold GF-RPMI containing 0.6 mM phloretin (Sigma-Aldrich, Castle Hill, Australia) and centrifuging through a cushion of 10% (w/v) BSA (50 µl) at 8880 x g for 1 min in a microcentrifuge tube. The cell pellet was washed with 250 µl GF-RPMI, lysed in 100 µl of 0.1% Triton X-100, and radioactivity determined using a liquid scintillation spectrometer. Results are presented as the average of duplicate determinations ± S.E.M.

**Cellular DNA content analysis.** Cells (1 x 10 6) were washed, fixed with 70% ice-cold ethanol and stored overnight at 4°C. Cells were pelleted and suspended in 1 ml 50 µg/ml propidium iodide (Sigma-Aldrich), 100 Kunitz units RNase A/ml (Sigma) in Mg2+ and Ca2+, free PBS pH 7.4 containing 1 mg/ml glucose and left at room temperature for at least 30 min. Samples were analysed within 24 h in a FACSscan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Cell cycle analysis was carried out using the ModFit LT version 1.0 cell cycle analysis software (Verity Software House, Topsham, ME, USA).

**Measurement of apoptosis.** Apoptosis was measured by flow cytometry using Annexin-V-FLOUOS (Boehringer Mannheim, Germany). Cell staining was carried out according to the manufacturer’s instructions, with minor modifications as follows: 106 cells were washed with PBS and centrifuged at 200 g for 5 min. Cells were incubated at 37°C for 10-15 min in 100 µl of buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl2) containing 1 µl Annexin-V-FLUOS labelling solution and 2 µl 50 µg/ml PI). Cells were analysed using FACSscan flow cytometer (Becton Dickinson) with FL1-H for Annexin-V-FLUOS and FL2-H for PI (30–32); sub-G1 cells were excluded from all analysis.

**Detection of c-MYC by Western blotting.** Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM PMSF, 30 KIU/ml Aprotinin) (106 cells/ml) and incubated at 4°C for 20 min. Lysates were clarified by centrifuging at 15,000 x g for 15 min in a Biofuge Fresco fixed rotor (Hereus, Hanan, Germany), mixed with Laemmli sample buffer and heated to 100°C for 3 min. Proteins were separated using 10% SDS-polyacrylamide gels and transferred electrophoretically to supported nitrocellulose membranes (Hybond-C, Amersham) at 150 V for 45 min. Membranes were blocked with Tris-buffered saline (TBS) (150 mM NaCl, 50 mM Tris base, pH 7.4) containing 0.1% Tween 20 and 5% BSA for 1 h at room temperature and then incubated with 5 µg/ml anti-c-Myc (c-33) mAb IgG (Santa Cruz, CA, USA) for 1 h. The membranes were washed using TBS-Tween 20 and incubated with rabbit anti-mouse Ig conjugated to horseradish peroxidase (1:4000; DAKO, Glostrup, Denmark). Membranes were again washed in TBS-Tween and proteins detected using enhanced chemiluminescence.
detection reagent (ECL, Amersham). Membranes were exposed to Hyperfilm-ECL* for 30 sec to 5 min and the film developed using a Kodak RP-OMAT processor.

**Results**

To investigate the effects of specific oncogenes on the apoptosis induced by glucose deprivation, oncogene-transformed cells on a common haemopoietic cell background were employed. Murine 32Dcl3 cells stably transformed with \( v\)-\textit{Hras}, \( v\)-\textit{src} or \( v\)-\textit{abl} had doubling times of 16 h, 15 h and 25 h compared with 17 h for 32Dcl3, while 32Dcl23 cells transformed with \( bcr\)-\textit{abl} and transfected with \( bcl-2\) had doubling times of 26 h and 24 h compared with 25 h for 32Dcl23. Thus, except for \( v\)-\textit{abl} which slowed cell cycling of 32Dcl3 cells, transformed cells showed comparable cell doubling times to parental cells.

**Effect of glucose deprivation on 2DOG uptake by growth factor-dependent and oncogene-transformed 32D cell lines.** Glucose uptake was measured in parental and oncogene-transformed 32D cells and the effects of glucose deprivation on 2DOG uptake determined (Figure 1). As expected, \( src\)-, \( ras\)- and \( abl\)-transformed 32Dcl3 cells exhibited 2-3.9 -fold greater 2DOG uptake than parental cells. Surprisingly, exponentially growing 32Dcl23\( bcr/abl\) cells exhibited only 23% of the 2DOG uptake observed with parental 32Dcl23 cells, whereas 32Dcl23\( bcl-2\) cells did not significantly differ from wild-type cells. Comparison of 2DOG uptake with cell doubling times showed no direct correlation, with 32Dcl23\( bcr/abl\) showing unexpectedly low and 32Dcl3\( ras\) unusually high 2DOG uptake. Thus, glucose uptake by oncogene-transformed 32D cells does not correlate with the predicted energy requirements for cell cycling.

The effect of glucose deprivation on glucose uptake by growth factor-dependent and oncogene-transformed 32D cell lines is also shown in Figure 1. 2DOG uptake declined by 20-63% following 2-h glucose deprivation and declined further after 5 h (50-91% of control). Reduced glucose uptake was not as pronounced with 32Dcl23 and 32Dcl23\( bcr/abl\) cells at 2 h, while uptake by 32Dcl3 and 32Dcl23\( bcl-2\) did not further decline at 5 h. In general, oncogenes did not maintain glucose uptake levels at those of glucose-maintained cells.

**Oncogene sensitisation to apoptosis following glucose deprivation.** The effects of specific oncogenes on glucose deprivation-induced apoptosis was investigated for periods of up to 72 h using Annexin V/propidium iodide staining and flow cytometry. Prior to glucose deprivation, cell viability was 90-98%. When 32Dcl3 and 32D cl23 cells were deprived of glucose in the absence of IL-3, cells rapidly became apoptotic with 20-25% survival at 24 h and 5-10% at 48 h (Figure 2). In the presence of IL-3, 56-60% of cell remained viable at 24 h and 25-40% at 48 h. Thus, IL-3 had a sparing effect on the apoptosis induced by glucose deprivation.

In the presence of specific oncogenes, glucose deprivation-induced apoptosis varied depending on the oncogene and whether growth factor was present. In the presence of IL-3, 32D cells transformed with \( ras\), and to a much lesser extent \( src\) and \( abl\), were more sensitive to apoptosis induced by glucose deprivation than were parental cells. In contrast, in...
the absence of IL-3, these oncogenes spared cells from glucose deprivation-induced apoptosis. With bcl-2, a small sparing effect on glucose deprivation-induced apoptosis was observed in the presence of IL-3 but no significant effect was seen in the absence of IL-3. In contrast to other oncogenes, bcr-abl-transformed 32D cells were remarkably resistant to glucose deprivation-induced apoptosis in the presence or absence of IL-3 maintaining 95% viability at 48 h and 85% viability at 72 h. This may be related to their low glucose uptake (22% that of parental cells (Figure 1), suggesting that these cells may utilize alternative energy sources.

Cell cycle changes following glucose deprivation in non-transformed and transformed cell lines. To investigate whether cell survival following glucose deprivation was associated with cell cycle arrest, cells were deprived of glucose for up to 72 h and cell cycle profiles determined by propidium iodide staining and flow cytometry. Cell cycle analysis was carried out on all viable cells and the percentage of viable cells is shown at the base of each histogram (Figure 3).

Following glucose deprivation, 32Dcl3 and 32Dcl3src arrested in G1, whereas 32Dcl23 arrested in both G1 and G2/M. 32Dcl3ras, 32Dcl3abl, 32Dcl23bcl-2 and 32Dcl23bcr/abl failed to consistently accumulate in G1, with all cells except 32Dcl3ras showing a small tendency to arrest in G2/M. Thus, in most cell lines examined, G1 and/or G2/M checkpoints appeared to be partially or fully intact. 32Dcl23bcl-2 and 32Dcl3abl did not show a significantly altered proportion of cells in S-phase following glucose deprivation, whereas 32Dcl3ras showed a small increase and 32Dcl23bcr/abl a small decrease. Loss of cells from one phase of the cell cycle is normally balanced by changes in other cell cycle compartments. With 32Dcl23, 32Dcl3 and 32Dcl3src cells, loss of cells in S-phase was associated with increased G1 and/or G2/M and apoptosis. However, 32Dcl3abl cells that do not appear to lose cells from S-phase also undergo apoptosis.

c-MYC expression in normal and oncogene-transformed 32D cells. C-MYC participates in the control of cell growth, proliferation, programmed cell death and differentiation (33). The expression of c-MYC has been shown to sensitise cells to apoptosis following glucose deprivation. Therefore, we investigated whether c-MYC expression correlated with glucose deprivation–induced apoptosis in growth factor-dependent and oncogene-transformed cells. Cells were lysed and proteins separated on SDS-PAGE gels, transferred to nitrocellulose and probed with anti-c-MYC antibody. Under normal culture conditions, 32Dcl23, 32Dcl3, 32Dcl3src and 32Dcl3abl expressed high levels of c-MYC, whereas 32Dcl3ras, 32Dcl23bcl-2 and 32Dcl23bcr/abl cells expressed considerably lower levels. Following 2 h of glucose deprivation, c-MYC expression decreased in all cell lines (Figure 4) and was barely detectable in 32Dcl23, 32Dcl3ras, 32Dcl3abl, 32Dcl23bcr/abl and 32Dcl23bcl-2. Interestingly, when compared with cell viability data (Figure 2), the low c-MYC-expressing cells, 32Dcl3ras and 32Dcl23bcr/abl were less sensitive to glucose deprivation-induced apoptosis than the high c-MYC-expressing cells (32Dcl3src and 32Dcl3abl).

Discussion

Many different cancer cells exhibit an increased rate of glucose uptake suggesting that this trait confers a survival benefit or proliferative advantage. Previous studies have demonstrated that glucose deprivation can cause cancer cells to apoptose...
Figure 3. Effect of glucose deprivation on cell cycle phase distribution in normal and transformed 32D cells. Cells were cultured in GF-RPMI 1640, 5% dialysed FBS and supplemented with 20 ng/ml IL-3 if required for normal cell growth (32Dcl23, 32Dcl3, 32Dcl23bcl-2). Cell samples were removed at the times indicated and cell distribution and DNA content determined by PI staining and flow cytometry and ModFit LT analysis. The percentage of viable cells is indicated at the bottom of each bar. The results of one experiment are shown and are representative of 3 separate experiments.
In this study we investigated whether transformation of 32D cells with specific oncogenes sensitised or protected cells from apoptosis induced by glucose deprivation and found that the effect observed depended on the particular oncogene.

Prior studies have shown that transfection of 32Dcl3 cells with constitutively activated v-Hras, v-src (36) bcr/abl (37), or v-abl (38) overcomes the need for IL-3 for survival and proliferation. Bcl-2 expression on the other hand did not confer growth factor independence on 32D cells (39). Transformation of 32D cells with ras, which encodes a GTP-binding protein, or src or abl, which encode tyrosine kinases, increased their rate of glucose uptake compared with parental cells. In contrast, 32D cells expressing bcl-2 did not alter glucose uptake, while 32D cells expressing bcr-abl exhibited decreased glucose uptake (22% of parental cells).

When parental 32D cells were deprived of glucose they underwent apoptosis and this was accelerated in the absence of IL-3. In contrast, 32D cells transformed with ras and to a much lesser extent src and abl in the presence of IL-3 were more sensitive to glucose deprivation-induced apoptosis than parental cells. In the absence of growth factor, ras, src and abl protected cells from glucose deprivation-induced apoptosis. Ras transformed cells were highly sensitised to glucose deprivation-induced apoptosis if IL-3 was present in the media. A similar phenomenon has been observed with MDA-468, human breast cancer cells, where treatment with EGF at low glucose concentrations appeared to sensitise these cells to undergo apoptosis (40). Interestingly, if 32Dclras cells were cultured in the presence of both IL-3 and glucose, differentiation into macrophage-like cells was induced (results not shown).

Bcl-2-transformed cells showed a similar loss of cell viability to parental cells when deprived of glucose or IL-3, but in the presence of IL-3, cell survival was enhanced. Previously bcl-2 has been shown to block apoptosis at a point downstream from the collapse of cellular homeostasis (41). In neurons and astrocytes, overexpression of bcl-2 and bcl-xL was shown to protect these cells from glucose deprivation-induced apoptosis (42). Thus, the protection offered by some bcl-2 family members appears to be cell-type specific and may confer greater protection to primary cells rather than cell lines.

Interestingly, bcr/abl-transformed 32D cells maintained 95% viability at 48 h and 85% following 72 h of glucose deprivation, regardless of whether IL-3 was present in the culture media or not. The rate of glucose uptake in these cells was only 22% that of parental cells, suggesting that these cells require little glucose and are able to employ alternative energy sources. Recent studies have indicated that rapidly proliferating cancer cells require a constant supply of glucose whereas slower growing cells may not be so dependent on glucose (43). However, the cell division rates for both bcr/abl and parental cells were similar, indicating that glucose uptake is not the primary determinant of cell proliferation rate in these cells. Several reports have shown that, in the absence of glucose, ATP can be generated from pyruvate oxidation or from the conversion of glutamine to lactate (44,45). In a preliminary study, culturing 32Dcl23bcr/abl cells in the absence of both glutamine and glucose for 48 h did not result in cell death, nor did exposure to 11 mM 2DOG for 48 h (results not shown). The possibility that 32Dcl23bcr/abl cells generate ATP by mitochondrial metabolism of amino acids remains to be investigated (46,47).

Growth factor-dependent and transformed cells deprived of glucose were unable to maintain 2DOG uptake. None of the cells examined maintained 2DOG uptake at levels observed prior to glucose deprivation. Several different primary cell types have been found to up-regulate 2DOG uptake 2- to 4-fold following glucose deprivation (48-52), by increasing glucose transporter mRNA and cell surface protein expression. Cancer cells on the other hand respond to glucose deprivation initially by expressing an array of stress-related genes and subsequently undergoing apoptosis (53). For example, early in glucose deprivation, human breast cancer cell lines exhibit increased levels of Lyn and JNK as well as increased expression of c-MYC mRNAs. This suggests that some cancers cells are uniquely sensitive to glucose deprivation-induced apoptosis. Interestingly, except for 32Dcl23bcl-2, which requires IL-3 for survival and growth, those cells most sensitive to glucose deprivation-induced apoptosis were found to have high c-MYC expression. This has now been shown for several cell types, and apoptosis appears to be p53-independent and possibly linked to increased LDH-A expression (34).
Following glucose deprivation both parental and transformed cells accumulated in G1 or G2/M. Thus, in all cell lines examined, G1 and/or G2/M checkpoints were partially or fully intact. Existence of an energy-dependent checkpoint at both the G1/S and G2/M boundary has been reported before (2). These checkpoints, in theory, would give cells the ability to detect nutritional availability throughout the cell cycle.

As discussed, one major biochemical difference between normal and cancer cells is the way they take up and utilise glucose and therapeutic strategies for targeting high glucose-consuming cells are being developed (24). Our work clearly shows that cells expressing different oncogenes, cultured in the absence and presence of growth factors, respond differently when deprived of glucose. Cells expressing constitutively active v-ras, and to some extent v-src and v-abl, were more susceptible to cell death following glucose deprivation in the presence of IL-3 than parental cells, suggesting that cancers with these oncogenes may respond to treatment with compounds which block glucose uptake or its metabolism i.e. 2DOG, while cancers expressing bcr/abl or bcl-2 may not be susceptible to these therapies.

It is important to note that the transforming events investigated in this study are on an immortalised cell background and that combined effects of oncogenes and/or tumour suppressors will be operative. The immortalisation events in 32Dcl23 and 32Dcl3 are unknown; it would be interesting to determine whether cancer cells known to carry specific oncogenes behave like oncogene-transfected 32D cells when deprived of glucose. Our study suggests that therapies which take advantage of increased glucose consumption (e.g., inhibition of glucose transport or non-metabolisable glucose analogues) should take into account the particular transforming event. Although full tumour genotypes are seldom known, some tumours are characterised by overexpression of specific oncogenes. For instance, bcr/abl is found in 90-95% of CML (54), and mutations of the ras proto-oncogenes (H-ras, N-ras and K-ras) are found in 20–30% of all human tumours including 90% of adenocarcinomas of the pancreas, and 50-70% of CML. Together, these data suggest that detailed knowledge of the cancer genotype and phenotype may benefit patients being treated with inhibitors of glucose transport, and that some tumours may be more or less responsive than others. Thus, under conditions where growth factor is limiting, cancers containing bcr/abl, ras and bcl-2 may be more resistant to therapies where glucose transport is inhibited. Ideally, an inhibitor of glucose uptake or metabolism that is toxic in high doses but does not affect normal tissues and does not pass through the blood brain barrier is desirable. Treatment of certain cancers with inhibitors of glucose uptake in conjunction with localised treatment with current therapies may prove to be highly beneficial in some situations.

Acknowledgements

This work was supported by the Cancer Society of New Zealand and its Wellington Division and by the Health Research Council of New Zealand.

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Received February 25, 2004
Accepted May 10, 2004