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Apoptosis and schizophrenia: A pilot study based on dermal fibroblast cell lines

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Abstract

Introduction: The aim of this study was to investigate whether there is an increased susceptibility to apoptosis in cultured fibroblasts from patients with schizophrenia.
Method: Dermal fibroblasts were collected and cultured from three groups: patients with schizophrenia, patients with non-schizophrenic psychosis, and healthy comparison subjects. Susceptibility to apoptosis was measured at the level of degradation product (proportion of cells in the sub-G0 cell cycle fraction in which apoptotic bodies accumulate), pro-apoptotic effector (activated caspase-3), and molecular regulators (P53, Bax and Bcl-2). Cell lines were studied under both basal culture and cycloheximide (an apoptotic inducer) exposure conditions.
Results: Consistent with increased susceptibility to apoptosis, the proportion of sub-G0 cells under basal conditions was significantly larger in the schizophrenia group, compared to the non-schizophrenic psychosis group. However when apoptosis was stimulated with cycloheximide, the schizophrenia group showed an attenuated caspase-3 response. The pattern of correlations between regulators, caspase-3 and the proportion of sub-G0 cells was different in the schizophrenia group, consistent with group-specific apoptotic pathway dysregulation.

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Conclusion: The study demonstrated anomalous apoptotic mechanisms in schizophrenia, which appear not to affect non-schizophrenia psychosis patients. The detection of these anomalies in fibroblasts suggests that altered apoptosis may be observable in all somatic cell types in schizophrenia.

Keywords: Schizophrenia; Bipolar disorder; Cell cycle; Apoptosis; Fibroblast

1. Introduction

The evidence of reduced incidence rates of cancer in schizophrenia (for a recent review, see Grinshpoon et al., 2005) was advanced in support of the hypothesis that increased susceptibility to apoptosis is implicated in the pathophysiology of schizophrenia (Catts and Catts, 2000). The rationale for this proposal was based upon the role of apoptosis in protecting against malignancy. Apoptotic regulators check DNA integrity at all stages of the cell cycle. If DNA damage is detected, cell cycle progression is arrested to allow repair of damaged DNA. If DNA repair is not possible, the potentially pre-cancerous cell normally undergoes apoptosis and is eliminated.

The current study was designed to carry out a multi-level functional assessment of apoptosis in living cells—at the level of regulators (P53, Bax and Bcl-2), effectors (caspase-3), and degradation products (apoptotic bodies). Phosphorylation of the transcription factor P53 increases levels of the pro-apoptotic protein Bax. Homodimerisation of Bax results in mitochondrial release of cytochrome C, which activates proteolytic enzymes called caspases in the cytosol. Caspase activation leads to DNA fragmentation and cell death. As the dying cell shrinks, the plasma membrane forms convolutions around cellular contents ('blebbing'), which sequester to form 'apoptotic bodies' containing DNA fragments. The anti-apoptotic protein, Bcl-2 keeps this process in check by forming Bax-Bcl-2 heterodimers, preventing Bax–Bax Homodimerisation.

We measured apoptotic markers in cultured dermal fibroblasts, based on the assumption that systemic cancer resistance would be related to altered apoptotic mechanisms in all cell types. Cultured dermal fibroblasts have been demonstrated to be a convenient and useful model for investigating schizophrenia (Mahadik and Mukherjee, 1996). The primary culture can be maintained in vitro for many cell passages overcoming the confounding effects of medications taken by the patients at the time of biopsy, barring prolonged medication-induced gene expression changes via epigenetic modification. By assessing cells under basal culture conditions, apoptotic mechanisms could be studied when not challenged by an apoptotic stimulus. By exposing cells to cycloheximide, mechanisms could be assessed when cells were undergoing an active apoptotic response.

2. Materials and methods

2.1. Subject recruitment and assessment

The study was approved by Griffith University and Wolston Park Hospital Institutional Ethics Committees. Subjects provided written informed consent. Full details of the subject recruitment procedures are provided elsewhere (McGrath et al., 2000). Subjects were assessed with the Diagnostic Interview for Psychosis (Jablensky et al., 1999) and diagnosed according to DSM-III-R. Patients in the schizophrenia group ($n=10$) were diagnosed with schizophrenia (7 males, 3 females; mean age 38 ± 10). The non-schizophrenic psychosis (NSP) group ($n=11$) included six patients with Bipolar Disorder (Episode(s) With Psychotic Features), four with Major Depressive Episode With Psychotic Features, and one patient with Psychotic Disorder Not Otherwise Specified (Atypical Psychosis) (6 males, 5 females; mean age 41 ± 10). There were 10 healthy controls (6 males, 4 females; mean age 39 ± 13). Groups did not differ significantly in terms of age ($F=0.261$, $df=2, 28$, $p=0.772$) or sex ($p=0.764$).

2.2. Specimen collection and cell culture

Human dermal fibroblasts were cultured by explant growth from skin obtained by biopsy from the upper

inside arm, under local anaesthesia using a 5 mm disposable punch. Initial cell line culture procedures were based on those of Edelstein and Breakefield (1980). Fibroblasts were then cryogenically stored in liquid nitrogen.

2.3. Experimental procedures

Full details of laboratory procedures are available on request. The experimenter (VSC) was blind to group membership of cell lines. Cell lines were cultured under proliferating conditions in complete DMEM (containing 10% fetal bovine serum and 50 ng/ml gentamycin) over a period of about two to three weeks. An aliquot of about 6.5 million cells was plated into each of 6 culture flasks at a density of 10,000 cells/cm^2. After 48 h incubation, cycloheximide (200 μg/μl methanol; Calbiochem) at a concentration of 200 μg/ml of culture medium was added to three flasks. Nothing was added to the three flasks containing the untreated cell lines. All experimental procedures were carried out in duplicate.

2.4. Flow cytometry analysis

Flow cytometry measures the distribution of cells in the different cell cycle phases by sorting and counting cells according to DNA content. Cells were fixed in 250 μl ice-cold PBS followed by a 250 μl ice-cold 60% ethanol and stored at 4 °C. Prior to analysis, the cells were resuspended in 500 μl warm PBS, 1 μg RNase and 25 μg propidium iodide for 30 min in the dark. Flow cytometric analysis was performed on a BD Calibur flow cytometer. Percentage of cells was determined for each of the following cell cycle phases: G0/G1 (46 chromosomes); S (greater than 46 chromosomes, less than 92 chromosomes); G2/M (92 chromosomes); and polyploid (greater than 92 chromosomes). Cells with a DNA content of less than 46 chromosomes were designated as being sub-G0 (including apoptotic bodies).

2.5. Western blot analysis of Bax, Bcl-2, phosphorylated $P53^{P392Ser}$ and actin protein

Western blot analyses of whole cell lysates were performed using standard procedures. Protein was extracted in an ice-cold lysis buffer (50 mM Tris.HCl, pH 7.5; 150 mM NaCl; 1% Nonidet P40; 0.5% sodium deoxycholate; 0.1% SDS; 1% protease inhibitor cocktail [Sigma]). To enable comparison across gels, an aliquot of cell lysate from the breast cancer cell line MCF-7 was included in each blot. The primary antibodies used were rabbit anti-Bax 1:20 (Oncogene); rabbit anti-Bcl-2 1:50 (Oncogene); rabbit anti-actin 1:200 (Sigma); mouse anti-phosphorylated $P53^{P392Ser}$ 1:200 (Alexis) and horseradish peroxidase conjugated secondary antibodies used were anti-mouse (BioRad); anti-rabbit (BioRad), both 1:50,000. Band volumes were determined using Quantity One 4.4.0. Band volumes for Bax, Bcl-2, phosphorylated $P53^{P392Ser}$ and actin protein were divided by the corresponding band volume of the MCF-7 cell lysate; and, loading errors of Bax, Bcl-2 and phosphorylated $P53^{P392Ser}$ were controlled for by dividing with the adjusted actin band volume.

2.6. Caspase-3 activity assay

Levels of caspase-3 activity were derived from a fluorometric assay involving the cleavage of the synthetic substrate, DEVD-AFC, by caspase-3 according to the manufacturers instructions (Clontech).

2.7. Data analysis

Data analysis of laboratory measures was performed using the mean of the two independent determinations of each measure. Significant outliers ($p < 0.01$) were assessed within each diagnostic group using Grubb's method. Three outliers were detected and replaced with the highest value plus one unit for that variable within that diagnostic group, in accordance with standard procedure (Tabanick and Fidell, 2001). Across the matrix there were less than 3% missing data, missing at random due to technical errors. Estimation maximisation was used to replace these missing values using all categorical and continuous data to perform this procedure.

We conservatively conceptualised the experiment as a two-way factorial design, with one between-groups (diagnosis) and one within-groups (treatment) independent variable. Diagnosis had three levels: schizophrenia group, NSP group, and healthy com-

parison group. Treatment had two levels: cycloheximide exposure and no treatment (basal condition). A multivariate analysis of variance (MANOVA) was performed to assess overall effects of group and treatment. While one of the assumptions of MANOVA (that there be more subjects in the smallest cell than DVs) is violated (the smallest cell contains the same number of subjects as DVs), overall this technique was considered a conservative preparatory analysis for the univariate post-hoc testing. Significant effects detected by the MANOVA were explored using univariate ANOVA and Tukey's post-hoc test. Correlations between markers were assessed using Pearson's coefficients.

3. Results

3.1. Overall effects on laboratory measures

The MANOVA including laboratory measures showed a significant overall effect of treatment

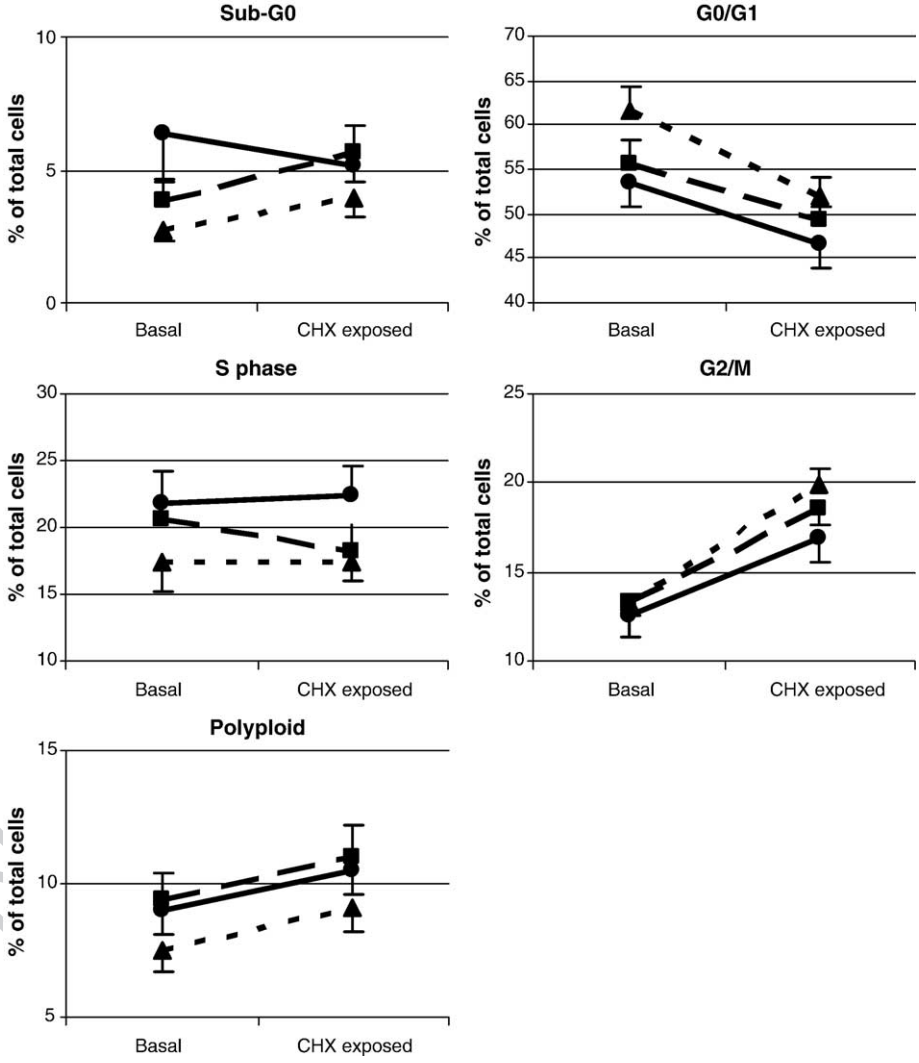

Fig. 1. Cell cycle distribution. The basal and cycloheximide (CHX) induced average proportions ± S.E.M. of cell cycle distribution were determined for fibroblast cells from patients with schizophrenia (continuous line/circles), patients with non-schizophrenic psychosis (dotted line/triangles) and non-affected controls (dashed line/squares).

($F=10.704$, $df=1$, 60, $p=0.000$) and diagnostic group ($F=1.715$, $df=2$, 59, $p=0.044$).

3.2. Treatment effects

The results of flow cytometry analysis for each diagnostic group are presented in Fig. 1. Points on the left of each graph are values for cell lines under basal conditions. Points on the right of each graph are values for cell lines exposed to cycloheximide. The MANOVA detected a significant effect of cycloheximide treatment on: 1) the proportion of cells in G0/G1 ($F=14.843$, $df=1$, 29, $p=0.000$), 2) the proportion of cells in G2/M ($F=49.589$, $df=1$, 29, $p=0.000$), and 3) the proporti on of polyploid cells ($F=4.115$, $df=1$, 29, $p=0.047$). Cycloheximide exposure was associated with an across-group reduction in the proportion of cells in G0/G1 from 57% to 49%; and, increases in the proportion of cells in G2/M from 13% to 18%, and polyploid phase, from 9% to 10%. There was no significant effect of cycloheximide exposure on sub-G0 ($F=0.543$, $df=1$, 29, $p=0.464$) or S phase ($F=0.088$, $df=1$, 29, $p=0.768$).

Treatment effects on caspase activity levels for each diagnostic group can be seen in Fig. 2. Cycloheximide exposure had a significant effect on caspase activity ($F=27.823$, $df=1$, 29, $p=0.000$). The average caspase activity across-groups increased from 4 to 11 μM AFC/h/μg protein with cycloheximide exposure. There was no statistically significant change in levels of phosphorylated $P53^{P392Ser}$ with treatment ($F=2.714$, $df=1$, 29, $p=0.105$), although it increased numerically from 0.7 to 0.9 optical density units. Also, there was no statistically significant effect of cycloheximide exposure on Bcl-2 or Bax protein levels, or on Bcl-2:Bax ratios (see Fig. 3).

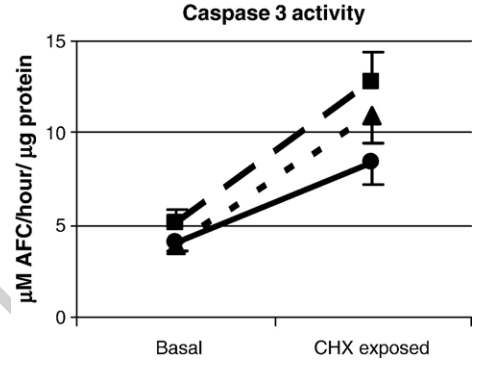

Fig. 2. Caspase-3 activity. The basal and cycloheximide (CHX) induced mean ± S.E.M. caspase-3 levels were determined for fibroblast cells from patients with schizophrenia (continuous line/circles), patients with non-schizophrenic psychosis (dotted line/triangles) and non-affected controls (dashed line/squares).

3.3. Diagnostic group effects

The MANOVA detected a significant effect of diagnostic group on the proportion of cells in sub-G0 ($F=3.487$, $df=2$, 59, $p=0.037$). Tukey's post-hoc test revealed that this effect was due to a significant increase ($p=0.029$) in the proportion of cells in sub-G0 in the schizophrenia group compared to the NSP group (basal and cycloheximide exposure conditions combined), whilst there was no difference between the healthy comparison group and the NSP group on this measure ($p=0.515$). As can be seen in Fig. 1, this result was mainly attributable to a significant increase in the basal level of sub-G0 cells ($t=2.117$, $p=0.024$, one-tailed) in the schizophrenia group (6.4%) compared with the NSP group (2.8%), though this effect was apparent at the level of a trend in the cycloheximide exposed cells (5.1% versus 3.9%; $t=1.443$, $p=0.083$, one-tailed). There was a trend for the basal level of sub-G0 cells to be increased in the schizophrenia group (6.4%) compared with the healthy comparison group (3.8%), though again this trend did not achieve conventional levels of statistical significance ($t=1.395$, $p=0.090$, one-tailed). In the cycloheximide exposed cells, there was no difference in the proportion of sub-G0 cells between the schizophrenia and healthy comparison groups (5.1% versus 5.6%; $t=-0.443$, $p=0.33$, one-tailed).

Although the MANOVA did not detect a statistically significant effect of diagnostic group on caspase activity, there was evidence of an effect at trend level ($F=2.994$, $df=2$, 59, $p=0.06$). Visual inspection of Fig. 2 suggested that there was no difference in caspase-3 activity levels between the three diagnostic groups under basal conditions, but there was a difference in the cycloheximide exposure condition. An exploratory post-hoc t-test revealed, contrary to hypothesis, that the schizophrenia group had significantly less caspase-3 activity compared to the healthy comparison group in the cycloheximide exposed samples ($t=-2.246$, $p=0.038$, two-tailed).

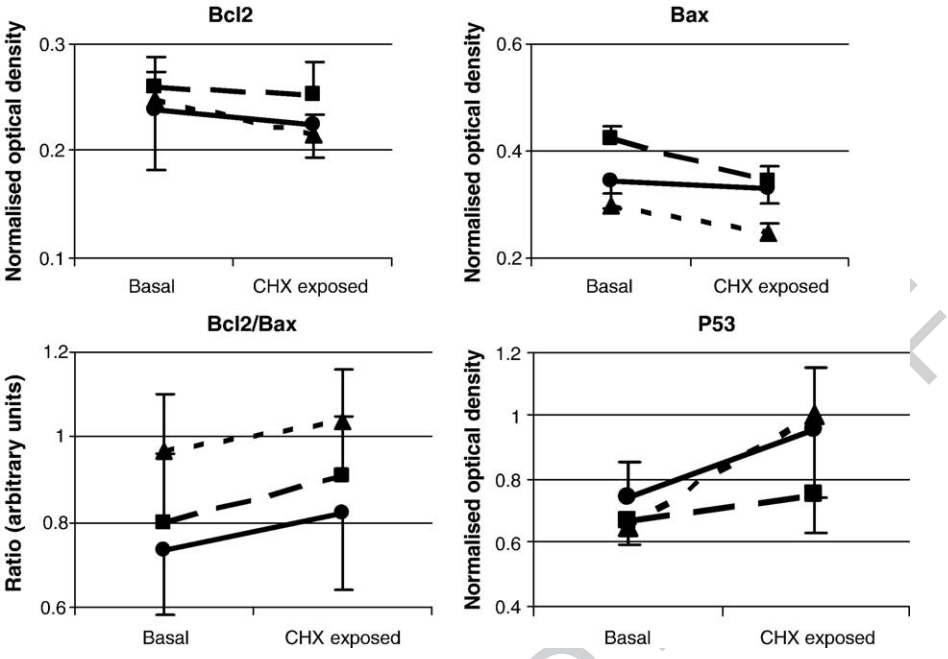

Fig. 3. Phosphorylated $P53^{PSer392}$, Bcl-2, Bax levels and Bcl-2:Bax ratio. The basal and cycloheximide (CHX) induced mean ± S.E.M. phosphorylated $P53^{PSer392}$, Bcl-2, Bax levels and Bcl-2:Bax ratio were determined for fibroblast cells from patients with schizophrenia (continuous line/circles), patients with non-schizophrenic psychosis (dotted line/triangles) and non-affected controls (dashed line/squares).

There was no statistically significant effect of diagnostic group for the level of phosphorylated $P53^{PSer392}$ protein ($F=0.448$, $df=2, 59$, $p=0.64$), level of Bcl-2 protein ($F=0.394$, $df=2, 59$, $p=0.68$), level of Bax protein ($F=2.089$, $df=2, 59$, $p=0.13$), or for the ratio of Bcl-2:Bax protein ($F=1.177$, $df=2, 59$, $p=0.32$). Inspection of the Fig. 3 graph headed Bcl-2:Bax reveals that the Bcl-2:Bax ratio was numerically lower in cells under both basal and cycloheximide exposure conditions in the schizophrenia group compared with the other two groups, but post-hoc testing did not detect significant group differences.

3.4. Intercorrelations between apoptotic markers

Assay intercorrelations were determined to assess whether the relationships between markers conformed to what is known about apoptotic pathways, and to explore group differences in these relationships. When bivariate correlation coefficients were calculated for apoptotic markers across diagnostic groups separately for cells under basal and cycloheximide exposure conditions, there were no significant correlations between: phosphorylated $P53^{PSer392}$ and Bax levels; Bax levels and caspase-3 activity; and caspase-3 activity and the proportion of cells in sub-G0.

Within-group intercorrelations between apoptotic markers are presented in Table 1. There are distinct diagnostic group differences in the overall pattern of relationships. Under basal conditions there are significant negative correlations between Bax levels and the Bcl-2:Bax ratio (and the absence of such a relationship between Bcl-2 levels and the Bcl-2:Bax ratio) in the healthy comparison group and the NSP group; whereas in the schizophrenia group, there is a significant positive correlation between Bcl-2 levels and the Bcl-2:Bax ratio (and the absence of such a relationship between Bax levels and the Bcl-2:Bax ratio). In the cycloheximide exposure condition, Bax levels are significantly negatively correlated with the Bcl-2:Bax ratios in each of the three subject groups. However, the expected positive correlations between Bax levels and caspase-3, and caspase-3 and sub-G0, are only evident in the healthy comparison group and the NSP group. In the schizophrenia group correla-

Table 1
Inter-assay correlations for diagnostic groups

	Basal					Cycloheximide exposed cells				
	Bcl-2	Bax	Bcl-2:Bax	Caspase-3	Sub-G0	Bcl-2	Bax	Bcl-2:Bax	Caspase-3	Sub-G0
Healthy controls										
P53	−0.15	−0.32	0.09	0.24	0.43	0.20	0.20	0.24	0.08	−0.27
Bcl-2		0.59	0.03	0.34	0.38		0.31	0.17	−0.16	−0.38
Bax			−0.63*	0.06	0.58			−0.76*	0.33	−0.07
Bcl-2:Bax				0.52	−0.40				−0.40	−0.25
Caspase-3					0.31					0.37
Non-schizophrenic psychosis										
P53	0.41	−0.13	0.59	−0.36*	−0.15	0.32	−0.22	0.32	−0.46	−0.34
Bcl-2		0.35	0.41	−0.79*	−0.03		0.33	−0.07	−0.32	0.02
Bax			−0.64*	−0.36	0.72*			−0.83**	0.49	0.68*
Bcl-2:Bax				−0.25	−0.59				−0.44	−0.40
Caspase-3					0.03					0.71*
Schizophrenia										
P53	0.74*	0.53	0.49	0.10	0.18	0.26	0.22	0.01	0.24	0.36
Bcl-2		0.21	0.87**	−0.01	−0.07		−0.31	0.86*	0.43	−0.19
Bax			−0.26	−0.45	−0.01			−0.71*	−0.25	0.69*
Bcl-2:Bax				0.30	0.01				0.27	−0.41
Caspase-3					0.47					−0.62

* $p<0.05$
** $p<0.01$.

tions between Bax and caspase-3, and caspase-3 and sub-G0, are both negative.

4. Discussion

The central aim of the present study was to test in a dermal fibroblast cell model the hypothesis that there is an increased susceptibility to apoptosis in schizophrenia. Supporting this hypothesis, we found an increased proportion of cells in the sub-G0 fraction of the cell cycle under basal culture conditions in the schizophrenia group compared to the NSP group. This measure was not significantly different in the NSP group compared to the healthy comparison group. As it is well established that apoptotic cell bodies accumulated in sub-G0, we interpreted this finding as being consistent with patients with schizophrenia showing increased basal susceptibility to apoptosis. This interpretation is supported by the finding that the Bcl-2:Bax ratio was numerically lower in the schizophrenia group compared to the other groups, consistent with an increased proneness to apoptosis in the schizophrenia patient fibroblasts (Adams and Cory, 1998).

There were no differences in caspase-3 activity across the three diagnostic groups under basal conditions. This finding is comparable to Jarskog et al. (2004), who did not find a significant difference in the level of caspase-3 in a postmortem tissue study of temporal cortex in patients with schizophrenia compared with healthy controls. In the cycloheximide exposed cell lines there was a strong trend (statistically significant on post-hoc testing) for patients with schizophrenia to have decreased caspase-3 activity compared with healthy controls. Hence, our findings concerning caspase activity are not consistent with increased susceptibility to apoptosis in patients with schizophrenia, unless this susceptibility is caspase-3 independent. For instance, this form of susceptibility to apoptosis could be mediated by another caspase. Alternatively, caspase-independent susceptibility could be mediated by the apoptosis-inducing factor (AIF), which is essential for programmed cell death during cavitation of embryoid bodies (Joza et al., 2001).

Further convergent evidence of schizophrenia-specific altered apoptotic mechanism was seen in the pattern of within-group correlations between apoptotic pathway markers. Under basal conditions

the Bcl-2:Bax ratio was primarily determined by Bcl-2 levels in the schizophrenia group, in contrast to the two control groups where Bax was the primary determinant of the Bcl-2:Bax ratio. In the cycloheximide exposure condition the expected positive correlations between Bax levels and caspase-3, and caspase-3 and sub-G0, found in the two control groups, were both negative in the schizophrenia group. This pattern of findings may account for the unexpected reduction in caspase-3 activity, and the attenuated increase in the sub-G0 cell fraction, in the schizophrenia group cultures under cycloheximide exposure conditions.

There were limitations with the cell model used. Dermal fibroblasts were chosen because they can be induced to proliferate in vitro, and antipsychotic medication effects can be minimised or eliminated by maintaining the culture for many cell passages. However, they do not appear to be readily susceptible to apoptotic cell death. In pilot testing, hydrogen peroxide in doses of 50, 100, 200 μM; UV radiation (dose 19.6 J/m^2); ethanol at concentrations of 1%, 2%, 3%, 4%, and 5%; and 100 μg cycloheximide/ml of media, were ineffective in inducing cell death in the adult human dermal fibroblasts, although it has been reported that high concentrations (1000 μM) of H_2O_2 do induce apoptosis in these cells (Uberti et al., 2002). We chose to use a relatively high dose of cycloheximide (200 μg/ml) which successfully induced apoptosis but which also impacts many biologically significant processes in addition to those directly involved in apoptotic or cell cycle mechanisms. Another issue with dermal fibroblasts is that they appear to be sensitive to small differences in experimental procedures since we found that correlations between duplicate estimates of laboratory measures were relatively low.

The authors acknowledge that there are reasons to view the results as preliminary and in need of confirmation. Nonetheless, our results are in accord with those of Jarskog et al. (2000, 2004). We found, as this other research group found, significant evidence of anomalous apoptotic mechanisms that is relatively specific for schizophrenia. Investigations of apoptosis tend to include a limited number of molecules and use a single type of assay, for example gene expression or protein measures. This approach restricts interpretation, especially in relation to determining whether a change is primary or secondary. Benes (2006) recently reported down-regulation of pro-apoptotic markers in a postmortem gene expression study of hippocampal tissue in schizophrenia. However, as noted by Weinberger and McClure (2002), it is impossible to determine from gene expression studies alone whether reduced mRNA levels of pro-apoptotic markers represents secondary molecular compensation to cell loss from apoptosis or primary reduction in apoptotic signaling. However, comprehensive assessment of apoptotic mechanisms will generate a large number of dependent variables, especially if proneurotrophins and neurotrophins are included (Lu et al., 2005; Weickert et al., 2005). This suggests that functional studies informed by genotyping data in large subject samples (see Harris et al., 2005 for exemplar) may accelerate the understanding of the significance of altered apoptosis in schizophrenia.

In conclusion, the results of the current study provide further evidence of disease specific aberrant regulation of apoptosis in schizophrenia, which may be observed in somatic cell lines, not just in brain tissue. Although our study did not conclusively support or refute the hypothesis, the proposal of increased susceptibility to apoptosis in schizophrenia continues to have heuristic value. As well as accounting for the putative cancer resistance in schizophrenia, it could also explain the negative disease association between schizophrenia and rheumatoid arthritis (Oken and Schulzer, 1999). The hypothesis has generated interesting genetic candidates (e.g. adenomatous polyposis coli [APC] and P53, Cui et al., 2005; Ni et al., 2005). Although apoptotic mechanisms operate mainly to cause cell death, there is evidence that they may occur in neurons as a sub-lethal cellular process, called synaptic apoptosis (Mattson and Duan, 1999; Mattson et al., 1998) or, simply as a modulator of neuroplasticity (see Lu et al., 2005, in reference to proneurotrophins). In schizophrenia, abnormalities in synaptic apoptosis are ideal candidates to account in part for putative anomalies in dendritic pruning (Glantz et al., 2006) and the reported loss of prefrontal neuropil (Selemon et al., 1995). We believe further that large scale studies of apoptotic mechanisms in schizophrenia are justified.

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