Tumour cells surviving in vivo cisplatin chemotherapy display elevated c-myc expression

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Summary: The c-myc oncogene has been extensively implicated in cell proliferation, cell differentiation and programmed cell death (Spencer and Groudine, 1991). Expression of c-myc is induced in proliferating cells following mitogenic stimulation (Kato et al., 1992) but is down regulated in quiescent cells following factor withdrawal (Lotem and Sachs, 1993; Hermeteking and Eick, 1994).

Activation of c-myc has been associated with tumours of the breast (Watson et al., 1993), colon (Smith et al., 1993), ovary (Tashiro et al., 1992) and squamous cell carcinomas (Ogumbi et al., 1993). Significantly, c-myc gene activation has been correlated with poor clinical prognosis for aggressive tumours including bladder cancer (Kotake et al., 1990) and human non-small-cell lung carcinoma (Oendl et al., 1993).

Several in vitro studies of tumour cell lines suggest that elevated c-myc expression can confer resistance to cisplatin (Sklar and Prochownik, 1991; Niimi et al., 1991; Mitzuani et al., 1994). Cisplatin (cis-diaminedichloroplatinum; CDDP) is an antineoplastic agent with demonstrated clinical effectiveness against hormone-resistant prostate cancer (Yagoda and Petrylak, 1993), ovarian carcinoma (Markman, 1993) and as a radiosensitizer for advanced solid head and neck tumours (Chougule et al., 1994; Nakata et al., 1994).

Resistance to cisplatin and other chemotherapeutic agents represents a major obstacle to effective cancer therapy as clinically significant levels of resistance emerge rapidly following treatment (Andrews and Howell, 1990; Kashani-Sabet et al., 1990). Chemoresistance to cisplatin is typically generated by exposure of tumour cells in vitro to gradually increasing concentrations of the drug (Brennan et al., 1991; Christen et al., 1993) or fractionated X-irradiation (Eicholtz-Wirth et al., 1993; Taverna et al., 1994). Reported mechanisms for acquired cisplatin resistance include increased mRNA and enzyme activity of dTMP synthase (Scanlon and Kashani-Sabet, 1988), induction of DNA repair enzymes (Scanlon and Kashani-Sabet, 1989; Kelland et al., 1992), decreased drug accumulation (Gately and Howell, 1993), increased levels of glutathione (Godwin et al., 1992), hsp60 chaperonins (Nakata et al., 1994) and metallothioneins (Kasahara et al., 1991). Cisplatin chemoresistance is not part of the multidrug resistance phenotype mediated by the mdr1 gene (Toffoli et al., 1991), although a number of in vitro studies have implicated roles for genes such as c-fos (Funato et al., 1992), c-jun (Rubin et al., 1992), c-Ha ras (Isono et al., 1991) and c-myc (Mizuati et al., 1994). However, to date there is a dearth of in vivo investigations attempting to examine the relationship between cisplatin therapy and the expression of specific cellular oncogenes (Sklar and Prochownik, 1991; Osmak et al., 1993; Taverna et al., 1994).

Antisense oligodeoxyribonucleotides are being applied to modulate the expression of specific genes (Stein and Cheng, 1993) with phosphorothioate analogues preferred over the conventional phosphodiester analogues because of their superior hybridisation affinities and resistance to degradation by nucleases (Iversen, 1991). In vitro studies using antisense c-myc analogues have confirmed the role of c-myc in cellular proliferation (Pariia et al., 1992), signal transduction pathways (Biro et al., 1993) and differentiation (Yang and Yang, 1995).

We have examined quantitative expression of the rat c-myc gene following cisplatin therapy using a solid tumour model and we have also extended our studies to investigate the role of c-myc in cellular proliferation of the tumour cells and have discovered that a significant reduction in cell growth rate is achievable in vitro. We hypothesise that the antisense therapy directed at c-myc in combination with cisplatin may achieve therapeutic efficacies in vivo that greatly exceed those displayed by either agent in isolation.

Materials and methods

Probe cDNAs

A 1.8 kbp EcoRI-bound murine c-myc cDNA cloned into the EcoRI site of pBluescript plasmid was provided by Dr Suzanne Cory, WEHI, Melbourne, Australia. Murine β-actin...
cDNA (1.1 kbp PstI-bound fragment in pUC19) was donated by Dr Ismail Kola (CEHD, Monash University, Melbourne, Australia).

Animals
Syngeneic DA rats (10–16 weeks old) were housed 3–4 per cage, sex segregated in temperature-controlled rooms with a 12 h light/dark cycle. Food (crude pellet) and water were provided ad libitum through wire-roofed plastic cages. Animals were randomised by weight and sex into control and treatment groups (ten animals per group).

Tumour implantation, treatment and removal
A solid transplantable rat salivary adenocarcinoma obtained from the Lions Cancer Institute, Royal Perth Hospital, was used as the experimental tumour model. Tumour studies were conducted on the lateral aspect of the hind limbs of DA rats. A small incision was made through the skin and a 1 mm³ piece of healthy tumour was implanted subcutaneously. Tumour growth was assessed daily using calibrated vernier calipers and expressed as the product of the minimal and maximal length of tumour axes. This method has been used extensively by this and other groups (Napoli et al., 1992; Burton et al., 1990). The chemotherapeutic drug, cisplatin, was administered on day 10 of tumour growth to the treatment group. This point coincides with commencement of the proliferative period of tumour growth. An intravenous administration of cisplatin, equivalent to a low dose used in the clinical setting (1 mg kg⁻¹), was delivered via the inferior vena cava and was performed during a laparotomy under general anaesthesia. In both control and treatment groups tumours were dissected free from the hind limbs after 6, 12 and 17 days growth and processed for RNA analysis. These sample points were selected from data on in vivo tumour growth kinetics (Figure 1) as a pretreatment time point (day 6) and two post-treatment time points, one during the phase of tumour growth retardation (day 12) and the other well after resumption of tumour growth (day 17).

Cell Cultures
Primary cell cultures were established from the in vivo passed tumour by seeding cells into fresh RPMI-1640 medium containing 5% fetal calf serum (FCS) (Trace Biosciences) incubated at 37°C in 5% carbon dioxide. Media were supplemented with antimicrobial/antimycotic PSN antibiotics (Gibco-BRL). Cells were harvested with trypsin-versene, washed three times with Hanks’ balanced salt solution (Trace) before pelleting by centrifugation (3000 r.p.m.; 10 min) and processing for RNA extraction.

Antisense trials
The established in vitro cell culture was treated on day 3 of cell growth with 5 µM dosages (selected from preliminary studies, data not shown) of a 15-residue phosphorothioate antisense oligodeoxyribonucleotide (5'-CACCAGTTAGGGCCCAT-3'). The antisense transcript was directed at the translation initiation codon and next four codons (MPLNV) of exon 2 in the rat c-myc gene (Hayashi et al., 1987) and delivered upon the fifth passage of cell culture. In addition, a scrambled sequence (mismatch) oligomer (5'-AGCGTAGGCTAGGCT-3') was employed to confirm gene specific inhibition of tumour cell growth. Cell numbers and cell viability were monitored daily in triplicate.

Total RNA isolation
Total RNA was extracted from fresh tumour tissue samples and harvested from cell cultures using TRIzol reagent (Gibco-BRL). For tissues, a sample of healthy tumour tissue (0.2 g) from freshly dissected tumours was homogenised to a fine slurry in TRIzol reagent using a Ystral X10/25 homogeniser (HD Scientific). For cell cultures approximately 2.3–4.6 x 10⁶ cell samples were pelleted (2000 r.p.m., 5 min), and lysed in TRIzol reagent for total RNA preparation. Both homogenised and lysed samples were then incubated at room temperature for 5 min. Chloroform was added, the solution gently shaken and incubated at room temperature for a further 2–3 min. After centrifugation (12 000 g, 10 min at 4°C), the RNA was precipitated from the upper aqueous phase with the addition of isopropanol (0.5 ml ml⁻¹ TRIzol used), and incubated at room temperature for 10 min. The RNA was pelleted (12 000 g, 10 min at 4°C) and washed in 1 ml of DEPC (diethyl pyrocarbonate, BPH Chemicals) treated 70% ethanol, repelleted (12 000 g, 5 min at 4°C) and dissolved in TE buffer before gel fractionation.

RNA fractionation
Briefly, a 1.4% agarose (Promega) gel was cast using DEPC-treated sterile water, 1 x MOPS [3-(N-Morpholinol) propane-sulphonic acid] buffer (20 mM MOPS, 5 mM sodium acetate, pH 7.0, 1.0 mM EDTA) and 6.3% formaldehyde (Sigma) in a fume hood. RNA samples were prepared by combining 20 µg total RNA in 2% denatured formaldehyde, 1 x MOPS buffer, 16% formaldehyde buffer and denatured for 5 min at 65°C before chilling on ice. Before loading on the gel, loading buffer (50% v/v glycerol, 0.1 mg ml⁻¹ bromophenol blue) and 0.5 µg ethidium bromide was added to each sample. The gel was run at 0.5 V cm⁻¹ for 4 h in 1 x MOPS running buffer. Fractionated RNA was transferred to Hybond-N membrane (Amersham) using established techniques (Sambrook et al., 1989). Fixation of Northern blots was carried out by exposing the membrane to ultraviolet light for 2–3 min.

Hybridisation
cDNA inserts were cleaved free of vector plasmids and purified with Bresclean (Bresatec Ltd) before radiolabelling with [32P]-dATP radioisotope by random priming with Klenow DNA Polymerase using a GIGAprime kit (Bresatec). Northern blots were hybridised with radiolabelled probes in glass bottles containing hybridisation buffer (10 mM Heps, pH 7.0, 0.4 mM sodium chloride, 0.04 mM trisodium citrate, 0.2% bovine serum albumin, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 2 µg ml⁻¹ herring sperm, 0.1% sodium dodecyl sulphate) which were rotated in a DNA HI-2001 hybridisation incubator (Bartlett Instruments) for 16 h at 65°C. All blots were washed under high-stringency conditions (0.015 M sodium chloride, 0.003 M trisodium citrate at 65°C) and dried before exposure to Kodak XRP film. Autoradiographs were scanned using a Hewlett Packard 8000 flatbed
c-myc expression

Statistical analysis

The mean and standard deviation of daily tumour sizes was calculated for each animal group and plotted against time. Raw data from each group was transformed (\(\sqrt{x}\)) and compared using linear regression analysis (Snedecor and Cochran, 1967). Appropriate differences between means were statistically examined using a t-test (Howell, 1982).

Results

In vivo tumour growth kinetics

The effect of cisplatin was examined by comparing the tumour growth kinetics for untreated and treated animals (Figure 1). The experiment was conducted five times, accounting for the ten tumours measured. Regression line analysis revealed that the untreated tumours had a doubling rate of 3.65 days, while the treated tumours had a doubling rate of 4 days. Cisplatin was administered on day 10, a period that corresponded with the early onset of the proliferative period of growth. Retardation of tumour growth was evident in the treated animals for a period of up to 48 h. Tumour growth rates identical to those of untreated animals following this 48 h period.

In vitro tumour growth kinetics

The tumour cell growth kinetics for control, myc antisense oligomer-treated and mismatch oligomer-treated samples are shown in Figure 2. This experiment was repeated in triplicate. Statistical analysis revealed no significant difference \((P>0.05)\) between control and mismatch treatment regression lines. However, the c-myc antisense treatment group clearly demonstrated significant difference in growth retardation of cell populations \((P<0.05)\) between control and mismatch groups. Indeed, 25% growth retardation of tumour cells was observed for a period of up to 4 days following the single 5 \(\mu\)M dose of naked c-myc phosphorothioate antisense oligomers. Maximal inhibition (42%) was observed 4 days after administration.

**Figure 2** Cell culture growth curves for c-myc antisense oligonucleotide-treated, mismatch oligomer-treated and control tumour groups. Fifth passage cell cultures were treated with 5 \(\mu\)M c-myc antisense on day 3 of cell growth. Each data point represents the mean of three counts on triplicate samples. (\(\pm\)1 s.d.).

**Figure 3** Relative expression of the c-myc gene as determined by Northern blot hybridisation analysis of RNA isolated from the in vivo serial transplantable tumour. Cisplatin was administered on day 10 of tumour growth. Mean blot intensities (following standardisation with values for \(\beta\)-actin expression) are shown for each group (\(\pm\)1 s.d.); E, Pretreatment, day 6 \((n=6)\); MC, control group, day 12 \((n=8)\); MT, treatment group, day 12 \((n=4)\); LC, control group, day 17 \((n=7)\); LT, treatment group, day 17 \((n=10)\). RNA degradation accounts for variations in sample numbers.

 Autoradiograph blot intensities from each experimental group for c-myc and \(\beta\)-actin were determined using the 'Blotscan' program. Following digitisation, the values were standardised with respect to values for \(\beta\)-actin, and mean blot intensity from each group in the in vivo study is shown in Figure 3. The results demonstrate that 2 days (48 h) after cisplatin treatment, a significant 2-fold rise \((100\%)\) in c-myc expression was observed between control and treatment groups \((P=0.01)\). This elevation in expression was also evident at the 7 day sample point \((P=0.01)\). Expression of the c-myc gene in tumour samples before treatment showed no significant difference with the 2 day \((P=0.81)\) or 7 day \((P=0.06)\) control sample points.

Relative expression of the c-myc gene from in vitro cell cultures for control, myc antisense oligomer-treated and mismatch oligomer-treated groups are shown in Figure 4. There were no significant differences in the level of c-myc expression between untreated and mismatch treatment groups \((P>0.05)\). c-myc transcripts were undetectable (at the sensitivities afforded by this system), following c-myc phosphorothioate antisense treatment suggesting virtually complete inhibition of c-myc expression.

Discussion

Our in vivo studies demonstrate that a single low-dose cisplatin treatment results in both tumour growth retardation and a 2-fold elevation in the level of c-myc expression. Comparison of the 7 day sample points for untreated and treated tumours suggests that the rise in expression may be a constitutive feature of the surviving cells and not a transient rise as the cells begin to recover from cytotoxic insult. This reproducible 2-fold elevation in the expression of c-myc is mirrored by reports of analysis conducted on both in vitro cultured tumour cell lines (Marazzi et al., 1991) as well as freshly isolated colon carcinoma tissues from patients with failed cisplatin therapy (Kashani-Sabet et al., 1990).

The effect of specific induction of myc gene expression may be explained by the presence of non-saturating dosages of cisplatin reaching cells and random mutagenic action of the drug upon the c-myc regulatory region. Cells whose myc expression becomes elevated in this fashion may attain a selective advantage in cell proliferation and survive...
chemotherapy treatment. The effect of this elevated expression upon tumour response to subsequent cisplatin insult warrants further investigation.

Our in vitro studies have demonstrated that upon suppression of c-myc expression using phosphorothioate oligodeoxynucleotides, tumour cell growth is suppressed significantly for a period of up to 4 days. This time period of growth inhibition coincides with the average half-life (4–5 days) of phosphorothioate oligodeoxynucleotides in serum culture (Shaw et al., 1991). The most likely scenario is that the cells are held in stasis in the G0 or G1 phases of the cell cycle by suppression of c-myc expression as demonstrated in other systems (Heikkila et al., 1987). In addition, Myc protein is now known to modulate expression of the cyclin E gene whose product mediates the G0 to S-phase transition of the cell cycle (Shichiri et al., 1993; Hanson et al., 1994). The c-myc gene product appears to act as a transactivator, controlling genes that mediate the transition from G0 to S-phase (Hanson et al., 1994). Treatment with c-myc antisense oligomer may result in cells being held in the G1 phase of the cell cycle, which has been shown to be cisplatin sensitive (Fralav and Roberts, 1978). Indeed, recent evidence from cell culture studies favours this hypothesis. Mizutani et al. (1994) reported a synergistic cytotoxic effect for c-myc antisense in combination with cisplatin therapy for the T24 bladder tumour line and two freshly derived urinary bladder cells in culture. Interestingly, chemoresistance in these cells was completely reversed by c-myc antisense treatment.

The molecular basis for chemoresistance to platinum-based drugs is poorly understood. The rapid onset of cisplatin chemoresistance is well documented in vitro and parallels clinical observations (Andrews et al., 1990; Howell et al., 1992). Although the basis for this resistance has been variously ascribed to several candidate genes (Funato et al., 1992; Rubin et al., 1992; Isonishi et al., 1991; Nakata et al., 1994), no one gene has been consistently implicated, with the exception of the c-myc gene (Kashani-Sabet et al., 1990; Marazzi et al., 1991; Sklar and Prochownik, 1991; Nimi et al., 1991; Mizutani et al., 1994). The presence of a cisplatin-responsive element within the human c-myc gene promoter was demonstrated by Spanidios et al. (1991), who defined it within the region 290 and 350 bp upstream of the c-myc PI cap site. Using transfected plasmids carrying the S' c-myc linked to the chloramphenicol acetyltransferase (CAT) reporter gene, the authors were able to demonstrate that cisplatin stimulated a 9- to 11-fold elevation in activity of the CAT gene. Although our data demonstrate that relatively low doses of cisplatin can evoke a significant rise in c-myc expression, it is premature to suggest there is a direct link between cisplatin use and c-myc-modulated chemoresistance. Clearly, the mechanism for cisplatin chemoresistance remains to be investigated.

Targeted genetic disruption of c-myc gene expression represents an attractive new cancer treatment modality that in combination with classical anti-cancer therapies offers the potential for greatly enhanced therapeutic efficacies for certain cancers. In addition, chemoresistance may be reversed by gene-targeted antisense therapy directed at the c-myc gene. The use of antisense technology may have clear benefits particularly when used in conjunction with conventional chemotherapies. We are presently attempting to characterise c-myc expression in relation to the development of cisplatin chemoresistance in vivo as a foundation for evaluation of combination therapies in this tumour model.

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