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Association of c-Raf expression with survival and its targeting with antisense oligonucleotides in ovarian cancer

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Summary c-Raf is an essential component of the extracellular related kinase (ERK) signal transduction pathway. Immunohistochemical staining indicated that c-Raf was present in 49/53 ovarian adenocarcinomas investigated and high c-Raf expression correlated significantly with poor survival (P = 0.002). c-Raf protein was detected in 15 ovarian cancer cell lines. Antisense oligodeoxynucleotides (ODNs) (ISIS 5132 and ISIS 13650) reduced c-Raf protein levels and inhibited cell proliferation in vitro. Selectivity was demonstrated by the lack of effect of ISIS 5132 on A-Raf or ERK, while a random ODN produced only minor effects on growth and did not influence c-Raf expression. ISIS 5132 produced enhanced apoptosis and cells accumulated in S and G2/M phases of the cell cycle. In vivo, ISIS 5132 inhibited growth of the s.c. SKOV-3 xenograft while a mismatch ODN had no effect. These data indicate that high levels of c-Raf expression may be important in ovarian cancer and use of antisense ODNs targeted to c-Raf could provide a strategy for the treatment of this disease. © 2001 Cancer Research Campaign http://www.bjcancer.com

Keywords: Raf; antisense; ovarian; cancer; oligodeoxynucleotide

c-Raf is a ubiquitously expressed 74 kDa serine/threonine protein kinase which is central to the ERK pathway (Daum et al, 1994) and has long been implicated in oncogenesis (Rapp et al, 1988; Storm et al, 1990). Despite this, little information on c-Raf protein expression in ovarian cancer is available. c-Raf is activated by phosphorylation after translocation to the plasma membrane by a mechanism that involves Ras (Avruch et al, 1994). Mutations in ras genes have been frequently detected in ovarian tumours and Ras protein is downstream of receptors such as the EGF/erbB receptor tyrosine kinases (Rozakis-Adcock et al, 1993; Teneriello et al, 1993). Compared to normal ovary and benign ovarian tumours, malignant ovarian tumours have increased expression of EGF receptor (erbB-1) (Stewart et al, 1992) and erbB2 (Meden and Kuhn, 1997). The erbB receptors and Ras are currently being investigated as potential targets for growth inhibitors in cancer (Cowsert, 1997; Fan and Mendelsohn, 1998; Witters et al, 1999). Targeting growth inhibitors to c-Raf in cancer cells would eliminate c-Raf-mediated cell proliferation signals from these upstream effectors and prevent cell proliferation arising from over-expression of c-Raf.

Targeting genes using antisense technology provides a highly selective, sequence-specific mechanism for inhibiting the expression of a chosen gene product. A number of antisense oligodeoxynucleotides (ODNs) are currently undergoing clinical trials for the treatment of cancer (Cho-Chung, 1999; Nemunaitis et al, 1999; Yuen et al, 1999). ISIS 5132, a 20-mer phosphorothioate antisense ODN (first generation compound) that targets the 3′ untranslated region of c-Raf mRNA, inhibits the expression of c-Raf mRNA and protein in lung, colon, cervical and prostate cancer cells (Monia et al, 1996a; Lau et al, 1998). Second generation antisense ODNs containing 2′-methoxyethyl sugar modifications, such as ISIS 13650, have also been developed and these may be more potent inhibitors of mRNA expression (Monia, 1997).

To investigate the role and function of c-Raf in ovarian cancer, we have measured c-Raf expression in primary tumours and ovarian cancer cell lines and studied relationships between protein expression and histology, grade of differentiation, tumour stage and patient survival time. To explore the therapeutic potential of first- and second-generation c-Raf antisense ODNs in ovarian cancer, the extent of growth inhibition following antisense ODN treatment was investigated in ovarian cancer cell lines and a xenograft model. Our findings demonstrate that high levels of c-Raf expression are important in ovarian cancer and use of antisense ODNs targeted to c-Raf may provide a novel strategy for the treatment of this disease.

MATERIALS AND METHODS

Tumour samples

Fresh primary ovarian tumour tissue was obtained from 53 previously untreated patients with epithelial ovarian cancer at initial debulking surgery, transferred to liquid nitrogen, then formalin-fixed and embedded in paraffin. Tumour histology was assessed on paraffin embedded sections and classified according to WHO criteria (details in Table 1).

Cell lines

PEO1, PEO1CDDP, PEO4, PEO6, PEO14 and PEO16 were established as described previously (Langdon et al, 1988); SKOV-3 and CaOV3 cells were obtained from the American Type Culture
Collection (Manassas, VA, USA); OVCAR3, OVCAR4 and OVCAR5 were obtained from Dr TC Hamilton (Fox Chase Institute, Philadelphia, PA, USA); 41M, 59M, OAW42 and A2780 cells were obtained from the European Tissue Collection (Porton Down). All cell lines were routinely grown as monolayer cultures in RPMI 1640 (Life Technologies Inc, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal calf serum and 100 μM1 penicillin/streptomycin. Cells were maintained at 37°C in a 5% CO2 humidified incubator.

Immunohistochemistry
Sections (3 μm) were deparaffinised and rehydrated. Endogenous peroxidase activity was blocked by incubating sections in 3% H2O2 for 30 min immersing in citric acid buffer (0.005M, pH 6.0) and microwaving for 3 x 5 min. Slides were washed in 0.05M Tris/HCl buffer (pH 7.6) then incubated in 20% fetal calf serum in the above Tris buffer for 10 min. Anti-c-Raf antibody (R19120, Transduction Laboratories) was used at 1:10–1:20 dilution in 20% fetal calf serum and sections were incubated for 1.5–2 h. A streptavidin–biotin multilink method (StrAviGen Multilink kit; Biogenex, San Ramon, CA) was used to detect reactivity. Sections were stained with a secondary multilink antibody at a 1:20 dilution for 30 min, then incubated with a horseradish-peroxidase-labelled streptavidin complex at a 1:20 dilution for 30 min.

Diaminobenzidine tetrachloride was used as chromagen and applied for 5 min. Sections were lightly counterstained in haematoxylin, dehydrated and mounted. Negative controls for each tumour section were included by replacing the primary antibody with Tris buffer. Immunoreactive scores between 0 and 12 were generated for each sample and represent the product of intensity (0 = negative, 1 = weak, 2 = moderate, 3 = strong) and percentage positive cell staining (0 = 0%, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, 4 = 76–100%).

Statistics
Relationships between variables were analysed using the Fisher’s exact test, the student t-test and the Mann–Whitney test where appropriate. Differences in survival were determined using the Kaplan–Meier method and groups were compared using the log-rank test and χ2 test.

Western blotting
Cells were grown to 70% confluence, washed twice with PBS, and lysed in ice cold hypotonic lysis buffer (50 mM Tris-HCl (pH 7.5), 5 mM EGTA (pH 8.5), 150 mM NaCl, 1% Triton X-100, 2 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM phenylmethylene- sulfonfluoride, 10 μg ml–1 leupeptin, 10 μg ml–1 aprotinin and 10 mM sodium molybdate). Lysates were centrifuged for 6 min at 13 000 rpm in a microfuge. Tumour samples (100 mg) were finely chopped, then homogenized on ice in a Silverson homogenizer in 1.8 ml lysis buffer (excluding Triton X-100). Samples were incubated on ice after the addition of 1% Triton X-100, then centrifuged 14 000 rpm for 30 min. Protein concentrations of supernatants were determined using the Bio-rad Protein Assay Kit (Bio-rad, Richmond, CA). Cell lysates (30 μg) or tumour lysates (50 μg) were resolved on 10% or 12% SDS-PAGE then transferred electrophoretically overnight onto Immobilon-P membranes (Millipore, Bedford, MA). After transfer, membranes were blocked with 1% blocking agent in TBS (20 mM Tris-HCI, 137 mM NaCl, pH 7.5) before probing with the appropriate primary antibody, anti-c-Raf (R19120, Transduction Laboratories, Lexington, KY), anti-A-Raf (R14320, Transduction Laboratories), anti-ERK (E16220, Transduction Laboratories) overnight at 4°C. Immunoreactive bands were detected using enhanced chemiluminescent reagents (1520709, Boehringer Mannheim) and Hyperfilm ECL (Amersham, Buckinghamshire, UK).

Antisense ODNs
Antisense ODNs targeted to the 3’ untranslated region of c-Raf mRNA (sequence: TCCGCCTGTGACATGCATT) were supplied by ISIS Pharmaceuticals (Carlsbad, CA). Two forms of the antisense ODNs were used: a first generation compound (ISIS 5132) which has a phosphorothioate backbone and a second generation compound (ISIS 13650) which also has a phosphorothioate backbone and the addition of 2′ methoxyethyl groups on the sugar moiety. Two control ODNs were available: a second generation random ODN (ISIS 16971; sequence, TCACATGCGGCTTAGCGCT) and a first generation mismatch ODN (ISIS 10353 sequence, TCCGGCAGTTCGATGCATT).

Growth and protein inhibition experiments
For growth inhibition experiments, log phase cells were trypsinized and seeded into 24-well tissue culture plates (1 x 104 in 1 ml) and incubated to reach 40–60% confluence. Cells were then washed with PBS before adding 250 μl of Optimum (Gibco-BRL) containing ‘Lipofectin’ (Gibco-BRL) (6 μl ml–1). Antisense and random ODNs were added (50 nM–200 nM) from 50 μM stock solutions. Cells were incubated at 37°C for 3 h, washed with PBS, replenished with RPMI (plus 10% fetal calf serum and 100 μM1 penicillin/streptomycin) and replaced in the incubator for the remainder of the time course. Cells were trypsinized and counted at the appropriate time point using a ‘ZM’ Coulter Counter.

c-Raf protein inhibition experiments were carried out as above except that cells (2.5 x 105 in 4 ml) were plated into 60 mm diameter petri dishes and washed with PBS (2 ml) prior to addition of Opti-phem/Lipofectin/ODN (1 ml). Cells were lysed and analysed by Western blotting as previously described.

Cell cycle analysis
DNA analysis of treated cells was carried out on a Becton Dickinson ‘FACSCalibur’ flow cytometer using methodology described by Levack et al (1987).

Apoptosis assay
SKOV-3 cells were treated with ODNs as described above and apoptosis was measured using the TACS Annexin V-FITC kit (R&D Systems) following the prescribed protocol.

Xenograft experiments
Female adult nude (nu/nu) mice were obtained from ICRF (Clare Hall, South Mimms, UK) and maintained in negative pressure isolators. SKOV-3 cells (5 x 108 cells/injection) were injected into both flanks of 2 groups of mice. The control group consisted of 10 mice (2 tumours/mouse) and the treatment groups
RESULTS

c-Raf expression in primary ovarian cancer

c-Raf expression was identified in 49 of 53 ovarian cancer sections and varied from weak to intense staining (examples are illustrated in Figure 1A). Immunoreactivity was found almost exclusively in the epithelial cells with only minor staining in the stroma. The relationships between immunoreactive scores and clinical and pathological parameters are represented in Table 1. Serous adenocarcinomas expressed higher levels of c-Raf than all other subtypes combined ($P = 0.005$, Fisher’s exact test). No significant associations between c-Raf expression level and either stage (I / II vs III / IV, $P = 0.18$ Fisher’s exact test) or grade of differentiation (poor vs moderate/well, $P = 0.17$, Fisher’s exact test) were observed.

Survival data were available for 52/53 patients. High c-Raf expression was linked with poorer survival in this group (Figure 1B). The survival of patients whose tumours had an immunoreactive score $\geq 7$ ($n = 14$) was significantly poorer than that of patients with scores $\leq 6$ ($n = 38$) ($P = 0.002$, log-rank test). Since histology and stage are major prognostic variables, we also analysed survival in patients having stage III serous adenocarcinomas which represented a majority sub-group ($n = 28$). Again patients whose tumours had a higher level of c-Raf expression (immunoreactive score of $\geq 7$) ($n = 12$) had significantly poorer survival ($P = 0.035$, log-rank test) than patients with tumours expressing lower levels of c-Raf ($n = 16$).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>c-Raf expression in primary ovarian cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoscore</td>
<td>Number of patients with immunoscore*</td>
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<tr>
<td>All tumours</td>
<td>39</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>I / II</td>
<td>13</td>
</tr>
<tr>
<td>III / IV</td>
<td>23</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
</tr>
<tr>
<td>Well / moderate</td>
<td>14</td>
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<tr>
<td>Poor</td>
<td>21</td>
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<tr>
<td>Histology</td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>16</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>17</td>
</tr>
<tr>
<td>Clear cell</td>
<td>5</td>
</tr>
<tr>
<td>Mucinous</td>
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The expression of c-Raf was determined by immunohistochemical staining as described in Materials and Methods and scores between 0 and 12 were generated for each sample. *53 patient samples were analysed for expression but information on stage and grade were available for 50 and 48 patients respectively. *Fisher’s exact test. *Fisher’s exact test (serous vs rest).
SKOV-3 cells. After 48 h, both ODNs reduced c-Raf levels at 100 nM and 200 nM (Figure 3A). In comparison, similar concentrations of the random control ODN ISIS 16971 had no effect on c-Raf levels. The specificity of ISIS 5132 for c-Raf was examined by measuring its effects on A-Raf and ERK and after 48 h, ISIS 5132 (200 nM) had no effect on the expression of either of these proteins (Figure 3B). To determine the time required for c-Raf antisense ODNs to exert their effects on c-Raf protein, the level of c-Raf present in SKOV-3 cells was assayed at various intervals following antisense treatment. Both ISIS 13650 (200 nM) and ISIS 5132 (200 nM) reduced c-Raf levels by > 50% at 24 h and almost complete removal of c-Raf protein was observed at 48 h (Figure 3C).

c-Raf antisense ODNs inhibit cellular proliferation in SKOV-3 cells

To evaluate the effectiveness of c-Raf antisense ODNs as anti-proliferative agents, SKOV-3 cells were treated with ISIS 13650, ISIS 5132 or random ODN and the cell number determined 72 h after treatment. At 200 nM both ISIS 13650 and ISIS 5132 demonstrated > 80% inhibition of cell growth, compared to < 30% inhibition shown by the random ODN (Figure 4A). The time required for c-Raf antisense ODNs to exert their effects on cell proliferation was investigated in SKOV-3 cells. Cells not exposed to ODN (control cells) and cells treated with mismatch ODN (200 nM) grew steadily over the time course demonstrating a 3-fold increase in cell number over the 72 h period (Figure 4B). In contrast, the addition of 200 nM ISIS 13650 or ISIS 13650 resulted in marked growth inhibition at all time points investigated, with both antisense ODNs preventing cell growth in the first 24 h after treatment (Figure 4B).

Effect of c-Raf antisense ODNs in a panel of ovarian cancer cell lines

The ability of c-Raf antisense ODNs to inhibit cell growth was further investigated in a panel of 12 ovarian cancer cell lines 48 h after treatment. Both ISIS 13650 and ISIS 5132 inhibited cell proliferation by ≥ 60% in 9/12 cell lines examined (Figure 4C) compared to < 30% inhibition shown by the random ODN in selected cell lines.

ISIS 5132 causes cell cycle arrest and enhanced apoptosis in SKOV-3 cells

DNA analysis showed an accumulation of cells arrested in the S and G2/M phases of the cell cycle with a concomitant reduction in the G0/G1 phase. These specific effects were dose-dependent and not seen in either untreated cells or cells treated with ISIS 16971 (Figure 5A). A dose-dependent enhancement of apoptosis was
similarly seen in cells treated with ISIS 5132 compared with untreated cells or cells exposed to ISIS 16971 (Figure 5B).

**ISIS 5132 inhibits growth of SKOV-3 ovarian cancer xenografts**

ISIS 5132 inhibited the growth of SKOV-3 cells implanted as a subcutaneous xenograft in the flanks of nude mice. Treatment initiated 24 h after implantation of cells reduced the growth rate of these tumours at both 10 and 25 mg kg\(^{-1}\) day\(^{-1}\) compared to a vehicle control (Figure 6A). A mismatch control (ISIS 10353) was available for these studies and had no effect on growth. When treatment was delayed until tumours had reached a median size of 4 mm, ISIS 5132 (25 mg kg\(^{-1}\) day\(^{-1}\)) again produced a significant effect on growth (Figure 6B).

**DISCUSSION**

In the present study, which is the first to look at the prognostic significance of c-Raf expression in ovarian cancer, we show that high c-Raf expression is a negative prognostic factor. c-Raf was present in 92% of the ovarian adenocarcinomas investigated and a high level of c-Raf expression correlated significantly with both poor survival and serious histology. Within the serous subset, there was again a significant correlation between expression and survival, indicating that high c-Raf expression is associated with poor survival irrespective of other parameters such as histology and stage. The epithelial tumour cells were highly positive for c-Raf staining compared to stromal cells, which showed little c-Raf expression.

The c-Raf signalling pathway plays an important role in the growth regulation of some ovarian cancers. ERK activation has previously been identified in ovarian cancer cell lines and attributed to an increase in MEK and c-Raf activity (Hoshino et al, 1999). In addition, ERK activation has been shown to correlate linearly with increasing concentrations of MEK (Zheng and Guan, 1993). These data in combination with our finding that c-Raf is highly expressed in most of the ovarian cancer cell lines suggest that an increase in c-Raf level may contribute to increased signalling through the ERK pathway. Further analysis of the ERK signalling pathway in ovarian cancer cell lines is essential to elucidate the role of c-Raf in this disease, and will form the basis of future studies.

The use of antisense ODNs has allowed us to establish that c-Raf plays an important role in the proliferation of ovarian cancer cell lines, supporting conclusions of other studies that c-Raf is a critical mediator of oncogenic transformation (Storm et al, 1990; Daum et al, 1994). We have shown that both ISIS 5132 and ISIS 13650 reduce c-Raf protein in SKOV-3 ovarian cancer cells, in line with observations in other disease types (Monia et al, 1996a; Monia, 1997; Lau et al, 1998). Evidence to support specificity was provided by the observations that while c-Raf was reduced, related signalling molecules were unaffected and a random ODN (ISIS 16971) had no effect on c-Raf protein expression.

Investigation of the effects of antisense ODNs in a panel of ovarian cancer cell lines has allowed an insight into the efficiency of these antisense compounds in a range of cell types with variable amounts of c-Raf protein. Despite a 24-fold variation in c-Raf
protein levels between the cell lines, both ISIS 5132 and ISIS 13650 substantially reduced cell growth. Most of the cell lines were > 60% growth inhibited, while 3 cell lines (A2780, OVCAR5 and CaOV3) were < 40% growth inhibited by antisense ODNs. These intercellular growth inhibitory differences cannot be accounted for by the variation in c-Raf protein levels between the cell lines. Explanations for these differences are likely to come from further analysis of the growth signalling pathways in these cells. It is also possible that some cell lines do not require c-Raf for cellular proliferation. Such observations have been reported in a study investigating constitutive activation of the ERK signalling pathway in over 100 different tumour cell lines, including OVCAR3, SKOV-3 and OVCAR5 (Hoshino et al, 1999). Both OVCAR3 and SKOV-3 demonstrated high constitutive activation of the ERK pathway and in our study these cell lines were growth inhibited to a great degree by c-Raf antisense ODNs. In contrast, OVCAR5 exhibited minimal constitutive ERK activation that was not increased upon stimulation with serum (Hoshino et al, 1999). This suggests that OVCAR5 may not signal predominantly through c-Raf, and may explain our inability to inhibit OVCAR5 cell proliferation with antisense ODN. Consequently, some ovarian cancer types may be highly responsive to therapies that target c-Raf, while others may not.

Finally, we have demonstrated that ISIS 5132 is effective in vivo, significantly inhibiting the growth of the SKOV-3 xenograft. ISIS 5132 is presently undergoing clinical trials and has been shown to significantly reduce levels of c-raf mRNA in peripheral blood mononuclear cells. This was associated with clinical benefits in 2 out of 14 patients with a mixture of advanced solid tumours (O’Dwyer et al, 1999). ISIS 5132 has been rigorously examined and was shown to significantly reduce levels of c-raf mRNA in peripheral blood mononuclear cells. This was associated with clinical benefits in 2 out of 14 patients with a mixture of advanced solid tumours (O’Dwyer et al, 1999). ISIS 5132 has been rigorously examined and was shown to significantly reduce levels of c-raf mRNA in peripheral blood mononuclear cells. This was associated with clinical benefits in 2 out of 14 patients with a mixture of advanced solid tumours (O’Dwyer et al, 1999).

In summary, our findings demonstrate an association between high levels of c-Raf expression and reduced survival time for patients with ovarian adenocarcinomas suggesting an important role for c-Raf in tumour growth, although the extent of tumour types that heavily depend on c-Raf remains to be explored. We have shown that ODNs that specifically target c-Raf are effective growth inhibitors for the majority of ovarian cancer cells. In addition, the use of antisense ODNs will help identify cells that rely on c-Raf for growth. Studies examining the effects of ISIS 5132 and ISIS 13650 in vivo and in combination with other standard chemotherapeutic agents will give further information on the practical application of antisense ODNs for the treatment of cancer.

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