Total inhibition of involucrin synthesis by a novel two-step antisense procedure

Further examination of the relationship between differentiation and malignancy in hybrid cells

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Summary

A novel procedure involving the sequential use of two different antisense constructs has been used to inhibit the synthesis of involucrin in a hybrid cell line formed by the fusion of a human cervical carcinoma cell with a normal human keratinocyte (ESH100P6). In this cell line, and other similar hybrids, malignancy, as measured by progressive growth in vivo, is suppressed; and it has been shown that the keratinocyte imposes its own programme of terminal differentiation on the non-malignant hybrid cell. In particular, involucrin, a precursor of one of the major components of the cornified envelope of mature keratinocytes, continues to be produced. When, however, malignant segregants arise in the hybrid cell population, the terminal differentiation programme of the keratinocyte is not expressed and involucrin ceases to be made. It seemed possible that if the synthesis of involucrin, a critical marker of keratinocyte terminal differentiation, could be completely inhibited, this differentiation programme might be disrupted, and the malignant phenotype might then reappear in the non-malignant hybrids. This question was investigated further in the present paper. Total, and specific, inhibition of involucrin synthesis was indeed achieved by a sequential two-step antisense procedure, which might provide a systematic general method for the complete inactivation of other selected target genes.

Key words: antisense RNA, involucrin, malignancy.

Introduction

When any of a large range of tumour cells is fused with a normal fibroblast, malignancy, as measured by the ability of cells to grow progressively in compatible hosts, is suppressed (for a recent review of this work, see Harris, 1990). Evidence has accumulated in support of the view that this suppression may be achieved by the imposition on the hybrid cell of the terminal differentiation pattern of the normal cell with which the tumour cell is fused (Harris, 1991). In the case of hybrids between a human carcinoma cell line (D98) and normal keratinocytes, Peehl and Stanbridge (1982) showed that the suppression of malignancy was associated with the execution in vivo of the terminal differentiation programme of the keratinocyte. Harris and Bramwell (1987) showed further that, in a series of such hybrids, involucrin production continued while malignancy was suppressed but ceased when the malignant phenotype reappeared in segregants that arose in the hybrid cell population. Immunohistochemical studies of tumours of the cervix and of the skin also indicate that a reduction in involucrin synthesis is associated with an increase in malignancy as judged by histological criteria (Warhol et al., 1982; Sassoon et al., 1985; Murphy et al., 1984).

Involucrin is an important marker of terminal differentiation in the keratinocyte (Watt, 1989). It is first expressed in the upper spinous layers in vivo and immediately above the basal layer in vitro. It is cross-linked in the cornified layer during the construction of the cornified envelope, of which it is a major component. Epithelia that do not possess a stratum corneum, however, still express involucrin (Banks-Schlegel and Green, 1981). This suggests that involucrin may have a function in addition to the formation of the cornified envelope, an idea that is supported by the observation that even in cornified epithelia not all the involucrin synthesised by the keratinocyte is incorporated into the cornified envelope. Involucrin is normally expressed in vivo when the cells have lost the ability to divide, but ESH100P6 cells are unusual in that they
express involucrin but continue to proliferate. These cells, however, do not differentiate completely in vitro as the involucrin remains in the cytoplasm and is not cross-linked to form a cornified envelope. When inoculated into nude mice, ESH100P6 cells can form keratin cysts. This may indicate that in vivo the cells respond to signals that induce further differentiation (Peehl and Stanbridge, 1981).

The aim of the present experiments was to see whether the synthesis of involucrin could be completely inhibited in D98 × keratinocyte hybrids in which malignancy was suppressed and, if so, whether this inhibition would result in the reappearance of the malignant phenotype. It seemed possible that if the synthesis of a cardinal early marker of keratinocyte differentiation such as involucrin could be completely abolished, the execution of the rest of the terminal differentiation programme of this cell type might be aborted. To inhibit the synthesis of involucrin we used a novel procedure in which the cells were transfected sequentially by two different antisense expression vectors. Complete, and specific, inhibition of involucrin synthesis was achieved.

**Materials and methods**

**Preparation of sense and antisense plasmids**

Two expression vectors were used in this study: pKG4 (Steel and Harris, 1989) and pBabe Hygro (Morgenstern and Land, 1990). pKG4 contains an ampicillin-resistance gene, the TK-neo selectable marker gene that confers resistance to the antibiotic G418 on mammalian cells, the SV40 early region promoter, and SV40 transcription-termination and polyadenylation signals. pBabe Hygro contains an ampicillin-resistance gene, the hph selectable marker gene that confers resistance to hygromycin B on mammalian cells, the Moloney murine leukaemia virus (MoMuLV) 5' long terminal repeat (LTR) promoter and enhancer sequences, and the MoMuLV 3' LTR that contains the transcription-termination and polyadenylation signals. The involucrin DNA fragments were excised from pAl-3H6B (Eckert and Green, 1986), which contains the translation initiation codon and the entire coding region.

A 1.835 kb HaeIII-HaeIII involucrin gene fragment (Fig. 1) was blunt-end ligated into the HindIII site of pKG4 in sense and antisense orientations with respect to the SV40 promoter. Standard cloning procedures were used (Maniatis et al., 1982) and all cloning enzymes were supplied by Boehringer Mannheim. The antisense construct was designated pEGAS24, and the sense construct, pEGS21. Both are 7.646 kb. The 1.835 kb involucrin gene fragment corresponds to part of the intron, the 3' splice site, the translation initiation codon and 1433 bp of coding sequence.

A second antisense involucrin expression vector, pEGAS3D (7.556 kb), was constructed. A 2.356 kb BglII-BamHI involucrin gene fragment (Fig. 1) was ligated into the BamHI site of pBabe Hygro in the antisense orientation with respect to the MoMuLV promoter. This 2.356 kb involucrin gene fragment consists of part of the intron, the 3' splice site, the translation initiation codon and the entire coding region. The antisense RNAs transcribed from pEGAS24 and pEGAS3D will therefore have the potential to exert their effects in a variety of ways by inhibiting transcription, splicing or translation.

**Cell lines**

ESH100P6 is a non-tumorigenic cell line formed by the fusion of a D98 cell (a HeLa derivative) with a normal human keratinocyte (Peehl and Stanbridge, 1981). The ESH100P6 TR1.2 cell line is a malignant segregant derived from ESH100P6 by passage through a nude mouse. Both cell lines were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 50 i.u./ml penicillin and 50 i.u./ml streptomycin.

**Transfection procedure**

Stable integration of the circular plasmid DNA into the target cells was achieved by a calcium phosphate transfection procedure based on that described by Graham and Van der Eb (1973). A total of 10^6 recipient cells were grown for 24 h before transfection. A 40 g sample of circular plasmid DNA was dissolved in 1 ml of 2 × HeBS (280 mM NaCl, 10 mM KCl, 1.5 mM Na2HPO4, 2H2O, 12 mM glucose, 15 mM Hepes) and 1 ml of 250 mM CaCl2 was added dropwise to the DNA solution, through which air was bubbled. A calcium phosphate/DNA precipitate was allowed to form for 20 min. It was then resuspended and added to the growth medium overlying the target cells. After 5-7 h incubation at 37°C, the medium was removed and the cells were exposed to 2 ml of 25% (v/v) glycerol in complete DMEM for 1 min. This was washed off with DMEM and replaced by complete growth medium; the cells were then incubated for 36 h. They were thereafter grown in selective medium until clones could be isolated.

**Assays for involucrin**

Confluent cells were harvested, pelleted and extracted with an equal volume of 0.2% (w/v) sodium deoxycholate in 10 mM Tris-HCl, pH 8.0. The cell extracts were subjected to electrophoresis on an SDS-polyacrylamide gel (Atkinson and Bramwell, 1981) in the running buffer system described by Laemmli (1970). The material separated on the gel was transferred to a nitrocellulose membrane by Western blotting and the involucrin bands, which appear at 170 kDa in this system (Harris and Bramwell, 1987), were identified by treating the membrane with a mouse monoclonal anti-human involucrin antibody and a 125I-labelled rabbit anti-mouse immunoglobulin (Harris and Bramwell, 1987). The membrane was exposed to X-ray film (XR, Fuji) at -70°C for 24 h.

The presence of involucrin in individual cells was detected by immunocytochemical assay on preparations of cells grown on coverslips. The cells were fixed in methanol and treated
with a mouse monoclonal anti-human involucrin antibody for 30 min. The location of the bound antibody was detected by a conventional immunoperoxidase technique (McGee et al., 1982).

Preparation and analysis of genomic DNA
Genomic DNA was prepared from confluent monolayer cultures, which were washed twice with PBS and then treated with lysis buffer (10 mM Tris- HCl, pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.4% SDS, 100 g/ml proteinase K) for 4-5 h at 37°C. The lysate was recovered and extracted twice with phenol, twice with phenol/chloroform and once with chloroform (1:1 in all cases). The DNA was precipitated by ethanol and resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

A 25 g sample of genomic DNA was digested with an appropriate restriction enzyme and the digestion products were separated by electrophoresis through an agarose gel. They were then transferred to a nylon membrane by Southern blotting (Southern, 1975). The membrane was pre-hybridised for 2-4 h at 42°C in a solution containing 50% (v/v) deionised formamide, 6 x SSPE (SSPE is 0.18 M NaCl, 10 mM NaH2PO4, 1 mM EDTA, pH 7.0), 5 x Denhardt’s reagent, 0.5% SDS, 100 g/ml denatured salmon sperm DNA). An involucrin gene fragment labelled with [α-32P]dATP by a random primed DNA-labeling reaction (Feinberg and Vogelstein, 1983), was denatured and added to the hybridisation fluid (50% (v/v) deionised formamide, 6 x SSPE, 0.5% SDS, 100 g/ml denatured salmon sperm DNA). The membrane was hybridised with the probe for 22 h at 42°C and was washed twice in 2 x SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% SDS at room temperature for 10 min; twice in 1 x SSC, 0.1% SDS at 65°C for 15 min; and twice in 0.5 x SSC, 0.1% SDS at 65°C for 15 min. The membrane was exposed to X-ray film (XR, Fuji) at -70°C.

Preparation and analysis of RNA
RNA was isolated from cells by the guanidinium method described by Chirgwin et al. (1979). Monolayer cells approaching confluence were washed twice with PBS and lysed in place with 3.5 ml of a guanidinium solution (4 M guanidinium isothiocyanate, 20 mM sodium acetate, pH 5.2, 0.1 mM dithiothreitol, 0.5% N-lauryl sarcosine). The lysate was passed through a 23 G needle five times and layered onto guanidinium isothiocyanate, 20 mM sodium acetate, pH 5.2, 0.5% SDS, 100 g/ml proteinase K. An involucrin gene fragment labelled with [α-32P]dATP by a random primed DNA-labelling reaction (Feinberg and Vogelstein, 1983), was denatured and added to the hybridisation fluid (50% (v/v) deionised formamide, 6 x SSPE, 0.5% SDS, 100 g/ml denatured salmon sperm DNA). The membrane was hybridised with the probe for 22 h at 42°C and was washed twice in 2 x SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% SDS at room temperature for 10 min; twice in 1 x SSC, 0.1% SDS at 65°C for 15 min; and twice in 0.5 x SSC, 0.1% SDS at 65°C for 15 min. The membrane was exposed to X-ray film (XR, Fuji) at -70°C.

Tumorigenicity assay
Cells to be tested for their ability to grow progressively in vivo were harvested and washed twice with PBS. Inocula of 106 cells in 1 ml PBS were injected subcutaneously into the scapula region of six-week-old congenitally athymic nude mice, six animals being used to test each cell line. Animals were scored as negative if they failed to develop a progressively growing tumour at the site of inoculation within 3 months. The latent period was the time between injection of the cells and the time the tumour was first identified.

Results
Transfection of pKG4, pEGS21 and pEGAS24 into ESH100P6 cells
The transfection of any foreign DNA sequence into cells may produce non-specific effects, which could result in decreased synthesis of involucrin. Control transfections of ESH100P6 cells were therefore done with the expression vector, pEGS21, in which the involucrin sequence is present in the sense orientation, and with the original pKG4 plasmid. A total of 56 clones of cells transfected with these control vectors were isolated by growth in medium supplemented with G418 at 1.2 mg/ml. All of them expressed unreduced amounts of involucrin, as determined by Western blotting. It is, therefore, very improbable that the clones expressing greatly reduced levels of involucrin that were isolated after transfection with the involucrin antisense expression vector could be variants that arose spontaneously.

To produce ESH100P6 cells that expressed antisense involucrin RNA, pEGAS24 was transfected into the cells. A total of 50 clones were isolated in medium supplemented with G418, and protein extracts were prepared from them. These were screened by Western blotting to identify clones with reduced involucrin levels. Twelve of the clones screened expressed less than 50% of the involucrin present in ESH100P6 cells. Five clones were selected for further study: clones 13.2.4, 13.4, 13.9, 14.3 and 18.7. These clones expressed between 3% and 100% of the amount of involucrin synthesised in untransfected ESH100P6 cells as determined by laser densitometry of Western blots (Fig. 2).

DNA analysis of ESH100P6 pEGAS24 clones
The stable integration of antisense involucrin gene sequences into the ESH100P6 genome was confirmed by Southern blot analysis. Fig. 3 shows a Southern blot of genomic DNA prepared from the ESH100P6 pEGAS24 clones and digested with EcoRI. The band at 801 bp was probed with an [α-32P]dATP-labelled TK-neo gene fragment. This indicates that pEGAS24 was stably integrated into the genome of this clone also.

Tumorigenicity of ESH100P6 pEGAS24 clones
The tumorigenicity of ESH100P6 cells, ESH100P6 pEGS21 and pEGAS24 was determined by injection into nude mice. The tumorigenicity data are
Fig. 2. Western blot of cell extracts of ESH100P6 cells transfected with pEGAS24. The presence of involucrin (at 170 kDa) is revealed by appropriate labelling with a radioactive antibody. (A) 10⁶ cells were used to prepare each protein extract. Lanes: 1, ESH100P6; 2, clone 13.9; 3, clone 13.4; 4, clone 14.3; 5, clone 18.7. (B) Lanes: 1, ESH100P6 extract prepared from 5 × 10⁶ cells; 2, clone 13.2.4 extract prepared from 5 × 10⁶ cells; 3, clone 13.2.4 extract prepared from 5 × 10⁶ cells.

Fig. 3. Southern blot of ESH100P6 pEGAS24 clones. Genomic DNA samples were digested with EcoRI, electrophoresed through an agarose gel and blotted onto a nylon membrane. The blot was probed with an α-³²P-labelled 1.835 kb HaeIII-HaeIII involucrin gene segment. The size of the restriction fragment containing the involucrin gene introduced in pEGAS24 is indicated in parentheses. Lanes: 1, ESH100P6; 2, clone 13.2.4 (10 kb); 3, clone 13.4 (8.5 kb); 4, clone 13.9 (4.9 kb); 5, clone 14.3 (8.5 kb); 6, clone 18.7.

Table 1. Tumorigenicity of ESH100P6 pEGAS24 clones

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Take incidence</th>
<th>Latent period (days)</th>
</tr>
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<tbody>
<tr>
<td>ESH100P6 TR1.2</td>
<td>6/6</td>
<td>9</td>
</tr>
<tr>
<td>ESH100P6</td>
<td>0/6</td>
<td>–</td>
</tr>
<tr>
<td>Clone 13.2.4</td>
<td>2/6</td>
<td>83</td>
</tr>
<tr>
<td>Clone 13.4</td>
<td>0/6</td>
<td>–</td>
</tr>
<tr>
<td>Clone 13.9</td>
<td>0/6</td>
<td>–</td>
</tr>
<tr>
<td>Clone 14.3</td>
<td>0/6</td>
<td>–</td>
</tr>
<tr>
<td>Clone 18.7</td>
<td>1/6</td>
<td>83</td>
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shown in Table 1. Clone 18.7, which expresses 47% of the original level of involucrin, produced a single tumour. This was a non-progressive keratin cyst, which only grew to a diameter of 2 mm even after many weeks. The cyst contained layers of differentiated epithelial cells and a keratinised centre. Clone 13.2.4, which expresses just 3% of the original level of involucrin, produced a tumour in two mice. These tumours grew progressively. They were disorganised, undifferentiated carcinomas composed of islands of tumour cells within a connective tissue stromal reaction, and they invaded muscle in some areas.

These results perhaps suggested that a decrease in involucrin expression to 3% of the original level increased the tumorigenicity of ESH100P6 cells, but the
findings were obviously inconclusive because only two out of the six animals inoculated with clone 13.2.4 cells developed tumours, and these appeared only after a long latency.

Transfection of pEGAS3D into clone 13.2.4 cells

Clone 13.2.4 cells, which express 3% of the original level of involucrin, were transfected with a second antisense involucrin expression vector, pEGAS3D, in the hope that subclones could be isolated that expressed no detectable involucrin. A total of 57 subclones were isolated by growth in medium supplemented with 200 \( \mu \text{g/ml} \) hygromycin B and protein extracts prepared from 10^6 cells of each were screened by Western blotting. Involucrin could not be detected in 17 subclones and 7 of these were selected for further investigation: subclones 6, 7, 14, 26, 27, 41 and 79 (Fig. 4). No involucrin could be detected in protein extracts prepared from even 10^7 cells of each of these subclones. Amido Schwartz staining of the Western blots indicated that protein synthesis in general had not been reduced by this antisense treatment, but that the inhibition was specific to involucrin.

DNA and RNA analyses of clone 13.2.4 pEGAS3D subclones

Southern blot analysis confirmed that the involucrin-negative subclones had stably integrated a copy of pEGAS3D into their genome (Fig. 5). The band at 2.3 kb, which is present in all cell lines, represents the DNA fragment containing the endogenous involucrin alleles. The band at 5.2 kb represents the involucrin gene fragment derived from pEGAS24 which is stably integrated into the genome of clone 13.2.4. The third band present in the subclones corresponds to the involucrin gene fragment introduced by pEGAS3D. The intensities of the bands indicate that this third gene fragment is present at 5.2 kb in subclone 6 and 2.3 kb in subclone 7; a fourth gene fragment is present at 2.3 kb in clone 14. The variation in size of the third band indicates that pEGAS3D has integrated into different sites in the clone 13.2.4 genome. The subclones contain just one copy of pEGAS3D, except subclone 14, which contains two copies.

Northern blot analysis of total RNA from the different cell lines proved difficult to interpret. Endogenous involucrin RNA could be detected in ESH100P6 cells as a faint band, but it could not be identified in any of the pEGAS24 clones or pEGAS3D subclones. Involucrin antisense RNA produced from pEGAS24 could be detected with any certainty only in clone 13.2.4 cells; some of the subclones transfected with EGAS3D contained involucrin antisense RNA. There appears, therefore, to be no consistent relationship between the levels of involucrin sense RNA, involucrin antisense RNA and the quantity of involucrin produced in these cell lines. This is in agreement with many other studies that show no simple stoichiometric relationship between the amount of antisense...
RNA produced and the degree of inhibition of synthesis of the relevant protein (Holt et al., 1986; Rivera et al., 1989; Munir et al., 1990).

Immunoperoxidase staining of cell lines for involucrin

The synthesis of involucrin in individual cells was detected by an immunocytochemical assay on preparations of control and transfected cell lines. The presence of involucrin was revealed by the brown pigment generated by the immunoperoxidase reaction. ESH100P6 cells all reacted strongly with the anti-involucrin antibody (Fig. 6A). The tumorigenic ESH100P6 TR1.2 cells showed no staining with this antibody. These results confirm the findings of Harris and Bramwell (1987).

Clone 13.2.4 cells synthesise involucrin at 3% of the original level as determined from Western blots. This is reflected by the immunoperoxidase staining of these cells (Fig. 6B). The reduction in involucrin expression is more or less uniform within the cell population.

Immunoperoxidase staining of the seven selected clone 13.2.4 pEGAS3D subclones failed to detect any involucrin. Fig. 6C shows one of these subclones, subclone 7. This can be compared with Fig. 6D, which shows subclone 7 cells stained by the standard immunoperoxidase technique, but with the omission of the specific anti-involucrin monoclonal antibody. These immunoperoxidase stains support the Western blot results and show that involucrin is, indeed, completely absent from the pEGAS3D subclones.

Tumorigenicity of clone 13.2.4 pEGAS3D subclones

The tumorigenicity of clone 13.2.4 involucrin-negative subclones was assayed. No tumours grew in any of the experimental animals, even after a long latency. Tumours were produced in control mice injected with ESH100P6 TR1.2 cells. These results clearly indicate that the selective total extinction of involucrin expression in ESH100P6 cells is not in itself enough to induce the reappearance of the malignant phenotype.

Discussion

The present study has demonstrated that the sequential use of two antisense expression vectors can completely block the expression of a targeted mammalian gene. Although this approach has so far been used for only one gene, that specifying the protein involucrin, there seems to be no reason why it should not provide a systematic method for inactivating any desired gene.

Southern blots of the genomic DNA of the clones in which involucrin synthesis was inhibited revealed that they had all retained both endogenous involucrin alleles. The inhibition of involucrin synthesis is not, therefore, the result of loss of the endogenous involucrin genes. With one exception, all clones contained only one integrated copy of the involucrin antisense plasmid. This should, in principle, produce the minimum degree of disruption of the host genome and the minimum perturbation of the expression of other genes. Izant and Weintraub (1985) transfected mouse cells with an antisense expression vector and isolated cell lines that contained about 20 stably integrated copies of the plasmid. Although such a high copy number increases the total amount of antisense RNA produced, it also increases the probability of interfering with the activity of genes other than the target gene, so that observed phenotypic effects might be difficult to interpret.

The mechanism by which the antisense construct inhibits involucrin synthesis cannot be deduced from the present study. The reduction in the level of endogenous (sense) involucrin RNA and the failure to detect involucrin antisense RNA consistently in the clones harbouring antisense vectors could be the result of the sense and antisense strands forming duplexes that are degraded rapidly by double strand-specific RNases (Crowley et al., 1985; Kim and Wold, 1985; Paulssen et al., 1990). It is, in any case, clear that inhibition of the synthesis of involucrin is not simply a question of how much antisense RNA is available to complex with the involucrin mRNA. Since it is difficult to know in advance the best region of the involucrin RNA molecule against which to target the antisense strand, the antisense RNAs used in the present experiments were selected for their potential to exert an inhibitory action at any of the steps from transcription of the gene to the synthesis of the protein.

It will be noted that the degree of inhibition of involucrin synthesis in cells transfected with a single antisense plasmid varies from clone to clone. One possible explanation for this is that the antisense plasmid may be integrated in a different nuclear compartment from the endogenous sense gene. In situ hybridisation has revealed that specific RNA transcripts are present within the nucleus in only one or two well-defined channels, which extend from the active gene to the nuclear membrane (Lawrence et al., 1989). This partitioning implies that RNA molecules do not diffuse freely throughout the nucleus, so that those transcribed from antisense genes may have difficulty in encountering those transcribed from the endogenous sense genes unless they happen to be in close proximity, for example on the same chromosome. Even so, there is likely to be some variation in the efficiency of hybridisation.

Concerning the relationship between the imposition of terminal differentiation and the suppression of malignancy the present experiments are not very informative. Preliminary experiments with specific anti-keratin antibodies suggest that abrogation of involucrin synthesis does not abort the rest of the keratinocyte terminal differentiation programme. It appears that clones in which involucrin synthesis has been inhibited continue to make normal amounts of keratins that are produced late in the terminal differentiation programme. We now propose to use this novel two-step antisense procedure to eliminate the production of other markers of keratinocyte differentiation to see whether we can impair the overall process effectively.
Fig. 6. Immunoperoxidase staining of cells for involucrin. The presence of involucrin is revealed by the generation of a brown pigment. Lanes: A, ESH100P6; B, clone 13.2.4; C, subclone 7; D, subclone 7 control: the cells were not treated with the specific anti-involucrin antibody.
enough to permit the malignant phenotype to reappear in the non-tumorigenic hybrids.

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