Quiescence and increased adhesion after mammalian DNA transfection of human fibrosarcoma cells

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Summary

When a population of human fibrosarcoma HT1080 cells was transfected with a mammalian expression vector and DNA pieces representing either the human whole genome or mouse bulk cDNA, there was a transient increase in the number of adhesive cells in the population. The number of cells with increased adhesion was proportional to the amount of transfected DNA; the increase occurred at a maximal frequency of between 10^{-4} and 10^{-5} per cell. Increased adhesion in these cells persisted for 9–12 days, corresponding to the period of highly efficient transient transfection, and was accompanied by arrest in cell division. Transfection of non-mammalian DNAs, reduction of transfected mammalian sequence length by restriction enzyme digestion, or omission of expression vector DNA did not permit these shifts in phenotype. The effects seen suggest that expression of specific transfected mammalian DNA sequences suppresses certain phenotypic characteristics in these transformed mammalian cells.

Key words: transfection, suppression, adhesion, fibrosarcoma cells.

Introduction

Some types of cultured transformed mammalian cell lines, particularly fibroblasts and endothelial cells, are less adhesive than their counterpart normal cells (Hynes, 1976). Loss of adhesion in the transformation process apparently results from a decrease in the concentration of large glycoprotein molecules, fibronectins, from the cell surface (Hynes *et al.* 1979). Reversion of a transformed cell phenotype to a more normal phenotype, either *via* a spontaneous process (Vogel & Pollack, 1974) or by acquisition of a chromosome from a non-transformed cell through cell hybridization (Harris *et al.* 1969), may result in an increase in cell surface adhesiveness (Stanbridge *et al.* 1982; Shin *et al.* 1975). In addition, the loss or gain of adhesiveness is sometimes coupled pleiomorphically to other transformation-specific phenotypes (Shin *et al.* 1975). Thus, the non-transformed revertant cell may not only be more adhesive than its counterpart transformed version, but also flatter, more serum-dependent, more contact-inhibited, and less able to divide in a semi-solid support medium (Shin *et al.* 1975).

The transformation-coupled property of decreased cell adhesiveness in certain types of cells has allowed us to separate more-adhesive normal or revertant cells from less-adhesive transformed cells in mixed populations. In turn, quantification of the number of recovered revertants has allowed us to ask (1) whether greater adhesiveness as a cellular property can be effected by introduction of mammalian DNA segments into transformed cells, and (2) whether other phenotypic changes are observed if increased adhesiveness results.

In attempting to assay for genes that increase adhesiveness, we have transfected human fibrosarcoma HT1080 cells (Rasheed *et al.* 1974) with expression vector DNA ligated or mixed with either human genomic DNA or bulk poly(A)$^+$ RNA-derived mouse cDNA. Evidence to show that greater cell adhesiveness is effected by these transfected nucleic acids has been sought during the period of transient transcription that immediately follows transfection by the DEAE–dextran method (Lopata *et al.* 1984). DNA-mediated transfection that results in transient phenotypic expression is vastly more efficient than transfection that results in stable transfectants. Because chromosomal integration is not necessary for expression, transient transfection is efficient in allowing the simultaneous

expression of a diverse set of cotransfected gene sequences in any cell that takes up DNA. During this period, as much as 80% of a transfected population of cells potentially express a diverse set of transfected genes; any one cell may transiently express greater than 5% of a transfected mammalian genome (Sussman & Milman, 1984; Lopata *et al.* 1984; Berman *et al.* 1984).

Assaying a transfected population of transformed cells for an increase in the number of cells that have become more adhesive during the brief period of transient transfection requires a positive assay for transformation-revertants that can be completed within a few days following the transfection. Previous assays for revertants having specific non-transformed phenotypes have generally utilized slow negative cell-selection methods (usually on the basis of killing cells that were able to grow in medium having a low serum concentration) that spare non-dividing revertants (Vogel & Pollack, 1974).

Materials and methods

Human placental, human lymphocyte, *Strongylocentrotus purpuratus* (sea urchin), and *Escherichia coli* DNAs were isolated by standard methods and then digested with *Sau*3A to sublimit; the generated pieces with 8–17 kb (10^3 bases) or shorter ranges, as indicated in Table 1, were recovered after electrophoresis in 0·4% agarose gels or centrifugation through salt gradients (Maniatis *et al.* 1982). These pieces were mixed in a 1:1 weight ratio with pZipNeoSV(X)1 expression vector DNA (Cepko *et al.* 1984) that had been linearized, at a cloning site between long terminal repeats (LTRs), with *Bam*HI and then dephosphorylated with calf intestinal alkaline phosphatase. After ligation, 10 μg of the DNA mixture was transfected into ≈10^6 HT1080 cells. In other transfections, ≈1 μg of cDNA, prepared (Gubler & Hoffman, 1983) from bulk poly(A)$^+$ mRNA obtained from normal NIH/3T3 cells (Chirgwin *et al.* 1979), was cotransfected with 2 μg linearized pZipNeoSV(X)1 DNA. Transfections were in the presence of 200 μg of DEAE–dextran (Pharmacia, M_r = 500 000) per ml of Dulbecco-modified Eagle's medium (DMEM) without serum, at 37°C for 10–12 h (Lopata *et al.* 1984). At the end of this period, the DNA/dextran solution was removed and the cells were exposed for 2 min to 10% dimethyl sulphoxide (DMSO) in 140 mM-NaCl, 25 mM-Hepes, 0·75 mM-Na_2HPO_4, pH 7·1. The cells were then rinsed three times with phosphate-buffered saline (PBS) and covered with DMEM containing 8% heated-inactivated calf serum.

HT1080 cells with increased adhesiveness were selected 24 h after transfection by the following method. After trypsinization at room temperature for 5 min with 0·05% trypsin, 2 mM-EDTA in PBS, trypsin was inactivated by the addition of 1 vol. of 8% calf serum in DMEM. The cells were pelleted twice in PBS and then suspended in PBS and allowed to attach for 1 h at room temperature, undisturbed, to the surface of a fresh tissue culture flask (Corning no. 25116). The flask surface was then rinsed three times with PBS and covered with conditioned DMEM containing 8% calf serum. The remaining attached cells were again selected, as above, for adhesiveness. For ease of counting adhesive cells and comparing cell or colony morphologies, the twice-selected cells were trypsinized and transferred to a plastic centrifuge tube by flushing the trypsinized culture flask four times with DMEM containing serum; the cells were then pelleted and replated in a microtitre plate well of small surface area. After replating, the cells were again exposed to conditioned medium.

Table 1. *Transfection of HT1080 cells with DNA*

Transfected DNA	No. of adhesive cells selected per ≈10^6 transfected HT1080 cells in different transfections
Non-cleaved human placental or lymphocyte DNA pieces	0, 0, 1, 2, 3, 3, 5
*Sau*3A-cleaved human placental or lymphocyte DNA pieces (8–17 kb)	0, 0, 0, 0, 1, 2, 3
Linearized pZipNeoSV(X)1	0, 0, 0, 1
pZipNeoSV(X)1 circles	0, 0, 0, 1
*Sau*3A-cleaved human placental or lymphocyte DNA pieces (0·75–2 kb) ligated to pZipNeoSV(X)1	0, 0, 3, 3, 4, 4
*Sau*3A-cleaved human DNA pieces (8–17 kb) further cleaved with *Eco*RI to 2–5 kb and cotransfected with linearized pZipNeoSV(X)1	0, 0, 2, 2
*Pst*I-cleaved human lymphocyte DNA pieces (2–5 kb) cotransfected with linearized pZipNeoSV(X)1	1, 1, 3, 3
*Sau*3A-cleaved human placental DNA pieces (8–17 kb) ligated to linearized pZipNeoSV(X)1	9, 12, 12, 23, 27, 29, 31, 35, 44
*Sau*3A-cleaved human lymphocyte DNA pieces (8–17 kb) ligated to linearized pZipNeoSV(X)1	27, 37, 39, 56
Non-cleaved mouse NIH/3T3 cDNA (<1·5 kb) cotransfected with linearized pZipNeoSV(X)1	87, 103, 126
Mouse NIH/3T3 cDNA cleaved to limit with *Sau*3A, cotransfected with linearized pZipNeoSV(X)1	0, 0, 2, 3
*Sau*3A-cleaved sea urchin DNA pieces (8–17 kb) ligated to linearized pZipNeoSV(X)1	0, 0, 1, 3, 3, 3
*Sau*3A-cleaved *E. coli* DNA pieces (8–17 kb) ligated to linearized pZipNeoSV(X)1	0, 0, 0, 2, 3, 3

Results

Transfection of populations of HT1080 cells with DNA pieces (unaccompanied by vector DNA) resulting from partial restriction enzyme digests of *E. coli*, sea urchin, or human whole cell DNA, did not noticeably increase the number of cells that had increased adhesiveness (Table 1). However, when whole genomic human 8–17 kb DNA, resulting from partial restriction digestion with *Sau*3A, was mixed and ligated with linearized pZipNeoSV(X)1 retroviral expression vector (Cepko *et al.* 1984), a portion of a transfected HT1080 cell population acquired a more adhesive phenotype within 24 h after transfection (Table 1). Similar results were seen when NIH/3T3 mouse cell cDNA, derived from bulk poly(A)$^+$ RNA, was cotransfected with linearized pZipNeoSV(X)1 vector DNA; this response was abolished when the bulk mouse cDNA was cleaved with *Sau*3A prior to cotransfection with linearized pZipNeoSV(X)1 DNA (Table 1). Non-mammalian DNAs, mixed and ligated with the linearized vector DNA, did not increase the number of adhesive cells (Table 1). In addition, after reduction of the length of human DNA pieces to a range of: (1) 0·75–2 kb by digestion with *Sau*3A; or (2) 2–5 kb by *Pst*I alone; or (3) 2–5 kb from 8–17 kb *Sau*3A pieces further cleaved by digestion with *Eco*RI, an increase in adhesion was not observed on transfection of ligated (Table 1) or non-ligated mixtures (data not shown) of these shortened DNAs and linearized vector DNA.

In these experiments, flasks containing $\approx 10^6$ HT1080 cells were transfected with a ligated mixture of 5 μg of linearized pZipNeoSV(X)1 vector and 5 μg of human DNA or, separately, 2 μg of linearized pZipNeoSV(X)1 vector mixed with 1 μg of mouse cDNA. Selection for adhesive cells during the transient period (24 h after DMSO shock) showed that 10–40 cells (human 8–17 kb DNA) or 90–120 cells (mouse non-cleaved cDNA) had become more adhesive compared with 0–5 cells in populations that had been transfected with other DNAs (Table 1). If any of the selected adhesive cells had divided during the 24-h period between transfection and selection, then the number of adhesive cells selected (Table 1) must reflect sibling as well as primary non-divided cells. However, as described below most of the adhesive cells selected were quiescent at 24 h after transfection. This suggests that few if any sibling cells were selected. In other experiments, selection at 8 h after transfection gave approximately the same frequency of occurrence of adhesive cells as was seen at 24 h (data not shown). Thus, it is unlikely that the transfection-driven increases in the number of adhesive cells seen were inflated by the presence of sibling cells.

Table 2. *Transfection dose response in HT1080 cells*

Transfected DNA (μg)	No. of adhesive cells selected per $\approx 10^6$ transfected HT1080 cells in different transfections
*Sau*3A-cleaved human lymphocyte DNA pieces (8–17 kb) ligated to linearized pZipNeoSV(X)1	
10	12, 21, 34
5	18, 25, 38
2·5	7, 16, 18
0	0, 1, 3
Non-cleaved mouse NIH/3T3 cDNA cotransfected with two parts by weight of linearized pZipNeoSV(X)1	
1	63, 112, 115
0·5	69, 83, 106
0·25	37, 51, 68
0	0, 3, 4

Reduction of the amount of transfected DNA, below about one half of the standard transfected mixture of pZipNeoSV(X)1 vector and human 8–17 kb DNA or below about one half of the standard mixture of mouse non-cleaved cDNA and linearized vector DNA, reduced the number of adhesive cells spared in the adhesion selection process; below these saturation levels, the DNA dose response was approximately linear (Table 2).

Greater than 95% of the twice adhesion-selected cells that had been transfected with (1) vector DNA ligated to or cotransfected with human 8–17 kb DNA or (2) vector DNA cotransfected with mouse cDNA became non-mitotic. The few cells continuing mitosis in these adhesion-selected populations may have resulted from inefficiency in discriminating against cells having less adhesion. Alternatively, cell cycle quiescence may not be coupled to increased adhesion in these few cells. Although we cannot rule out the latter case, it seems more likely that there is simply inefficiency in the selection process; when $\approx 10^6$ cells were transfected with non-mammalian DNAs or biologically inactive shorter pieces of mammalian DNAs, there was no post-transfection quiescent period in selected (Table 1) *or* non-selected cells (data not shown). Typically, fewer than six mitotically active cells were spared in selection; thus, this background level of mitotic cells was essentially the same in all transfections, including those utilizing the biologically active mammalian sequences (Table 1). The immediate use of conditioned media and replating at higher cell densities did not induce cell division in the selected transfection-specific quiescent cells. However, within 9–12 days after transfection, these cells again became mitotic and regained the less-adhesive phenotype of the original transformed HT1080 cell line. Stable HT1080 cell lines with increased adhesion have thus far not been isolated. Although pZipNeoSV(X)1 vector DNA

contains a Tn*5* element conferring G418 antibiotic resistance on cells expressing this vector, none of the cells regaining mitotic activity showed antibiotic resistance. This is consistent with low efficiency of chromosomal integration of DNA transfected by the DEAE–dextran method and loss of expression of transfected extrachromosomal DNA after a period of highly efficient transient transfection.

Discussion

It has been hypothesized that activation of oncogenes leads to tumorigenicity in mammalian cells (Land *et al.* 1983; see also Duesberg, 1985; Harris, 1986, for critical reviews). An alternative hypothesis is that deactivation of transformation-suppressor genes is required for tumorigenesis (reviewed by Klein & Klein, 1985). The presence of such suppressor genes in normal cells has been inferred on the basis of the findings that (1) cells having homozygous chromosomal deletions at specific loci occur in specific neoplastic diseases (Comings, 1973; Klein & Klein, 1985); and (2) both intraspecies and interspecies somatic cell hybrids, resulting from fusions of neoplastic mammalian cells with normal fibroblastic cells, often have a non-transformed phenotype (Jonasson & Harris, 1977; Stanbridge *et al.* 1982). In the latter case, comparison of hybrids having different combinations of normal cell chromosomes suggests that there may be specific suppressor genes on particular normal cell chromosomes (Jonasson *et al.* 1977; Evans *et al.* 1982; Stohler & Bouck, 1985). The suppression seen in interspecies hybrids also argues that there is evolutionary conservation of suppressor sequences and their targets.

In the experiments reported here we have seen an increase in cell adhesion and also mitotic quiescence within 24 h following transfection of mammalian DNA sequences ligated to or cotransfected with expression vector. These transient, coupled phenotypic changes occurred at a frequency of 10^{-4} to 10^{-5} per cell. We believe that the cellular effects seen were mediated by specific DNA sequences entering the transfected cells. The evidence for this conclusion is the finding that no phenotypic effect resulted if the transfected DNA (1) was non-mammalian, (2) was mammalian, but cleaved to 2–5 kb by digestion with restriction enzymes, (3) was mammalian cDNA, but cleaved with *Sau*3A, or (4) contained no expression vector DNA sequences (Table 1); in addition, the number of adhesive revertant cells in a population increased in proportion to the amount of transfected biologically active DNA (Table 2).

The phenotypic changes observed when the cells were transfected with mammalian DNA and expression vector could reflect sequence-induced complex cellular effects including expression of many different transfected mammalian sequences. In this respect, transient transfection by the DEAE–dextran/DMSO shock method used here may enable as much as 80% of a population of cells to receive DNA and, of these cells, each cell may carry (and transiently express as non-integrated DNA) as much as 5% of a transfected mammalian genome (Lopata *et al.* 1984; Berman *et al.* 1984; Sussman & Milman, 1984). It is therefore possible that phenotypic change required the presence of more than one type of transfected sequence per cell.

In other studies on suppression of fibrosarcoma cells, pseudodiploid HT1080 cells, whose transformed phenotype was suppressed by fusion with normal fibroblasts, became non-proliferating non-tumorigenic hybrids, whereas tetraploid HT1080 cells fused to normal fibroblasts remained transformed and mitotic (Benedict *et al.* 1984; Stanbridge *et al.* 1982).

We do not know the mechanism by which transfection induced the observed transient increase in cell adhesion and also quiescence of cell division. The requirement, that the transfected DNA be of mammalian origin and also attached to or cotransfected with the expression vector sequence suggests that specific DNA transcriptions were necessary for suppression of the transformed cell phenotype. Conceivably, antisense transcription of transfected mammalian sequences could block maturation or translation of cellular RNA and perhaps give rise to the suppression observed. Alternatively, translation of sense transcripts may produce proteins that act to cause suppression. In favour of suppression by translation of transcripts is the finding that neither 8–17 kb genomic DNA pieces nor intronless cDNA caused suppression if either had been cleaved by digestion with restriction enzymes prior to transfection (Table 1). It seems likely that antisense RNA would not be as length-sensitive as sense RNA since its biological activity does not depend upon translation of intact open reading frames.

We have observed a period of 9–12 days of suppression of particular phenotypic characteristics in transfected, adhesion-selected HT1080 cells. This period corresponds to the length of time that transient expression is normally seen when particular genes attached to expression vectors are transfected by the DEAE–dextran method into various types of mammalian cells (Lopata *et al.* 1984; Sussman & Milman, 1984). Although transfection can produce a high efficiency of DNA uptake per cell, stable expression through chromosomal integration may hardly occur at all (Sussman & Milman, 1984; Berman *et al.* 1984). Similarly, in our experiments the resumption at the end of this period of the less-adhesive, mitotic, transformed phenotype in the mammalian DNA-transfected HT1080 cells suggests that there has been a cellular loss or inactivation of transfected, non-integrated, DNA

sequences. This transient expression of phenotype, as well as the necessity of an expression vector and either non-cleaved mammalian cDNA or long (≥8–17 kb) mammalian genomic DNA, argues that the effects seen result from expression of a transfected specific gene(s). The resumption of transformed cell phenotypes at the end of the transient period suggests that transfected suppressor sequences were not integrated into the cellular chromosomes and therefore were not expressed after the transient period. Quiescence and increased adhesion may thus have resulted from a *trans* effect caused by expression of transfected mammalian suppressor genes present or active only during the transient phase of transfection.

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