J Oral Pathol Med (2006) 35: 146–54

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Analysis of apoptosis-associated genes and pathways in oral cancer cells

Sandra Gibson[1], Edward J. Shillitoe[2]

Departments of [1]Radiation Oncology and [2]Microbiology and Immunology, SUNY Upstate Medical University, Syracuse, NY, USA

BACKGROUND: Cancer cells can resist apoptosis that is induced by stimuli such as detachment or differentiation, but may be more susceptible to apoptosis when exposed to chemotherapeutic drugs. The pattern of gene expression that produces this phenotype is unknown.
METHODS: We compared gene expression patterns of normal human epidermal keratinocytes (NHEK cells) and the oral cancer cell line Tu183 when the cells were exposed to different apoptosis-inducing stimuli.
RESULTS: Pathway analysis revealed that the phenotype difference could be best explained by the simultaneous existence of both proapoptosis and antiapoptosis signals in the cancer cells. Microarray analysis, supported by immunoblotting, showed that one gene that was likely to be involved in the proapoptosis signal was *TNFRSF5*, which encodes the receptor CD40. When Tu183 cells were exposed to the CD40 ligand they showed apoptosis, while NHEK cells did not.
CONCLUSIONS: The effects of different apoptotic stimuli on normal cells and oral cancer cells can be explained by expression of proapoptosis genes, including the gene that encodes CD40.
J Oral Pathol Med (2006) **35**: 146–54

Keywords: apoptosis; *BCL2*; CD40; microarray; oral cancer

Introduction

An essential feature of cancer cells is a series of changes in the way that apoptosis is regulated. Normal epithelial cells have a limited lifespan in culture, and eventually undergo spontaneous differentiation and die by apoptosis (1). Prior to senescence, normal epithelial cells can be induced experimentally to differentiate and die; this also has features in common with apoptosis (2) and can be prevented by high levels of expression of the antiapoptosis gene *BCL2* (3). Malignant cells on the other hand, usually resist both spontaneous apoptosis (4) and differentiation-induced apoptosis; this may be associated with the fact that many tumor cells have an increased ratio of antiapoptotic to proapoptotic proteins (5). Another stimulus for apoptosis is detachment from a surface, which affects normal epithelial cells and is termed anoikis. It is regulated by the cell membrane, which can sense the matrix through the binding of specific integrins (6). When contact is lost the stress-activated protein kinase/Jun amino-terminal kinase pathway is activated which promotes apoptosis in a manner that again depends on expression of *BCL2* (7). Malignant cells are less likely to undergo apoptosis after detachment. This is probably due to changes in their use of surface receptors, although it may be associated with expression of proapoptosis and antiapoptosis proteins in a way that is only partially understood (8).

Yet another stimulus for apoptosis is exposure to some anticancer drugs. As cancer cells tend to resist apoptosis it would be expected that they would resist anticancer drugs. Paradoxically, the opposite is often true. For example, carcinoma cells are 10^3- to 10^4-fold more sensitive to methotrexate or fluorodeoxyuridine than are normal keratinocytes and this cannot be explained by their growth rate, or expression level of target proteins (9). The synthetic retinoid CD437 produces apoptosis in oral cancer cells, but causes only growth arrest in normal keratinocytes (10). Similarly, norcantharadin (11) and selenium compounds (12) induce apoptosis in oral cancer cells more than in normal oral cells. Although the unexpected susceptibility of cancer cells to apoptosis-inducing drugs has been discussed frequently, it still lacks a convincing explanation (13–15). One possibility is that it is a dysregulation of apoptosis-associated genes that causes such a change. Breast cancer cells appear to succumb to the apoptosis that is due to retinoids because they cannot raise their level of the protein Bcl-2 appropriately (16). Other cancer cells appear to modify the antiapoptotic protein Bcl-X_L in an inappropriate way (17). Nonetheless, this does not explain why the cancer cells would resist apoptosis that is due to other stimuli. We therefore undertook to compare directly, in

Correspondence: Edward Shillitoe, Department of Microbiology and Immunology, SUNY Upstate Medical University, 750 East Adams Street, Syracuse, NY 13210, USA. Tel: (315) 464-5453. Fax: (315) 464-4417. E-mail: shillite@upstate.edu
Accepted for publication October 13, 2005

a single study, the differences in patterns of gene expression in apoptosis that was induced by different stimuli in normal and in cancer cells.

Material and methods

Cells

Normal human epidermal keratinocyte (NHEK) cells were purchased from Cambrex BioScience (Walkersville, MD, USA) and were used within five passes. The oral cancer cell line Tu183 was obtained from Dr P. Sacks (M.D. Anderson Cancer Center, Houston).

Differentiation

To observe the effects of terminal differentiation the concentration of calcium in the culture medium was raised from the usual concentration of 0.2 mM (for NHEK cells) or 0.4 mM (for Tu183 cells) to 2.0 mM. Cells were harvested after 48 h, and parallel cultures were examined by phase-contrast light microscopy.

Detachment

To observe the effect of detachment from a surface, cells were transferred to 96-well plates that had been precoated with poly(2-hydroxyethyl methacrylate) (Sigma, St Louis, MO, USA). At various times cells were removed and assayed for apoptosis as described below, after 24 h.

Suppression of BCL2

To find the effect of reducing the expression of the gene *BCL2*, cells were exposed to the anti-*BCL2* ribozyme, Rz279. The gene for the ribozyme was cloned into an adenovirus vector adjacent to a cytomogalovirus (CMV) promoter, and the vector was used to introduce the ribozyme gene to oral cancer cells. The vector was the same as the vector Ad-MMTV-Rz279 that we described earlier (18) except that it uses the CMV promoter instead of the mouse mammary tumour virus (MMTV) promoter. For comparison, an adenovirus vector that expressed the *lac* z marker gene was used to confirm that the majority of cells had been transfected.

Paclitaxel

Paclitaxel was obtained from Sigma and added to cultured NHEK and Tu183 cells at a concentration of 10 μM. After 2 days apoptosis was assessed as above.

CD40 ligand

Recombinant soluble human CD40 ligand and a cross-linking antibody that potentiates its effects ('Enhancer') were obtained from Alexis Biochemicals (San Diego, CA, USA). These were combined for 30 min at 8 μg and 1 μg/ml respectively and 100 μl was added to wells of 96-well plates that had been seeded with 5000 cells. After 48 h the level of apoptosis was assessed as below.

Assessment of apoptosis

Cells were assayed for apoptosis using an antibody assay for detection of histone-associated DNA fragments. The cells were lysed and supernatants were transferred to a Cell Death Detection ELISA Plus plate (Roche Diagnostics, Indianapolis, IN, USA) where the level of apoptosis could be indicated by the optical absorbance ($A_{405\ nm}-A_{492\ nm}$). The positive control preparation always gave values over 1.5 and the background level was always <0.05. Assays were performed on at least two occasions with at least two replicates on each occasion and data were pooled.

Assessment of viability

To confirm that apoptotic cells had indeed lost viability, their ability to replicate was assessed by their ability to cleave the tetrazolium salt WST1[(4-[3–4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate] to its product, formazan as before (18). The conversion was evaluated as the A_{440}–A_{650} value and was assessed at two time points. The value at the second time point was then expressed as a percentage of the value at the earlier time. Assays were performed on at least two occasions with at least three replicates on each occasion, and data were pooled.

Pathway analysis

Conclusions from the above procedures were organized in the form of a truth table that was then interpreted by numerically simulated network analysis using a quantitative matrix (19). The analysis was performed by a custom-written computer program that simulated the result of allowing any item in the matrix to influence any other item. Resulting pathways were then imaged as pathway diagrams with items shown as nodes and influences shown as edges.

Microarray analysis of gene expression

To find the likely identity of genes that contribute to each pathway the Affymetrix proprietary microarray system was used according to the manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). Cells were grown and then either detached or exposed to calcium, ribozyme or paclitaxel as above. At the conclusion of the exposure period the cells were washed and scraped into lysis buffer. Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was precipitated with ethanol and re-extracted using the RNeasy Total RNA Isolation kit (Qiagen, Valencia, CA, USA).

The RNA was then used as a target in microarray analysis. The RNA from 5 μg of total RNA was processed by first synthesizing double-stranded cDNA followed by an *in vivo* transcription reaction and a fragmentation reaction. A hybridization mixture containing the cDNA, probe array control (Affymetrix), and herring sperm DNA was prepared and hybridized to the probe array at 45°C for 16 h. The hybridized array was then washed and bound biotin-labeled cRNA was detected with a streptavidin-phycoerythrin conjugate. Subsequent signal amplification was performed with a biotinylated antistreptavidin antibody. Each probe array was scanned twice (Hewlett-Packard GeneArray Scanner, Palo Alto, CA, USA), the images were overlaid and the average intensities of each probe cell

were compiled. Each cell type and experimental condition was tested three times.

Data analysis was performed with the AFFYMETRIX MICROARRAY SUITE version 5 software. To find the apoptosis-related genes that are differentially expressed between any two cell types or condition the lists of genes that were called as present were compared using the software default settings. A gene was recorded as showing different expression between any two samples if the $\log_2$ change was calculated as >1.0 or $-<1.0$ with a significant value of >0.99 or <0.01. Further analysis was then limited to genes that have previously been shown to be associated with apoptosis in human cells as defined in the GENECARDS database and its links to other databases (20). This was followed by manual editing to remove genes whose products are known only to be substrates for the apoptotic process. The final list comprises 260 apoptosis-associated genes.

To assess the reproducibility of the technique, the expressed genes of three independent cultures of Tu183 cells were compared with each other, in a total of three comparisons (A vs. B, A vs. C, B vs. C) and the same was carried out for three independent cultures of NHEK cells. Because each experimental sample was tested three times it was possible to make nine comparisons between experimental samples (E) and untreated samples (U) in each experiment (E1 vs. U1, E1 vs. U2, E1 vs. U3, E2 vs. U1, etc.).

Confirmation of gene expression by immunoblotting

To find if the changes in RNA expression that were reported by microarray analysis were reflected in changes in expression of proteins, 15 proteins were selected from the lists of differentially expressed genes on the basis of the commercial availability of antisera. The level of expression of each protein in cells was examined by immunoblotting of cell extracts after cells had been exposed to the same proapoptotic stimuli. Cells were grown and treated in 25 cm^2 culture flasks. Tu183 and NHEK cells that were detached were incubated for 14 h. Those exposed to either low or high calcium medium were incubated in the specified medium for a total of 42 h. Those treated with either paclitaxel or Rz279 were exposed to the treatment for 48 h prior to protein extraction. Protein was extracted with Cytobuster protein extraction reagent (Novagen, Madison, WI, USA) with an added cocktail of protease inhibitors. The total protein concentration was determined by the CB-X protein assay (Geno Technologies, Inc., St Louis, MO, USA). Proteins were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels loaded with 10–20 μg protein per lane, transferred to polyvinyliden virus (PVDF) membranes, and hybridized with specific antibodies or antisera. Detection was by an ECF Western Blotting Kit (Amersham Biosciences, Piscataway, NJ, USA). Stained blots were scanned on a Storm Phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA). Blots were re-stained with an anti-α actin antibody (Sigma Chemical Company) to control for equivalent loading. Bands were evaluated using IMAGEQUANT software (Molecular Dynamics).

The activity of each antibody was confirmed by inclusion of a positive control specimen that consisted of either a recombinant protein or an extract of a cell that is known to express that protein. Each immunoblot was performed at least twice.

Statistical analysis

Data were compared with a two-tailed unpaired *t*-test. Data were analyzed with assistance of the computer program GRAPHPAD PRISM, v4.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

Effects of differentiation

When the level of calcium in the medium was raised, apoptosis was induced in NHEK cells but not Tu183 cells (Fig. 1a). The NHEK cells developed an appearance consistent with epithelial differentiation (not shown).

Effects of detachment

When NHEK cells were detached from a surface they showed apoptosis while Tu183 cells were not affected (Fig. 1b).

Effects of Rz279

The ribozyme did reduce the level of the Bcl-2 protein (not shown) and this was accompanied by apoptosis of Tu183 cells. However, apoptosis was not seen in NHEK cells under these conditions (Fig. 1c).

Effects of paclitaxel

NHEK cells were resistant to paclitaxel at the concentrations that were tested, with apoptosis being undetectable. Tu183 cells showed a high level of apoptosis with 10 μM of the drug (Fig. 1d).

Pathway analysis

A truth table was constructed to represent the above data (Fig. 2a). As the results of differentiation and detachment were the same, they were considered as a single item in the table. Similarly the results of paclitaxel and the ribozyme were the same and they were combined into a single item. The difference between NHEK and Tu183 cells was also considered as being a single item, and was described as a 'cancer phenotype'. Numerical pathway simulation failed to recreate the truth table when each item influenced only the apoptosis item or the proapoptosis or antiapoptosis items directly. However, when each item was also permitted to facilitate or block the interaction between any other two items a number of possible pathways was found. The most simple is illustrated in Fig. 2b. All of the predicted pathways had one feature in common; the cancer phenotype stimulated both the proapoptosis and antiapoptosis signals.

Microarray analysis of gene expression

When the expression levels of genes in three independent NHEK cell cultures were compared with each other a

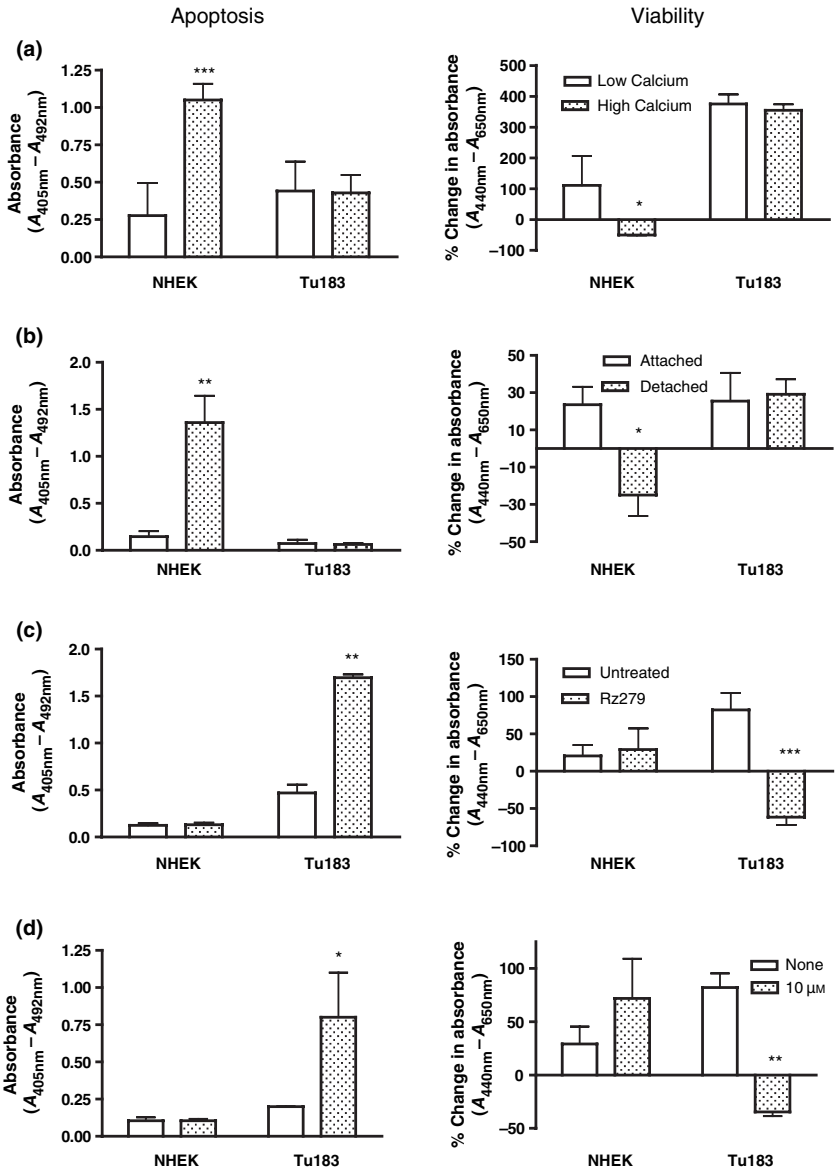

Figure 1 (a) Effect of the differentiation-inducing agent calcium on apoptosis in normal human epidermal keratinocyte (NHEK) and Tu183 cells. Cells were passed to 96-well plates at 10 000 cells per well and allowed to attach for 8 h. Medium was then replaced with either low calcium (0.2 mM for NHEK and 0.4 mM for Tu183 cells), or high calcium (2.0 mM) to induce differentiation. (Left) After 48 h cells were harvested for assessment of apoptosis by an immunoassay for detection of histone-bound protein. (Right) The viability of the cells was assayed by comparison of levels between day 1 and day 3 by a WSTI assay. Columns indicate the mean values of two to three replicates of two to three experiments and error bars indicate the standard error of the mean. Asterisks indicate the value of P when data from one column was compared with the one immediately to the left using a two-tailed t-test for unpaired samples (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). (b) Effect of detachment on apoptosis in NHEK and Tu183 cells. Cells were passed to 96-well plates, which had been pre-coated with methacrylate in 95% ethanol. (Left) Cells were harvested by centrifugation after 24 h and assayed for apoptosis as described in (a). (Right) The viability of the cells was assayed by comparison of levels between 2 and 14 h. (c) The effect of Rz279 on apoptosis in NHEK and Tu183 cells. Cells were transfected with the ribozyme-expressing vector or left unexposed as a control. (Left) The level of apoptosis was assayed after 48 h. (Right) The viability of the cells was assayed by comparison of levels between day 1 and day 3. (d) The effect of paclitaxel on apoptosis in NHEK and Tu183 cells. Cells were exposed to paclitaxel or left unexposed. (Left) The level of apoptosis was assayed after 48 h. (Right) The viability of the cells was assayed by comparison of levels between day 1 and day 3.

total of 11 apoptosis-related genes were reported as being differentially expressed in 2/3 cultures. A further 38 genes were reported as differentially expressed in only 1/3 comparisons between the three cultures. When three independent cultures of Tu183 cells were compared with each other, one apoptosis-related gene was reported as being differentially expressed in 2/3 cultures and a further 19 genes were expressed differently in 1/3 cultures. From this it was concluded that the spontaneous variation rate between cell cultures was unlikely to result in false reports if a gene was reported to be expressed differently only if found so in more than 2/3 comparisons. The expression differences in experiments were therefore designated as 'consistent' if they appeared

(a)

Cancer phenotype	Differentiation / detachment	Paclitaxel	Rz279	Apoptosis
F	F	F	F	**F**
F	T	F	F	**T**
F	F	T	F	**F**
F	F	F	T	**F**
T	F	F	F	**F**
T	T	F	F	**F**
T	F	T	F	**T**
T	F	F	T	**T**

(b)

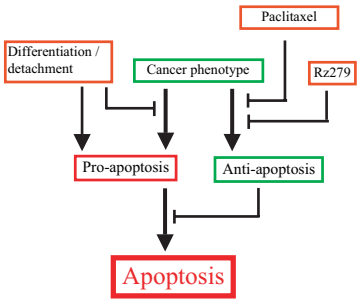

Figure 2 (a) Summary of experimental data, with the eight phenotypic states that were observed represented in the form of a truth table. The first four columns represent the status of the cell and the rightmost column represents the corresponding result (F, false; T, true). (b) The most simple model of phenotype interactions that is consistent with the truth table. The model requires six signals as shown.

in 8/9 or 9/9 comparisons and as 'inconsistent' if they appeared in 1/9 to 7/9 comparisons.

When levels of expression of apoptosis-associated genes were compared between untreated NHEK and Tu183 cells, 40 genes were found to be differentially expressed consistently. These are listed in Table 1A along with an indication of whether they were recorded in the database as being proapoptosis, antiapoptosis, or uncertain.

Increasing the concentration of calcium led to consistent changes in the expression of three genes in NHEK cells and two genes in Tu183 cells (Table 1B), while detachment from a surface caused consistent changes in expression level of 10 genes in NHEK cells and four genes of Tu183 cells (Table 1B).

Exposure to paclitaxel or Rz279 produced no consistent changes in gene expression of NHEK cells, although in Tu183 cells paclitaxel caused consistent changes in expression of two genes and Rz279 affected expression of two genes consistently (Table 1B). In addition to these changes that were considered 'consistent', many other changes in gene expression were observed 'inconsistently' (Table 2).

Confirmation of gene expression by immunoblotting

When the protein products of eight genes that were differentially expressed between NHEK and Tu183 cells were examined by immunoblotting, each of the eight was confirmed as being differentially expressed (Fig. 3). For all analyses, genes that were changed consistently were confirmed by immunoblotting in a total of 14 of 17 cases (82%) while for genes whose changes had been designated as 'inconsistent', differences in expression were confirmed in 21 of 39 cases (54%, Table 2).

Effects of CD40 ligand

The CD40 ligand did not induce apoptosis in NHEK cells. It did increase the level of apoptosis in Tu183 cells but the increase was smaller than that seen with the other apoptosis-inducing agents and did not reach a high level of statistical significance ($t = 2.01$, $P = 0.07$, d.f. $= 10$). The effect was, however, sufficient to reduce the growth of the treated Tu183 cells at a highly significant level (Fig. 4).

Discussion

This study yielded a proposed pathway that provides an overview of the various signals that control apoptosis in oral cancer cells when compared with normal epithelial cells, along with listings of the genes whose expression is likely to constitute such pathways. Although experiments were based on comparison of only one normal and one oral cancer cell line it is evident that if they displayed the appropriate differences in apoptotic responses, then they could be used to discover the genotypic differences that were responsible for the phenotypic differences.

The experiments of Fig. 1 confirmed that the prototypical differences in induction of apoptosis between normal and cancer cells were indeed demonstrated by comparison of NHEK with Tu183 cells. The NHEK cells showed greater susceptibility to apoptosis following detachment or differentiation, while Tu183 cells showed greater susceptibility to apoptosis following exposure to the drug paclitaxel or to the ribozyme Rz279. These observations are typical of comparisons between normal and cancer cells (8, 14, 21) and therefore justified further analysis of the mechanisms involved.

The method of pathway analysis that was chosen uses a straightforward numerical matrix (19, 22). The cancer phenotype was represented as only one node of the matrix. Similarly, detachment and differentiation were considered as being a single node because their effects were similar in the experiments that were performed. We also considered the effects of paclitaxel and of the anti-*BCL2* ribozyme as consisting of one node because they also produced similar experimental results. The assumption was adopted that the effect of these nodes was to influence the balance between two further nodes downstream, named 'proapoptosis' and 'antiapoptosis', which is a well-established concept (23). In the future all of these nodes can be subdivided into their component parts.

The most simple solution of the pathways that was found is depicted in Fig. 2b. Unexpectedly, the cancer phenotype was predicted to stimulate both proapoptosis and antiapoptosis signals. Overexpression of proapoptotic proteins has been reported in cancer cells before, such as Myc (24), Abl (25), caspases (26), and death receptors (27). However, beyond the suggestions that such features might be exploited for cancer therapy,

Table 1. Apoptosis-associated genes that were expressed at different levels in Tu183 cells than in normal human epidermal keratinocyte (NHEK) cells. (A) Genes whose expression was constitutively different between Tu183 and NHEK cells. (B) Genes whose expression was changed by calcium, detachment, paclitaxel or Rz279 in NHEK or Tu183 cells

(A) Constitutive expression in Tu183 cells

API5	↑	***LGALS1***	↑
ASC	↓	**MAP3K5**	↑
AXL	↑	*MCL1*	↓
BAG3	↓	**MYLK**	↑
BAG5	↓	*NME5*	↑
BCL10	↓	***OAZ2***	↑
BCL2	↑	**PDCD10**	↑
CASP1	↓	**PDCD4**	↓
CASP2	↑	**PIG8**	↓
CASP3	↓	**PIGPC1**	↓
CASP4	↓	**PLAGL1**	↑
CAV2	↓	***PLSCR3***	↓
CDK2	↑	**REQ**	↑
CDKN1A	↓	*TEGT*	↓
CLU	↑	**TNFRSF10B**	↓
ETS2	↓	**TNFRSF5**	↑
FXR1	↑	**TNFRSF6**	↓
GADD45B	↑	***TRPS1***	↑
HTATIP2	↓	*YWHAE*	↓
IER3	↓	*YWHAH*	↑

(B) Induced expression changes in:

	NHEK cells		Tu183 cells	
calcium	*BIRC3*	↑	*NRP1*	↓
	MMP9	↑	**PDCD4**	↓
	OAS1	↑		
detachment	*BIRC5*	↓	**GADD45B**	↑
	CIDEA	↑	**MYC**	↓
	MYC	↓	**MYLK**	↑
	NALP2	↑	*NRP1*	↓
	NFKBIA	↑		
	NOTCH3	↑		
	OAS1	↑		
	PDCD5	↓		
	TNFSF10	↑		
	TNFRSF10B	↓		
paclitaxel	None		**CDKN1A**	↑
			MDM2	↑
Rz279	None		*BIRC5*	↓
			CDKN1A	↑

The Affymetrix U133A Chip was used and the Affymetrix MAS-5 software package was used to identify genes whose expression was changed at a level of over twofold and with $P > 0.99$ or $P < 0.01$. The list of genes was then refined by eliminating those that have not previously been shown to participate in apoptosis of any human cell, as recorded in the GENECARDS database and its associated links (20). Each of the two cell types was examined on three occasions, thus allowing nine comparisons between them. Only genes that were identified as changes in 8/9 or 9/9 comparisons are shown. Genes that are generally classified as promoting apoptosis are shown in bold (e.g. **ASC**) and those that oppose apoptosis are shown in italics (e.g. *AP15*). Those that are undefined are shown in bold italics (e.g. ***CAV2***). Arrows indicate the difference in expression of each gene, i.e. a gene indicated with an arrow pointing up was found to be expressed at a higher level in Tu183 cells. Genes are identified by their UniGene symbol.

their significance appears to have remained unexplored. The present findings suggest that the proapoptosis signal is an integral part of the malignant process, and explains part of the cancer phenotype.

The effect of differentiation and detachment were predicted to have two simultaneous effects: stimulation of the proapoptosis signal, and the inhibition of the proapoptotic signal that is derived from the cancer phenotype. In cancer cells the effects of Rz279 and paclitaxel were predicted by the model to act by inhibition of the antiapoptosis signal of the cancer phenotype. It is already known that the effect of the ribozyme is to inhibit the expression of *BCL2*, which is an antiapoptosis protein whose expression is higher in the cancer cells. Thus, in this one path that can be compared with existing data, the pathway analysis produced the correct assignment. The prediction that paclitaxel also operates through suppression of an effect of the cancer phenotype is also consistent with expectations; if paclitaxel acted directly on the proapoptosis phenotype then it would also induce apoptosis in non-cancer cells at the dose used.

To find which genes could be assigned to each of the signals we performed microarray analysis of gene expression in NHEK and Tu183 cells which were either untreated, or were exposed to the different situations that induced apoptosis. In order to focus the analysis on apoptosis the analysis was restricted to 260 apoptosis-associated genes. To avoid the poor reproducibility that characterizes microarray studies (28, 29) genes were accepted for consideration only if their differential expression met very stringent criteria. These included that the observation of its change should be detected at least eight times of nine comparisons. When immunoblotting was used to examine expression of eight genes that were found by these criteria to be differentially expressed between NHEK and Tu183 cells their different expression was confirmed in every case. When the reproducibility criteria were relaxed so as to accept inclusion of genes with less reliable detection, the rate of confirmation by immunoblotting also fell (Table 2). These data imply that highly stringent criteria are necessary for this type of microarray analysis, and when they are applied confidence may be placed in the gene lists that are generated. In apoptosis-induced changes in gene expression the level of confirmation by immunoblotting was rather less, although it is possible that differences in gene expression might not always be expected to conform changes in protein levels.

The apoptosis-related genes that were found to be differentially expressed between NHEK and Tu183 cells are listed in Table 1. These represent a complex list of 40 genes of different functions. The upregulation and downregulation of so many genes could be the result of many independent mutations in the cancer cell which together cause dysregulation of apoptosis, or possibly there could be a very small number of genes that are mutated, with the other changes in expression happening as a survival response.

The genes of Table 1 that are typically described as antiapoptosis and which were upregulated in Tu183 cells included *BCL2*, whose overexpression in some oral cancer cells is already known (18) but the other antiapoptosis genes that were upregulated (*AP15*,

Table 2 Confirmation of changes in expression of selected apoptosis-related genes by immunoblotting

	Consistently changed genes[a]		*Inconsistently changed genes*[b]	
Comparison between	*Indicated by microarrays*[c]	*Confirmed by immunoblots*[d]	*Indicated by microarrays*	*Confirmed by immunoblots*[e]
NHEK and Tu183	40	8/8[e]	169	0/1
NHEK and NHEK + calcium	3	1/1	29	2/3
NHEK and NHEK, detached	10	1/2	68	3/6
NHEK and NHEK + paclitaxel	0	–	52	2/4
NHEK and NHEK + Rz	0	–	57	2/7
Tu183 and Tu183 + calcium	2	–	27	5/6
Tu183 and Tu183, detached	4	2/2	20	1/2
Tu183 and Tu183 + paclitaxel	2	1/2	19	3/5
Tu183 and Tu183 + Rz	2	1/2	29	3/5
Total		14/17 (82%)		21/39 (54%)

[a]Apoptosis-associated genes that were expressed differently in 8/9 or 9/9 comparisons by microarray analysis.
[b]Apoptosis-associated genes that were expressed differently in some comparisons, but $<8/9$ comparisons by microarray analysis.
[c]The names of these genes are in Table 1.
[d]The number of genes that were selected for further analysis by immunoblotting/the number of such genes whose protein product was thus confirmed to be changed.
[e]The immunoblots are illustrated in Fig. 3.
NHEK, normal human epidermal keratinocyte.

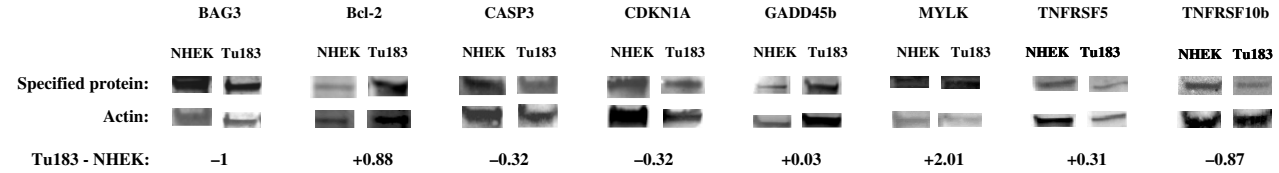

Figure 3 Western blots to confirm changes in protein expression that were suggested by microarray analysis. Immunoblots were prepared and imaged by fluorescent scanning. For each cell type the blot was imaged and the optical volume for the specific protein band was normalized by division by the volume of the corresponding actin band. The result for the normal human epidermal keratinocyte (NHEK) cells was subtracted from the result for Tu183 cells and the difference is shown in the bottom line of the figure.

AXL, *NME5*, *YWHAH*) do not appear to have been shown to be important in oral cancer previously.

The genes of Table 1 that are typically described as proapoptosis and were upregulated in Tu183 cells consisted of a caspase (*CASP2*), a cycle regulator (*CDK2*), a stress response gene (*GADD45B*), two signaling factors (*MAP35K*, *MYLK*), two transcription factors (*PLAGL1*, *REQ*), a gene of dubious function (*PDCD10*), and one member of the tumor necrosis factor receptor superfamily (*TNFRSF5*). We considered that the gene most likely to be of central importance in the putative proapoptosis phenotype would be *TNFRSF5*, which encodes CD40. CD40 is reported to be overexpressed in some carcinomas such as skin cancer (30). Ligation of CD40 usually causes apoptosis in carcinoma cells (31, 32), although it increases survival of B lymphocytes (33). One group has reported expression of CD40 in oral cancers with reduction in cell growth after ligation in some cell lines (34, 35).

Differentiation is predicted by the pathway of Fig. 2b to be accompanied by an increase in a proapoptosis signal in NHEK cells, and by an inhibition of a proapoptosis signal in Tu183 cells. In NHEK cells the microarray analysis did not show increased expression of any recognized proapoptosis gene in response to calcium, although in Tu183 cells there was a reduction of expression of one proapoptosis gene, *PDCD4*. Either this gene therefore contributes to the lack of effect of calcium in Tu183 cells, or the resistance of Tu183 cells to calcium-induced apoptosis is explained by the constitutive levels of apoptosis-associated genes in these cells.

Interestingly, although detachment of cells from a surface was predicted by pathway analysis to operate similarly to the effects of differentiation, the microarray analysis yielded quite different sets of genes. In NHEK cells four proapoptosis genes showed an increase in expression as did two antiapoptosis genes. Three proapoptosis genes showed a decrease in expression as did one antiapoptosis gene. Previous reports have shown that detachment of cells can activate several signaling events, but these do not seem to depend on changes in levels of gene expression (8). The present data suggest that in fact the onset of apoptosis following detachment of normal cells does involve significant changes in gene expression. In agreement with this, detachment of Tu183 cells that resisted apoptosis produced changes in only four genes. Thus, the role of changes in gene expression following detachment of normal and cancer cells is important and deserves further attention.

Exposure of cells to paclitaxel was predicted by the model of Fig. 2b to have no effect on gene expression patterns of NHEK cells and, in agreement with this, the

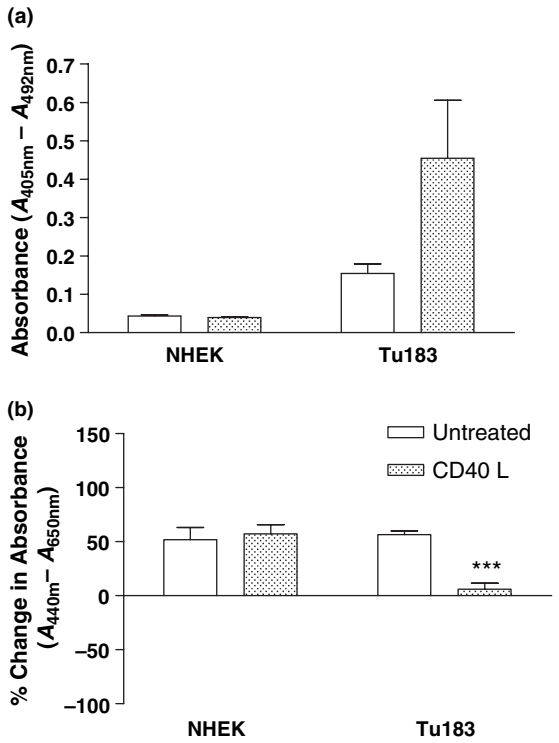

Figure 4 The effect of CD40 ligand on apoptosis in normal human epidermal keratinocyte (NHEK) and Tu183 cells. Cells were plated as described in Fig. 1, and then the medium was replaced with medium that contained recombinant soluble CD40 ligand at 8 μg/ml with enhancer at 1 μg/ml. (a) The level of apoptosis was assayed after 48 h. (b) The viability of the cells was assayed by comparison of levels between day 1 and day 3, as described in Fig. 1.

microarray analysis found no changes in expression of apoptosis-associated genes. In Tu183 cells the model predicted inhibition of the antiapoptosis phenotype, yet we found no decline in expression of any antiapoptosis gene. This is in contrast to data from laboratories that have reported that paclitaxel can induce a decrease in levels of P53 in other cancer cell types (36, 37). Instead, we found changes in only two apoptosis-associated genes, one of which was *CDKN1A*, encoding p21, and this could have been a result of the apoptotic process. The apoptosis of Tu183 cells after exposure to paclitaxel might therefore have been due to the pre-existing proapoptosis signal from the cancer phenotype in those cells.

The ribozyme 279 produced no changes in expression of apoptosis-associated genes in NHEK cells, and changes in only two genes of Tu183 cells (Table 1B). One of these was *CDKN1A* whose expression is known to be suppressed by the protein Bcl-2 (38) and which therefore might be expected to increase when expression of Bcl-2 is inhibited. The induction of apoptosis following reduction of the level of Bcl-2 might also depend on the pre-existing proapoptosis signal from the cancer phenotype in those cells. The lack of decline in detectable levels of the *BCL2* gene transcript is consistent with the earlier study that attributed the effect of the ribozyme to an antisense function rather than an enzymatic digestion of the transcript (18).

An important feature that emerged as part of a proposed proapoptosis pathway in the oral cancer cells, was expression of gene *TNFRSF5* which encodes CD40. A role for CD40 was supported by observation of increased expression of the protein by immunoblotting, and further supported by functional studies in which the CD40 ligand induced apoptosis in Tu183 but not NHEK cells. CD40 is overexpressed in cancer cells such as those from tumors of the breast (39), ovary (40), cervix (41), and lung (42). As a result, the ligand can cause apoptosis in some carcinoma cells (32) and can reduce the growth of some oral cancer cells (34, 35). The present data suggest that CD40 might play an important role in regulation of the cancer phenotype in oral cancer, including the response to anticancer drugs.

In conclusion, pathway analysis has proposed a broad scheme of signals that might be active in controlling the management of cell death in oral cancer cells, both spontaneously and in response to several stimuli. Analysis of gene expression can be expected to assign the genes that are responsible for each pathway. The expression of proapoptosis genes might explain some of the phenotypic changes of cancer, as well as assisting targeted therapy.

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Acknowledgments

The authors thank Dr Frank Middleton and Karen Gentile for assistance with the microarray analysis. This study was supported by the Department of Radiation Oncology and by PHS (grant R01DE13214).