Oncogenic transformation is a complex, multistep process, which goes through several stages before complete malignant transformation occurs. To identify early processes in carcinogenesis, we used an in vitro model, based on the initiating event in cervical cancer, papillomavirus transformation of keratinocytes. We compared gene expression in primary keratinocytes (K) and papillomavirus-transformed keratinocytes from early (E) and late (L) passages and from benzo[a]pyrene-treated L cells (BP). The transformed cells exhibit similar transcriptional changes to clinical cervical carcinoma. The number of transcripts expressed progressively decreased during the evolution from K to BP cells. Bioinformatic analysis, validated by detailed biochemical analysis, revealed substantial contraction of both pro- and antiapoptotic networks during transformation. Nonetheless, L and BP cells were not resistant to apoptotic stimuli. At doses of cisplatin that led to 30–60% apoptosis of K and E cells, transformed L and BP cells underwent 80% necrotic cell death, which became the default response to genotoxic stress. Moreover, appreciable necrotic fractions were observed in the cervical carcinoma cell line, HeLa, in response to comparable doses of cisplatin. The shrinkage of biochemical networks, including the apoptotic network, may allow a cancer cell to economize on energy usage to facilitate enhanced proliferation but leaves it vulnerable to stress. This study supports the hypothesis that the process of cancer transformation may be accompanied by a shift from apoptosis to necrosis.

Cancer is an evolving, complex process, which goes through several stages before full malignancy. In vivo, cell immortalization is followed by the development of benign lesions, which later progress into malignant tumors, finally metastasizing to other tissues. As it evolves, a cancer cell relinquishes pathways that interfere with proliferation and escapes from some of the restrictions of multicellular organisms. This process enables the cancer cell to proliferate in a broad range of naturally occurring microenvironments but may leave it vulnerable to rare or unexpected perturbations (e.g. genotoxic stress). The reactions of cancer cells, which acquire numerous molecular changes, may differ significantly from the reactions of normal cells to genotoxic stress.

Apoptosis is tightly controlled by the ability of the cell to integrate many pro- and antiapoptotic signals. Thus, the decision to live or to die in response to death signals is a choice the cell makes in the face of its cellular context. Molecular changes acquired by cancer cells may influence the connectivity of the signaling pathways and disturb the apoptotic network. As a result, the cancer cell may die by alternative death modes in response to genotoxic drugs.

In order to follow the changes that occur during the evolution of cancer, we use a model in which one can follow the progression from the normal phenotype all the way to the transformed phenotype, based on the natural evolution of cervical cancer. Infection with high risk human papillomavirus (HPV) causes immortalization of cells rather than malignancy. High risk HPV infections progress to cervical cancer in only a small percentage of infected women, after a long latency period (2). Risk factors such as smoking are associated with the progression of the infected cells to the tumor stage (3). Our model system consists of cultures of primary keratinocytes (K) and of HPV16-transformed keratinocytes (HF1 cells) from early (E) and late passage (L) (4). In an attempt to simulate the natural causes of transformation, we treated L cells with benzo[a]pyrene (BP), a carcinogen present in cigarette smoke. L and BP cells display enhanced growth rate (5), increased ploidy, and chromosomal translocations (data not shown), whereas BP cells also generate colonies in soft agar (Fig. S1). Furthermore, L and BP cells show a striking convergence of gene expression with published data from cervical carcinomas (6, 7), attesting to the biological relevance of this system to the development of human cervical cancer.

To gain a broad view of the molecular changes that occur during keratinocyte transformation, we compared K, E, L, and BP cell gene expression using microarray analysis. We found that the number of expressed transcripts progressively decreased during the evolution from K to BP cells, implying a contraction in the number of active cellular pathways. Among the biological categories that were significantly contracted during the progression from K to BP cells, we identified a large

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5 and Tables S1–S5.
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3 The abbreviations used are: HPV, human papillomavirus; CDDP, cisplatin; HF1 cells, HPV16-transformed keratinocytes; E cells, HF1 cells from early passage; L, HF1 cells from late passage; BP cells, HF1 cells from late passage treated with benzo[a]pyrene; K cells, primary keratinocytes; FACS, fluorescence-activated cell sorting; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; P38K, phosphatidylinositol 3-kinase; PKB, protein kinase B; FFL, firefly luciferase.
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group of pro- and antiapoptotic transcripts. Further bioinformatic and biochemical analysis of the extended apoptotic network, based on the recognition of hub proteins in each pathway, confirmed a substantial contraction of the pro- and antiapoptotic machinery. Nonetheless, L and BP cells were not resistant to apoptotic stimuli. On the contrary, L and BP cells displayed enhanced sensitivity to stress but showed a propensity to necrotic death, due to the absence of pro- and antiapoptotic avenues. Moreover, significant necrotic fractions were observed in the cervical cancer cell line HeLa in response to similar cisplatin (CDDP) concentrations. This study supports the hypothesis (8) that the process of cancer transformation is accompanied by a shift from apoptosis to necrosis.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents were purchased as follows: Dulbecco’s modified Eagle’s medium, Ham/F-12, and antibiotics from Biological Industries Israel Beit Ha’emek Ltd.; fetal bovine serum from Invitrogen; epidermal growth factor from Peprotech Inc.; insulin, T3, transferrin, cholera toxin, and hydrocortisone from Sigma; and TGFβ1 from R&D Systems. Inhibitors were purchased as follows: QNZ and U0126 from Calbiochem, and LY294002 from A.G. Scientific.

Cell Culture—Primary keratinocytes and HPV16-immortalized cells were maintained in keratinocyte growth medium, as described previously (5). Primary K cells were cultured from small biopsy specimens. The HPV16-immortalized keratinocytes had been transfected with a plasmid carrying the genome of the human papillomavirus, HPV16 (4). Early passage (passage 20; E) represents cells that underwent about 50 doublings after transfection. Late passage (passage 270; L) represents cells that underwent about 1000 doublings. BP cells were derived from L cells, by benzo[a]pyrene treatment, as follows. L cells were treated with the carcinogen benzo[a]pyrene (5 μM) for 30 days to establish a clone able to form colonies in soft agar. The established clone was suspended in soft agar for 2 weeks, and selected colonies were grown and then further cultured in soft agar for additional selection (BP cells). HeLa cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 100,000 units/liter penicillin, 100 μg/liter streptomycin.

Western Blot Analysis—Primary keratinocytes and transformed cells were plated in 6-well plates at the following concentrations: K (5 × 10^5 cells/well), E (1.2 × 10^5 cells/well), L (10^5 cells/well), BP (10^5 cells/well), and HeLa (0.8 × 10^5 cells/well). Where indicated, medium was replaced 72 h later with medium containing CDDP (25 μM). For inhibition of the apoptotic regulatory pathways, medium was replaced 72 h after initial plating with medium containing QNZ (30 nM), LY294002 (20 μM), and U0126 (10 μM). 1.5 h later, CDDP was added to a final concentration of 25 μM, and cells were further incubated for the indicated length of time. Cells were then washed and lysed, as described previously (5). For detection of HMGB1 release, medium from treated and untreated cells was collected and concentrated 40-fold using Amicon Ultrafree-MC filters. Sample buffer (5) was added to the medium, such that the medium was effectively concentrated 10-fold.

Western analysis was performed as described previously (5). The following antibodies were used: anti-Erk2, Smad4, and ICAD from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-phospho-Erk (Thr183, Tyr185) from Sigma; HMGB1 from BD Biosciences; caspase-3, cleaved caspase-3, caspase-6, caspase-9, and pGSK3 α/β from Cell Signaling Technology. Unless stated otherwise, each experiment was performed at least three times.

Flow Cytometric Analysis Using Annexin V and PI—K (5 × 10^5 cells/well), E (2.2 × 10^5 cells/well), L, BP (2 × 10^5 cells/well), and HeLa (10^5 cells/well) were seeded in 60-mm wells. 48 h later, the medium was replaced with medium containing CDDP, as indicated, and the cells were incubated at 37 °C. FACS analysis was performed on a FACScan flow cytometer (BD Biosciences) using the Vybrant apoptosis kit (Molecular Probes). Cells that were stained neither with annexin V nor with PI (annexin V−/PI−) were regarded as healthy. Cells that were stained with annexin V but not with PI (annexin V+/PI−) were considered to be in early apoptosis, and cells that were stained with both (annexin V+/PI+) were considered to be necrotic or late apoptotic. Cells that were stained with PI alone were necrotic. At least 10,000 cells for each sample were analyzed in each experiment. K and HF1 cells were analyzed several times in each of two variations. Samples were treated with CDDP at a fixed concentration (25 μM) and removed at various time points (0, 8, 12, 18, and 24 h); alternately, samples were treated with CDDP at various concentrations (0, 25, and 50 μM) for a fixed period of 24 h. HeLa cells were treated with CDDP at a fixed concentration (25 μM) and analyzed after 24 h.

Preparation of Labeled cRNA and Hybridization to Gene Chips—Three independent cultures of K and HF1 cells were grown. RNA was isolated using RNase kits (Qiagen) according to the manufacturer’s instructions. Approximately 10 μg of total RNA were reverse transcribed, amplified, and labeled as described. 8 μg of labeled cRNA were hybridized to Affymetrix Human Genome U133A microarrays, according to the manufacturer’s protocol.

Data Processing and Analysis—The gene expression data were analyzed using the Microarray Suite version 5.0 algorithm (Affymetrix). For each probe, a CHP output file contained a signal quantitative metric, which represents the relative level of expression of a transcript and a “detection” classification of each signal as present, marginal, or absent. To compare data from different arrays, the signal of each array was scaled to the same target intensity value. Further gene analysis was performed as described in the supplemental material.

Cell Transfections—K (5 × 10^5 cells/well), E (1 × 10^5 cells/well), L, and BP cells (8 × 10^5 cells/well) were seeded onto 6-well plates (Falcon). Cells were transfected 48 h later with 1 μg of DNA/well, as described previously (5). Cells were lysed after 36 h.

Reporter Assays—Cells were transfected with the appropriate reporter plasmids. In order to correct for transfection efficiencies, cells were co-transfected with pCMV-RENilla. After incubation for 36 h, cells were harvested, and protein extracts were prepared by lysis with reporter lysis buffer (Promega). Firefly luciferase and Renilla luciferase activities were assayed with a dual luciferase kit (Promega).
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The following constructs were used: 6AP-1 (9), HIV NFκB (10), and 3TP-luciferase (Prof. Yoav Henis, Tel-Aviv University, Israel).

RESULTS

Global Analysis of Microarray Expression Data—To unravel the complex interactions that contribute to the transformed phenotype, we analyzed gene expression in our model of cell transformation using gene microarrays. We isolated mRNA from three independent cultures each of K, E, L, and BP cells, grown under basal conditions. cDNA was prepared and hybridized to the human genome U133A array (Affymetrix). The overall picture shows that 1) there is a progressive divergence of later HF1 passages from K cells, and 2) the expression of more genes is reduced than induced, in the progressive passages (Fig. 1, A and B). Following paired t test analysis and using a cut-off of at least 2-fold change in the level of expression, 613 transcripts were induced in K (versus K), as compared with 906 repressed transcripts; 1424 transcripts were induced in L (versus K), as compared with 2597 repressed transcripts; and 2199 transcripts were induced in BP (versus K), as compared with 2902 repressed transcripts. The excess of repression versus induction becomes even more obvious as the cut-off is made more stringent (Fig. 1B). These findings indicate that there is a contraction in the number of genes transcribed as the cells become progressively more transformed, but certain genes are expressed at higher levels.

We compared our lists of genes with at least 2-fold changes in expression with the published data of Santin et al. (6) and Wong et al. (7). More than half of the genes that were repressed or induced in cervical cancer according to Santin et al. (6) were also progressively repressed or induced in HF1 cells (Fig. 1C). The probability of obtaining an overlap of 56% between our data for BP cells and the data of Santin et al. (6) randomly is 2.0 × 10^{-37} (see “Comparison of Overall Gene Expression in HF1 and Cervical Carcinoma” in the supplemental material and Fig. S2). Similarly, 64% of the genes that were modified in cervical cancer according to Wong et al. (7) were progressively modified in HF1 cells (see “Comparison of Overall Gene Expression in HF1 and Cervical Carcinoma” in the supplemental material). The probability of obtaining this overlap randomly in BP cells was calculated as explained in Fig. S2 and is 7.8 × 10^{-15}. These two sets of results show a marked convergence of the HF1 passages with cervical cancer and strongly support the relevance of the in vitro cellular system described here to cervical cancer.

Expression of Pro- and Antiapoptotic Genes Was Reduced during Transformation—In order to examine which biological processes were most affected by transformation, we used EASE software (11) to group the transcripts that were expressed at least 2-fold higher/lower in BP cells compared with K cells according to GO annotations. Biological categories that were significantly overrepresented, as defined by an EASE score of ≥0.05 and global false discovery rate = 0, are shown in Table S1 (supplemental data). Cell death was among the overrepresented biological categories with reduced gene expression (91 genes with reduced expression of 404 genes with this GO term on the chip; Table S1B) but was not among the overrepresented induced categories. Note that the EASE analysis yields biological categories with multiple overlaps (e.g. the down-regulated categories include cell death, regulation of apoptosis, negative regulation of apoptosis, and antiapoptosis; the up-regulated categories include cell cycle, mitotic cell cycle, M phase, and mitosis). Therefore, this analysis is useful for choosing biological processes of interest but cannot tell us how many cellular pathways were induced or repressed.

Although we anticipated the decrease in expression of proapoptotic genes, we were intrigued by the finding that antiapoptotic genes (27 of 88 genes in this category on the microarray; Table S1B) were also significantly overrepresented among genes with reduced expression. We therefore decided to focus in this study on the apoptotic network and how its contraction affects cellular behavior in the absence and presence of exogenous stress. To do this, we analyzed the extended apoptotic network, including regulatory pathways acting well upstream to the caspase cascade (although these were not defined by EASE as apoptotic pathways), reasoning that changes in the upstream pathways would influence the death response.

Reduced Expression of Major Pathways Controlling Apoptosis—The group of cell death genes with reduced expression in BP cells comprised 91 transcripts, including CASP3, CASP2, CASP6, CASP10, CASP1, BCL-X, BID, BCL2A1, DAPK1, and DAPK2 (Table S2). Most of the genes with reduced expression in L and BP cells showed a continuous decline in expression during the progression from K to BP cells, based on the microarray data. Several transcripts were induced slightly in E cells compared with K cells, but their expression decreased in L and BP cells.

Among the repressed genes classified by EASE as involved in cell death, we noticed transcripts participating in the MAPK pathway, such as GADD45A, RASA1, SERPINB2, IL1A, and IL1B (Table S2), although the members of the MAPK cascade themselves were not classified as involved in cell death. Similarly, several NFκB-regulated genes, such as BCL2L1, CFLAR, and BCL2A1, were classified as involved in cell death, although their upstream regulators were not. These findings prompted us to examine signaling pathways acting upstream to the apoptotic machinery. In particular, we looked at the expression of hub proteins (namely, proteins with multiple interaction partners or those that determine the fate of a certain pathway) in these pathways, reasoning that decreased expression of the hub proteins would affect the entire pathway.

In the extended analysis, we used GO and KEGG to identify members of the upstream pathways. Among genes with reduced expression, GO analysis identified 35 members of the NFκB pathway, although the pathway itself was not identified as overrepresented by GO analysis. Among the 35 down-regulated genes were key proteins like IKKβ, IKKBKE, RIPK, and Myd88 (Table S3). 10 members of the NFκB pathway, as defined by GO, showed induced expression. Using KEGG to categorize transcripts that were significantly induced/repressed, only 23 genes participating in the MAPK pathway were induced in BP cells, compared with 56 MAPK genes that were repressed in this category. Another five MAPK members were identified by a literature search (Table S3). All five had
reduced expression in L and BP cells. Using KEGG, 36 gene products that function in the TGFβ-Smad4 pathway were identified. Of these, the expression of 26 gene products (including Smad4, TGFBR1, TGFBR2; Table S3) was reduced, compared with 10 that were induced. In the JAK-STAT pathway, 28 genes were reduced in expression (including JAK2, STAT1, and STAT3; Table S4), compared with six that had increased expression. Moreover, we have shown in a parallel study that the antiapoptotic PKB pathway (12) is repressed in L and BP cells (5). Hence, components of at least five major pathways controlling apoptosis showed strongly reduced expression in transformed L and BP cells.

In order to validate the microarray data we investigated the biochemical status of the above mentioned pathways in K and HF1 cells, using Western blot and reporter assays, as follows. We first examined the substrates of the MAPK pathway, ERK, and AP-1. The involvement of ERK and AP-1 in both pro- and antiapoptotic processes has been well documented (12–15). The induction of apoptosis in response to treatment with CDDP has been shown to require AP-1 and ERK1/2 (15–17). ERK activation was assessed by the level of phosphorylated ERK in Western blots. There was a gradual decrease in ERK phosphorylation from K through E, L, and BP (pERK; Fig. 2A). AP-1 transcriptional activity, as assessed by a reporter assay, also declined with progressive transformation (Fig. 2B). This finding was consistent with the decreased expression of genes known to be induced by AP-1 (18–21) (Transcriptional Regulatory Element Database) in L and BP cells (Table S3).

NFκB also plays a dual role in the apoptotic process and promotes or inhibits apoptosis, depending on cell context and growth conditions (22). The transcriptional activity of NFκB was reduced progressively from K through E, L, and BP, as measured by a reporter assay (Fig. 2C). This result is consistent with
the reduced expression of 32 NFκB-regulated genes (22, 23) in L and BP cells (Table S3).

Smad4 is a central signal transducer of the TGFβ response, and its loss has been hypothesized to underlie the TGFβ resistance of some tumor cells (24). The microarray data showed that Smad4 mRNA levels dropped significantly in L and BP cells (Table S3). To confirm the biological significance of this, we first looked at the levels of Smad4 protein using Western analysis. Indeed, levels of Smad4 were reduced in L and BP cells (Fig. 2D). A reporter assay showed that the basal activity of the TGFβ-Smad4 pathway (i.e. in the absence of TGFβ) progressively declined during the progression K-E-L-BP, as did the activity induced by TGFβ (Fig. 2E).

**Comparison with Cervical Carcinoma at the Network Level**—We compared the output of the MAPK and NFκB pathways in the HF1 system with the data of Santin et al. (6) by comparing AP-1- and NFκB-regulated genes. AP-1- and NFκB-regulated genes were defined as described under "Microarray Data Processing and Analysis" in the supplemental material.

Fifteen genes that can be induced by AP-1 showed reduced expression in L and BP (Table S3); the expression of six of these was also reduced in cervical cancer according to Santin et al. (6) (Table S5A). Similarly, 1 of 2 AP-1-repressed genes that were induced in L and BP cells was also induced in the microarray set of Santin et al. (6) (Table S5A). Moreover, 9 of 32 NFκB-regulated genes that were repressed in L and BP cells were repressed in cervical cancer according to Santin et al. (6) (Table S5B).

Of the 214 genes classified by Santin et al. as repressed in cervical carcinoma, 79 were recognized by the KEGG data base. Of these, eight were categorized as members of the MAPK pathway. None of the 204 induced genes appeared in this category. Thus, expression of the MAPK pathway is significantly repressed in cervical cancer.

**Inactivation of the Caspase Cascade**—The shrinkage in apoptotic pathways also encompassed the caspase cascade, which we can think of as the direct executor of the apoptotic process. Caspase-3 protein is of fundamental importance in many forms of apoptosis (25). The levels of procaspase-3 mRNA (CASP3; Table S2) and protein (Fig. 3A) were lower in L and BP cells than in K and E cells. Likewise, the level of active, mature caspase-3 was reduced in CDDP-treated L and BP cells compared with CDDP-treated K and E cells. L and BP cells accumulated the inactive p19 intermediate (26) and were apparently unable to complete the conversion of procaspase-3 (p32) to active caspase-3 (p17), whereas K and E cells did make p17 (Fig. 3B). In order to evaluate caspase activity, we looked at levels of activated ICAD, p11 (Fig. S3A), a direct substrate of caspase-3/7. Less ICAD was activated induced following CDDP treatment in L and BP cells than in E cells (Fig. S3, A and B). Interestingly, the pattern of the cleaved product in apoptotic E cells was different from the pattern in L and BP cells, where an additional slowly migrating band appeared. We detected extremely low levels of the active ICAD in K cells (data not shown), possibly because keratinocytes do not always show all of the markers of apoptosis (27, 28).

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**FIGURE 2. Reduced activity of apoptotic regulatory network in the course of transformation.** A, ERK phosphorylation levels in K and HF1 cells were analyzed under basal growth conditions. Lysates were analyzed by immunoblot with anti-phospho-ERK (pERK) and pan-anti-ERK antibodies (ERK2). B, K and HF1 cells were transfected with firefly luciferase (FFL) reporter gene constructs (6AP-1) containing AP1 binding sites. FFL expression levels under basal growth conditions were measured 36 h after transfection. C and HF1 cells were transfected with FFL reporter gene constructs (HIV NFκB) containing an NFκB binding site. FFL expression levels under basal conditions were measured as in B. D and E, progressive reduction in TGFβ-Smad4 pathway activity in HF1 cells. D, Smad4 was measured by Western blot analysis of K, E, L, and BP cell lysates after growth under basal conditions. ERK2 served as a loading control. E, K and HF1 cells were transfected with FFL reporter gene constructs (3TP-luciferase) containing a Smad2/3 binding site in the presence or absence of TGFβ. FFL expression levels were measured as in B. The graphs show the calculated averages and S.D. values from four independent experiments.
As for procaspase-3, L and BP cells did not convert procaspase-9 (p47) to its most active form (p37), despite the total degradation of the procaspase form, but instead accumulated p35 (Fig. 3C). The inability to fully activate caspase-9 may be due to the failure to activate caspase-3 (29).

A deficiency in caspase-3 or caspase-9 can elicit compensatory activation of caspase-6 in vitro and in vivo (30). Therefore, we looked at the levels of caspase-6 protein in K and HF1 cells. L and BP cells had significantly lower basal levels of procaspase-6 (p33; Fig. 3D) than K and E cells and, unlike K and E cells, were unable to process procaspase-6 to the intermediate activated p30 form (Fig. 3D).

These data show that the caspase enzymes as well as both pro- and antiapoptotic upstream pathways that regulate apoptosis were diminished in the course of HF1 transformation. The reduction in proapoptotic pathways presumably contributes to the survival and increased proliferation of the transformed cells. The inactivation of antiapoptotic pathways in HF1 cells is more intriguing. Under basal growth conditions, L and BP cells survived and proliferated, despite the abrogated activity of several key antiapoptotic and prosurvival pathways.

Switch from Apoptosis to Necrosis in CDDP-treated L and BP Cells—
The shrinkage of the apoptotic network prompted us to examine the mode of cell death in K, E, L, and BP cells when exposed to stress, such as CDDP. In order to distinguish between apoptosis and necrosis, we performed time course analysis, using FACS. L and BP cells were more susceptible to cell death than K and E cells in response to the same concentration of CDDP (Fig. 4A). K cells had a gradual increase in the apoptotic fraction (represented by staining with annexin but not PI) after a 12-h exposure to CDDP (Fig. 4A), as did E cells, albeit to a lesser extent (Fig. 4A). The appearance of this fraction was followed by the even more gradual appearance of cells that stained with both annexin and PI, representing mainly late apoptotic (and perhaps some necrotic) cells. In contrast, the sudden jump in annexin/PI co-staining of L and BP cells at 18 h was not preceded by a correspondent fraction of early apoptotic cells, which should have been stained only with annexin (Fig. 4A). This indicates that the co-stained fraction seen for L and BP cells represents mainly necrotic cell death following CDDP treatment.

This finding was corroborated by the finding that CDDP induced the release into the growth medium of HMGB1 (high mobility group 1) protein from L and, to a greater extent, from BP cells but hardly from K or E cells (Fig. 4B). The release of HMGB1, a proinflammatory protein, to the extracellular environment is a hallmark of cells dying by necrosis and not by apoptosis (31). Levels of intracellular HMGB1 decreased in L and BP cells, following CDDP treatment. However, for K and E cells, intracellular levels of HMGB1 were unchanged upon

FIGURE 3. Reduced levels of caspase proteins in L and BP cells. A, K and HF-1 cells were cultured under basal conditions and analyzed by Western blot using anti-caspase-3. This antibody recognizes the prodomain of caspase-3 (Casp3; top). Procaspase-3 levels were normalized to ERK2 levels (bottom). The average value for each duplicate sample was determined in every experiment. The graph shows the calculated averages and S.D. from three independent experiments. B–D, HF1 cells were treated with CDDP (25 μM) for 24 h. B, procaspase-3 levels (p32) were detected by anti-caspase-3. Levels of the cleaved forms of caspase-3 were analyzed by Western blot using anti-cleaved caspase-3, which recognizes the two subunits (p19 and p17) generated during the process of caspase-3 maturation. C, levels of the cleaved forms of caspase-9 were analyzed by Western blot using anti-caspase-9 (Casp9), which recognizes the two subunits (p37 and p35) generated during the process of caspase-9 maturation as well as the procaspase (p47). D, procaspase-6 levels (p33) were detected by anti-caspase 6 (Casp6). 0h, without treatment; 24h, CDDP treatment for 24 h.
Inhibition of MEK/ERK, PI3K/PKB, and NFκB Pathways Led to Enhanced Necrosis in E Cells upon CDDP Treatment—To test whether the switch from apoptotic to necrotic cell death in L and BP cells could be attributed to repression of apoptosis, we repressed the apoptotic network in E cells and investigated their response to stress. To this end, we inhibited the major pathways acting upstream to the caspase cascade, the MEK/ERK, NFκB, and PI3K/PKB pathways, that we had shown were repressed in L and BP cells (Fig. 2; Tables S3 and (5)).

Fig. 5 shows the response of E cells to inhibitors of these pathways, in the presence or absence of the inducer of apoptosis, CDDP. CDDP has also been shown to lead to activation of antiapoptotic pathways (33). Treatment of E cells with CDDP alone led to the appearance of an annexin-positive apoptotic fraction. Treatment with the quinazoline compound QNZ alone (an NFκB inhibitor) or with LY294002 alone (a PI3K inhibitor) also led to some apoptosis. On the other hand, treatment with U0126 alone (a MEK1/2 inhibitor) led to the appearance of a significant annexin-negative PI-positive necrotic fraction. The combination of CDDP plus QNZ or CDDP plus LY294002 led to somewhat more apoptosis than CDDP alone. The combination of CDDP plus U0126 led to a decrease in necrosis, as compared with U0126 alone but a large increase in necrosis as compared with CDDP alone. The combination of CDDP plus U0126 plus QNZ led to an even larger increase in necrosis. Thus, inhibiting the antiapoptotic PI3K/PKB pathway led to increased apoptosis, but inhibiting the MEK/ERK pathway led to necrosis, and inhibiting NFκB led to increased apoptosis or necrosis, depending on which other pathways were active. The MEK/ERK and NFκB pathways have been shown to have both pro- and antiapoptotic effects, depending on cellular context (12–15, 22). Combination of CDDP plus all three inhibitors led to the appearance of a significant necrotic fraction, as compared with CDDP only. Moreover, in the presence of the inhibitors and CDDP, E cells were unable to convert pro-apoptotic P38 to active caspase-3 (p32) to active caspase-3 (p17), and consequently the levels of active ICAD were reduced (Fig. 5, D and E).

Treatment of K cells with the various inhibitors, singly or in combination, prior to CDDP treatment had little effect beyond the effect of CDDP alone. This may be because K cells are more robust than E cells and require more extensive inhibition of the apoptotic network to see the shift to necrosis. As expected, there was no difference in response between L and BP cells that were pretreated with the inhibitors or were not pretreated prior to CDDP application, since the relevant pathways were already repressed in these cells (Fig. S5).

Thus, the simultaneous inhibition of MEK/ERK, PI3K/PKB, and NFκB pathways in E cells was able to mimic at least partially the evolutionary depletion of the pro- and antiapoptotic network in L and BP cells and the consequent shift to necrosis rather than apoptosis in response to DNA damage. This finding supports the contention that the shift to necrosis during the course of transformation of L and BP cells was driven by the cellular ATP levels during CDDP treatment. As shown in Fig. S4, ATP pools were preserved in K and all HF1 cells during the course of CDDP treatment. Thus, the increased necrosis of L and BP cells was not caused by reduced ATP availability.

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CDDP treatment (Fig. 4C). We also measured LDH release from the cells after 12 h of CDDP treatment, when signs of cell death begin, and after 24 h of CDDP treatment, by which time cell death is evident. Whereas no release of LDH in K and a slight release in E cells was detected, L and BP cells released significant amounts of LDH to the medium (Fig. S3C). Thus, L and BP cells showed a marked increase in necrotic death upon CDDP treatment.

**Necrosis in L and BP Cells Was Not Induced by ATP Depletion—** Necrotic cell death has been reported to occur due to reduced availability of energy (32). To determine whether necrosis in L and BP cells was induced by ATP depletion, we measured intra-

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**FIGURE 4.** CDDP-induced death of keratinocytes and HF1 cells. A, cells were exposed to a fixed CDDP concentration (25 μM) for various lengths of time (0, 8, 12, 18, and 24 h). The apoptotic fraction (An+/PI−) gradually increased in K cells, starting at 12 h of treatment, whereas the necrotic fraction (An+/PI+) increased sharply in L and BP cells at 18 h. At least 10,000 cells were counted for each data point. The values are means ± S.D. of three independent experiments. B, detection of HMGB1 in culture medium from L and BP cells in response to CDDP. Keratinocytes and HF1 cells were treated with 25 μM CDDP for 24 h. Culture medium (S) was collected, and HMGB1 was detected by immunoblotting. HMGB1 levels were normalized to the intensity of Ponceau staining (middle) of the given lane and the protein concentration in the cell lysate (bottom; see “Experimental Procedures”). The graph shows data from three independent experiments; values are means ± S.D. C, HMGB1 was detected in cell lysates (pellet; P) of K and HF1 cells before (0h) and after (24h) CDDP treatment. The graph shows data from two independent experiments; values are means ± S.D.
decrease in entry into apoptosis and
the inability to activate the apopto-
ic machinery.

Appearance of Necrotic Fractions
in HeLa Cells in Response to CDDP—
In order to see whether the shift to
necrosis during HF1 transformation
is relevant to cancer development,
we looked at the effect of CDDP on
the cervical carcinoma cell line,
HeLa. Like L and BP cells (Fig. 3),
HeLa cells have lower levels of
caspases-3 and \( \frac{\beta}{\beta} \) than keratino-
cytes (34). We found that, like L and
BP cells, HeLa cells could not com-
plete the full conversion of pro-
caspase-3 to active caspase-3 (p17)
in response to CDDP (Fig. 6A).
Unlike L and BP cells, HeLa cells
were able to fully activate caspase-9
(Fig. 6A) in response to CDDP at
similar concentrations. Following
CDDP treatment, HMGB1 was
released into the medium (Fig. 6B).
FACS analysis showed the presence
of both apoptotic and necrotic cells.
24 h after exposure to CDDP (25
\( \mu \)M), \~{}40% of cells were dead; of
these, about two-thirds stained with
PI (as expected of necrotic cells) or
with PI and annexin (as expected of
necrotic or late apoptotic cells),
and about one-third stained with
annexin alone (Fig. 6C). Thus,
more than half of the dead HeLa
cells were necrotic. A similar per-
centage of keratinocytes was killed
by the same treatment, but no sig-
nificant necrotic fraction was
detected for these cells (Fig. 4A;
see above).

DISCUSSION

In order to follow the changes
that occur during HPV16-induced
transformation, we have used a
model system consisting of K cells,
HF1 cells, E cells, L cells, and BP
cells. L and BP cells display many of
the properties of transformed cells,
including enhanced growth rate (5).
Furthermore, BP cells are able to
form colonies in soft agar (Fig. S1).
The power of our model lies in the
fact that the initiating event in cer-
vical cancer of HPV integration is
known and has been reproduced
in vitro by many laboratories (35–39),

FIGURE 5. Increased necrosis of E cells upon inhibition of pathways upstream of apoptosis. E cells
were treated with 25 \( \mu \)M CDDP for 12 h (A), 18 h (B), and 24 h (C–E) after pretreatment for 1.5 h with or
without QNZ (Q; 30 \( \mu \)M), LY294002 (LY; 20 \( \mu \)M), and U0126 (U; 10 \( \mu \)M). A–C, FACS analysis of E cells upon
inhibition of pathways upstream of apoptosis. At least 10,000 cells were counted for each data point. Each
graph shows data from at least two independent experiments; values are means \pm S.D. D, levels of the
cleaved forms of caspase-3 were analyzed by Western blot using anti-cleaved caspase-3. E, levels of the
active (p11) and inactive forms (p45 and p35) of ICAD were analyzed by Western blot using anti-ICAD.
F, to verify inhibition by U0126 and LY294002, Western blotting for phospho-ERK (pERK) and phospho-GSK3
\( \alpha/\beta \) (pGSK3\( \alpha/\beta \)) was performed. To verify inhibition by QNZ, an FFL reporter gene construct (HIV NF\( \kappa \)B)
containing an NF\( \kappa \)B binding site was used. Lysates were prepared after treatment of E cells for 1.5 h with
LY294002, U0126, and QNZ. –, no treatment; \( U + LY + Q \), treatment with inhibitors.
promote or inhibit apoptosis, depending on cellular context or stimulus (12, 22), and the proapoptotic TGFβ-Smad pathway (Table S3 and Fig. 2). Comparison with the data of Santin et al. (6) suggested that these pathways are regulated in a similar manner during the development of cervical cancer (Table S5). In a parallel study, we have shown that the antiapoptotic PKB pathway (12) is inactivated in L and BP cells (5). Not only were pathways that regulate apoptosis repressed in L and BP cells under basal conditions; upon CDDP-induced DNA damage, L and BP cells did not activate the caspase cascade (Fig. 3) and thus could not undergo apoptosis (Fig. 4).

Necrotic cells release their contents into the extracellular space and create a proinflammatory environment, which induces an immune response in the dying cell. Macrophage activator HMGB1, which is released from L and BP cells during CDDP treatment, usually promotes inflammatory responses in vivo (42, 43). Thus, induction of necrotic cell death, which results in the induction of an immune response, may contribute to the effectiveness of CDDP as a therapeutic agent. Interestingly, it has been shown that BCR-ABL-positive leukemia cells can undergo caspase-independent necrosis, in response to treatment with Gleevec (imatinib) (44). Thus, the success of Gleevec in the clinic may be partly attributable to activation of the immune system by necrosis.

The cancer cell, which appears to be more robust in certain environments, appears to be more vulnerable than the noncancerous cell to unexpected perturbations (1). Indeed, cancer cells often display a heightened state of sensitivity to stress signals (45, 46). In the current study, upon CDDP treatment, L and BP cells underwent massive necrosis, which seems to have become the default mechanism in response to stress. Primary cells and cells at an earlier stage of transformation were more resistant at the same CDDP concentrations and retained their apoptotic response to CDDP. Nevertheless, when the pathways controlling entry into apoptosis were inhibited by pharmacological means, E cells were shifted toward necrosis. These results are supported by the finding that inhibition or absence of caspases can force cells to respond to stress by necrosis (reviewed in Ref. 47). Additionally, we have shown that a significant fraction of HeLa cells (cervical cancer line) undergoes necrosis in response to CDDP at a same concentration that leads to necrosis in L and BP cells. These findings support the hypothesis that the development of cancer cells is characterized by a shift from apoptosis to necrosis (8). Moreover, several publications have shown that cervical cancer cells undergo necrosis in response to various triggers (48–51).

Repression of the proapoptotic network may be advantageous for the cancer cell. Abrogation of proapoptotic pathways may enable the cancer cell to maintain its transformed phenotype and to escape from a wide range of apoptotic signals. The shrinkage of the antiapoptotic network, however, was a surprise. The loss of the pro- and antiapoptotic machinery in late stages of transformation during the evolution of HF1 cells appears to be part of the general decrease in the number of genes transcribed in L and BP cells (Fig. 1). Presumably, during the process of transformation, L and BP cells relinquish pathways that are not involved directly in
Apoptosis to Necrosis Shift in Transformation

proliferation. The elimination of the proapoptotic network apparently makes the antiapoptotic network redundant and allows its shrinkage in L and BP cells. This hypothesis is supported by a recent study, which has shown strong connectivity between the PI3K-PKB, TGFB-Smad, MAPK, and NFkB pathways, and suggests that they work together in a complementary way to generate the tumor phenotype (52). The contraction of cellular networks may reduce the energy requirements for cell maintenance, thereby diverting cellular resources toward rapid cell cycle progression and increased metabolism.

In summary, we have revealed a contraction in expression of the apoptotic network during HF1 cell transformation, which affected the ability of L and BP cells to execute apoptosis but did not lead to resistance to apoptotic stimuli. The contraction in the apoptotic machinery during the process of transformation was accompanied by a switch from apoptosis to necrosis in response to CDDP. The shrinkage of the pro- and antiapoptotic networks appears to be part of a general contraction in the number of genes transcribed in L and BP cells. We have also identified a large group of genes with induced expression, which are involved in cell metabolism and cell cycle, suggesting increased investment of the transformed cell in cellular proliferation (Table S1). We hypothesize that the decrease in expression of many diverse pathways, including the pro- and antiapoptotic networks, cuts the energy requirements for cell maintenance, allowing energy to be diverted toward rapid cell proliferation. Understanding the mode of cancer cell death will enhance our understanding of how tumors respond to genotoxic treatments.

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