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Retention of Cell Adhesion and Growth Capability in Human Cervical Cancer Cells Deprived of Cell Anchorage

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Cell adhesion is linked to various regulatory processes of growth as well as apoptotic cell death in normal and transformed epithelial cells. We investigated changes of cellular responses to the deprivation of cell anchorage associated with immortalization or malignant transformation. Normal human ectocervical keratinocytes (NCE cells) deprived of cell anchorage become susceptible to apoptosis, and in parallel they lose their adhesion to the culture substratum. The loss of cell adhesion is not directly due to apoptosis. NCE16 cells, an immortalized but not malignantly transformed subline of NCE, underwent apoptosis and lost cell adhesion in suspension, as the NCE cells did. By contrast, apoptosis was not inducible in human cervical cancer-derived C33A cells in suspension. Of other cell lines derived from human cervical cancer, SiHa cells showed a weak apoptotic response and Caski cells were highly sensitive to apoptosis in the absence of cell anchorage. Unlike NCE or NCE16 cells, all these cancer cells retained cell adhesion as well as growth capability in suspension cultures. These results indicate that retention of cell adhesion and growth capability in the absence of cell anchorage is more closely associated with cancer cell lines than resistance to apoptosis upon the deprivation of cell anchorage.

Key words: Epithelial cell — Transformation — Cell anchorage — Apoptosis — Cell adhesion

Normal, untransformed epithelial cells require cell anchorage for proliferation, and upon the deprivation of cell anchorage in suspension culture *in vitro*, these cells undergo apoptosis or become susceptible to apoptosis as indicated by the characteristic internucleosomal DNA fragmentation inducible by calcium.[1–4] This apoptotic response may be involved in terminal differentiation of epithelial cells,[3, 5] though this is controversial.[4] It may also be involved in the prevention of the inappropriate settling of the cells once detached from underlying matrix, termed *anoikis*.[2, 6] In addition to the apoptotic response, normal epithelial keratinocytes in suspension gradually lose cell adhesion as well as the ability to resume growth even if they can reattach to the culture substratum.[3, 7] We have previously shown that the loss of cell adhesion of normal human ectocervical keratinocytes in suspension proceeded without any obvious indication of apoptosis, but paralleled the acquisition of susceptibility to calcium-induced internucleosomal DNA fragmentation, and we suggested that the loss of cell adhesion may not be a consequence of execution of apoptosis.[3] Because both apoptosis and the loss of cell adhesion are active cellular responses depending on protein synthesis,[3] they may underlie the irreversibility of epithelial differentiation or, along with *anoikis*, the suppression of metastatic behavior of epithelial cells. This notion implies that disorders in these processes could be associated with the metastatic ability of cancer cells, at least in part. In support of this notion, the transformation of canine kidney epithelial cells (MDCK cells) with v-Ha-*ras* or v-*src* oncogenes was shown to abrogate sensitivity of the cells to apoptosis induced by the deprivation of cell anchorage.[2] In addition, the overexpression of p125 focal adhesion kinase noted in invasive cancers[8] was observed to prevent apoptosis of MDCK cells *in vitro*.[9] To date, however, no systematic investigation has been made on changes of cellular responses to the deprivation of cell anchorage during malignant transformation of epithelial cells. We studied the responses of primary-cultured human ectocervical keratinocytes (NCE cells), cell lines established from the NCE cells by transfection of human papillomavirus type 16 (HPV16) DNA,[10] and the human cervical cancer-derived cell lines SiHa (HPV16-positive), C33A (HPV16-negative)[11] and Caski (HPV16-positive)[12] to the deprivation of cell anchorage by the use of suspension culture.

MATERIALS AND METHODS

Cells and cell cultures Primary cultures of human NCE cells were prepared from normal uterine cervices removed owing to severe myomas, and were maintained in MCDB153 medium supplemented with growth hormones and antibiotics as described.[3, 13] The establishment of NCE16 cell line immortalized by transfection with full-length HPV16 DNA was previously reported.[10] The human cervical cancer cell lines SiHa, C33A and Caski

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were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and 10% fetal bovine serum. Suspension cultures of the cells were made by plating exponentially growing cells into dishes coated with 0.5% agarose saturated with the culture media.[3] Because Caski cells tended to form aggregates in suspension, they were dissociated with 0.1% trypsin at 37°C at the end of suspension cultures then used for replating or further analyses.

Analyses of DNA fragmentation For analysis of internucleosomal DNA fragmentation, cellular DNAs were prepared by the treatment of cell lysates with proteinase K, followed by extraction with phenol-chloroform and then precipitation with ethanol, as described.[3] After the digestion of RNA with RNase A, samples containing 2 to 5 μg of DNA were resolved by electrophoresis through 2% agarose gels for the detection of internucleosomal DNA fragmentation and fragments were visualized by ethidium bromide staining. For pulsed-field gel electrophoresis (PFGE) of chromosomal DNA, 1×10^6 cells were embedded in 75 μl of 0.8% low-melting-point agarose. Agarose plugs containing cells were treated with 1 mg/ml of proteinase K in 0.5 *M* EDTA (pH 9.0) containing 1% (w/v) sodium N-lauroyl sarcosinate at 37°C for 48 h. A half of the plug was subjected to PFGE in 1.5% agarose with 0.5× Tris-HCl boric acid EDTA (TBE) buffer using a rotary gel electrophoresis apparatus (ATTO, model AE-6800, Tokyo). Electrophoresis was done for 17 h at 8 V/cm with a pulse time of 20 s at 14°C.

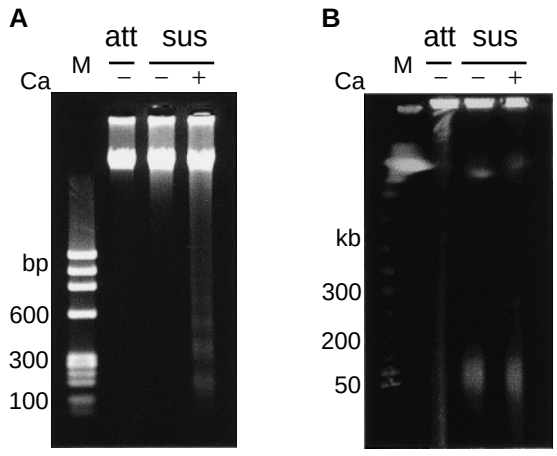

Fig. 1. DNA fragmentation assays of primary NCE cells in suspension using (A) gel electrophoresis through 2% agarose gel and (B) pulsed-field gel electrophoresis. The cells were cultured either on normal culture dishes (att) or on agarose-coated dishes (sus) with MCDB153 medium for 48 h. A final concentration 2 m*M* $CaCl_2$ (Ca) was added after 24 h of incubation. Lanes M are size markers, which are *Hae*III-digested øX174 DNA in (A) and a λ DNA ladder (Promega, Madison, WI) in (B).

Flow-cytometric analysis of apoptotic cells with the TUNEL technique The cells were fixed with 1% paraformaldehyde for 15 min on ice and permeabilized with 70% ethanol at –20°C for at least 12 h. The detection of fragmented DNA through terminal deoxynucleotidyl transferase-mediated BrdUTP nick end labelling (TUNEL) on flow cytometry[14] was achieved by using an APO-BRDU kit (Phoenix Flow Systems, San Diego, CA) following the manufacturer's instructions. Labelling of the DNA with BrdUTP was done for 3 h at 37°C. The cells were then reacted with an anti-BrdU monoclonal antibody conjugated with fluorescein isothiocyanate (FITC), and also stained with propidium iodide. The cells were analyzed on a flow cytometer (FACScan, Becton-Dickinson Immunocytometry Systems, Mountain View, CA) for both FITC and propidium iodide fluorescence.

Cell adhesion and cell growth For the cell adhesion assays, 1×10^5 cells suspended for the indicated periods were plated in 6 cm dishes in triplicate and incubated for 24 h. Then, both the attached cells recovered by trypsinization and the unattached cells were counted and the percentages of attached cells were determined. For the colony formation assays, 1×10^4 cells, either normally cultured or suspended for 48 h, were plated in 6 cm diameter dishes in triplicate. After 10 to 14 days, the cell numbers were counted.

RESULTS

Induction of 50 kb-scale chromosomal DNA fragmentation in normal keratinocytes by deprivation of cell anchorage in low calcium condition We have previously reported that normal human ectocervical keratinocytes (NCE cells) deprived of cell anchorage become susceptible to calcium-induced apoptosis as judged by the appearance of internucleosomal DNA fragmentation.[3] We first re-examined apoptosis of NCE cells by analyzing cleavages of chromosomal DNA into 300 kb and/or 50 kb lengths, since these large-scale DNA cleavages rather than internucleosomal DNA fragmentation have been shown to be essential for apoptosis.[15]

When NCE cells were suspended for 48 h in MCDB153 medium containing 0.03 m*M* calcium, internucleosomal DNA fragmentation was not observed (Fig. 1A). Exposure of the cells in suspension to a final concentration of 2 m*M* calcium chloride for the last 24 h resulted in induction of internucleosomal DNA fragmentation, as we have previously reported.[3] However, analysis of chromosomal DNA by pulsed-field gel electrophoresis revealed that cleavage of chromosomal DNA into 50 kb lengths was induced in the suspended NCE cells without addition of calcium (Fig. 1B). The 50 kb-scale DNA fragmentation appeared not to be augmented by addition of calcium, suggesting that calcium triggers internucleosomal DNA fragmentation but not the 50 kb-

scale DNA fragmentation. To see whether the 50 kb-scale DNA fragmentation is an active process that requires ongoing protein synthesis, we examined the effect of a protein synthesis inhibitor, cycloheximide (Fig. 2). Cycloheximide added at the onset of suspension culture inhibited induction of the 50 kb-scale DNA fragmentation, suggesting that this is an active response. In addition, benzyloxycarbonyl-Asp-CH_2OC(O)-2,6,-dichlorobenzene (Z-Asp-CH_2-DCB), a potent inhibitor of ICE-family proteases (caspases)[16] inhibited induction of the 50 kb-scale DNA fragmentation (Fig. 2), as well as internucleosomal DNA fragmentation upon addition of calcium (data not shown). From these results, we concluded that NCE cells deprived of cell anchorage undergo apoptosis as indicated by the 50 kb-scale DNA fragmentation and that addition of calcium triggers further degradation of DNA into oligonucleosomal fragments.

Dissociation of apoptosis and the loss of cell adhesion in normal epithelial keratinocytes deprived of cell anchorage Epithelial keratinocytes placed in suspension gradually lose cell adhesion.[3, 7] Because the loss of cell adhesion proceeded without internucleosomal DNA fragmentation, we had previously concluded that it may not be a direct consequence of execution of apoptosis.[3] But the finding that NCE cells in suspension undergo apoptosis as represented by the 50 kb-scale DNA fragmentation argued against the previous conclusion, since several of the proteins involved in cell attachment, as well as cytoskeletal proteins, have been shown to be cleaved by caspases upon apoptosis.[17] To determine whether the loss of cell adhesion is due to execution of apoptosis or not, we examined the effect of inhibition of apoptosis on the loss of cell adhesion in the NCE cells. The cells were suspended for 24 h either with or without Z-Asp-CH_2-DCB, which blocks induction of apoptotic DNA fragmentations (Fig. 2), and then replated to the culture dishes. At 24 h after replating, both reattached cells and unattached cells were counted to determine the attachment ability (Fig. 3). In the absence of Z-Asp-CH_2-DCB, 80% of the cells lost cell adhesion (Fig. 3; ref. 3). Z-Asp-CH_2-DCB added at the onset of the suspension culture did not prevent the cells from losing cell adhesion, suggesting that cell adhesion is lost independently of apoptosis, or at least independently of the induction of apoptotic DNA fragmentation. We further conducted a quantitative analysis of apoptotic cells by the TUNEL technique in combination with flow cytometry (Fig. 4). When NCE cells were suspended for 48 h with addition of calcium for the last 24 h, at most 30% of the cells were judged to undergo apoptosis on the basis of TUNEL positivity. Because more than 80% of the cells suspended for 48 h lost cell adhesion, the loss of cell adhesion can not be fully ascribed to execution of apoptosis. These results suggest that the loss of cell adhesion in NCE cells deprived of cell anchorage is independent of, or can be uncoupled from, apoptosis.

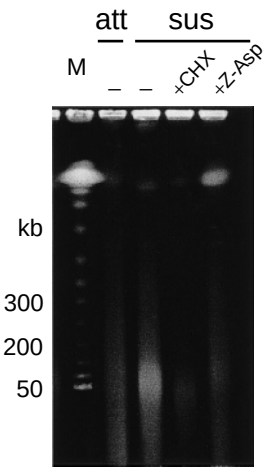

Fig. 2. Effects of inhibition of protein synthesis or caspases on the formation of large DNA fragments in NCE cells in suspension. The cells were cultured either normally (att) or on agarose-coated dishes (sus) with MCDB153 medium for 48 h. Cycloheximide (CHX, final concentration 10 μg/ml) or the caspase inhibitor Z-Asp-CH_2-DCB (Z-Asp, final concentration 100 μM) was added at the onset of suspension culture. Lane M is a λ DNA ladder (Promega) as a size marker.

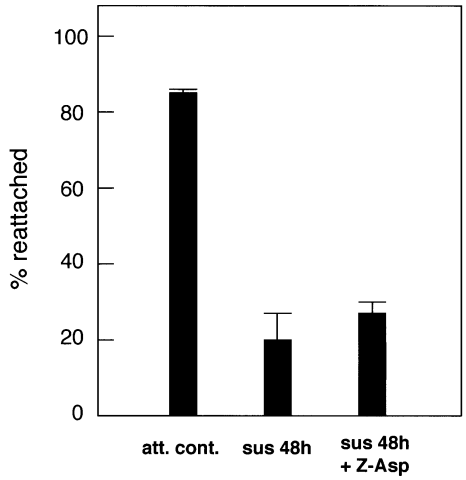

Fig. 3. Effect of inhibition of caspases on the loss of cell adhesion in primary NCE cells deprived of cell anchorage for 24 h. A final concentration of 100 μM Z-Asp-CH_2-DCB was added at the onset of suspension culture. The results represent averaged results of triplicate cultures and vertical bars indicate standard deviations.

Resistance of cancer cell lines to apoptosis in suspension We then examined whether human cervical cancer cell lines undergo apoptosis upon the deprivation of cell

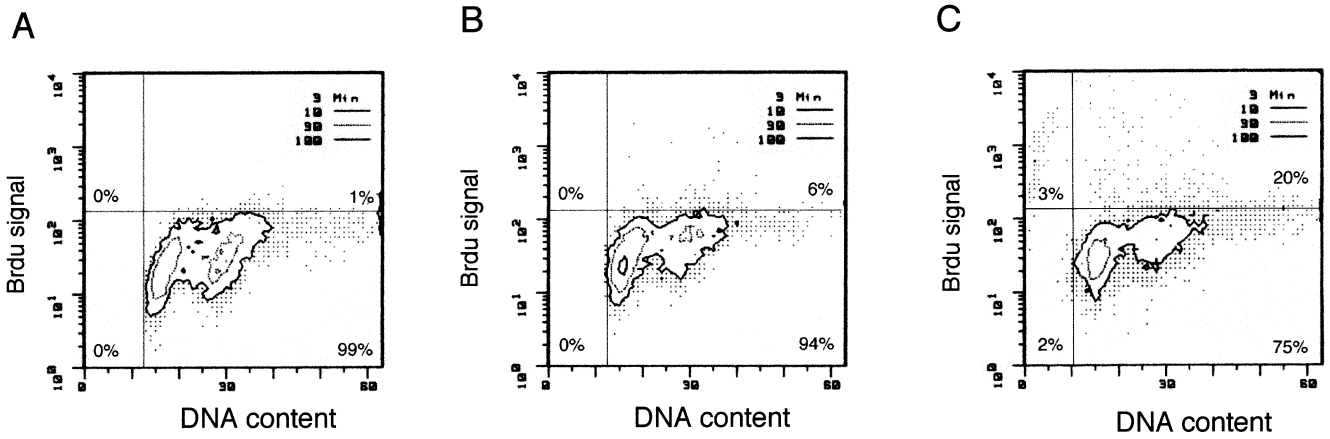

Fig. 4. Flow-cytometric TUNEL assay of primary NCE cells in suspension. The results are shown as isometric contour maps of DNA content vs. DNA strand break labelling. (A) Attached cells, (B) suspended for 48 h without addition of calcium and (C) suspended for 48 h with addition of calcium after 24 h of incubation. The relative percentage of positive cells is indicated in the respective quadrant.

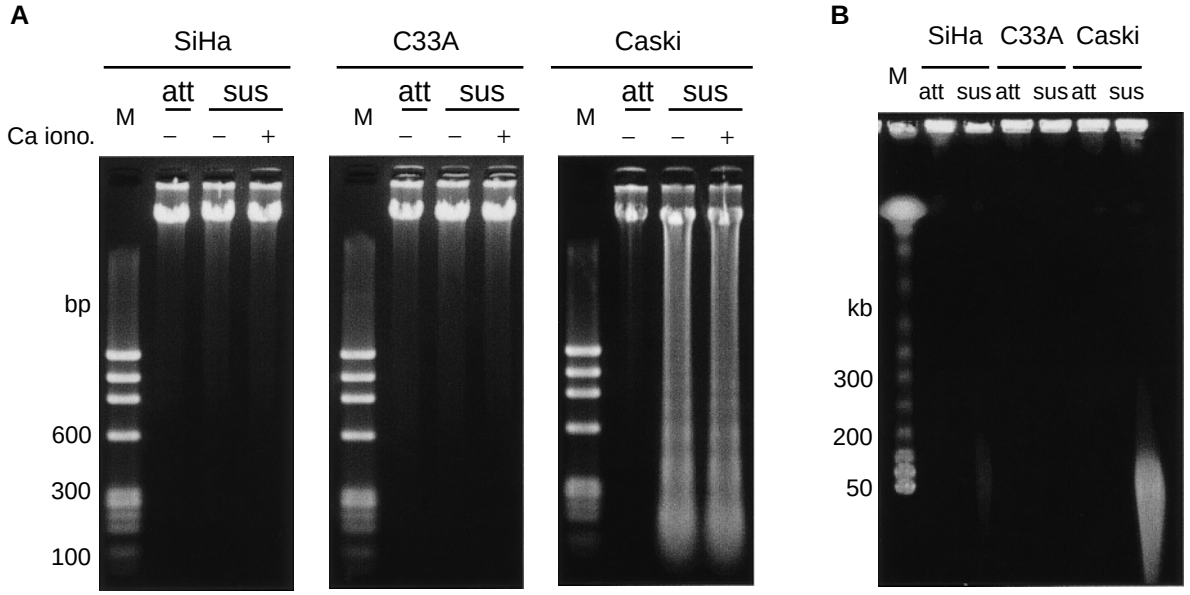

Fig. 5. DNA fragmentation assays of human cervical cancer cell lines. (A) Internucleosomal DNA fragmentation analyzed on 2% agarose gel and (B) 50 kb-scale DNA fragmentation analyzed by pulsed-field gel electrophoresis. The cancer cell lines SiHa, C33A and Caski were cultured on agarose-coated dishes with DMEM containing 2 m*M* $CaCl_2$ for 48 h, either with or without treatment with a final concentration of 1 μM calcium ionophore A23187 for 24 h.

anchorage. In contrast to NCE cells, SiHa and C33A cells suspended for 48 h in DMEM containing 2 m*M* calcium and 10% serum showed no internucleosomal DNA fragmentation, even when they were further treated with the calcium ionophore A23187 for 24 h (Fig. 5A). The 50 kb-scale DNA fragmentation was not observed in the suspended C33A cells (Fig. 5B). In the suspended SiHa cells, a small but distinct amount of 50 kb DNA fragments was observed, suggesting that these cells are not fully resistant to apoptosis induction (Fig. 5B). The Caski cells were found to have undergone extensive apoptosis in the absence of cell anchorage, as evidenced by induction of both internucleosomal DNA fragmentation and the 50 kb-scale DNA fragmentation (Fig. 5). To determine whether immortalization of the cells accompanies the appearance of resistance to apoptosis in suspension, we also examined

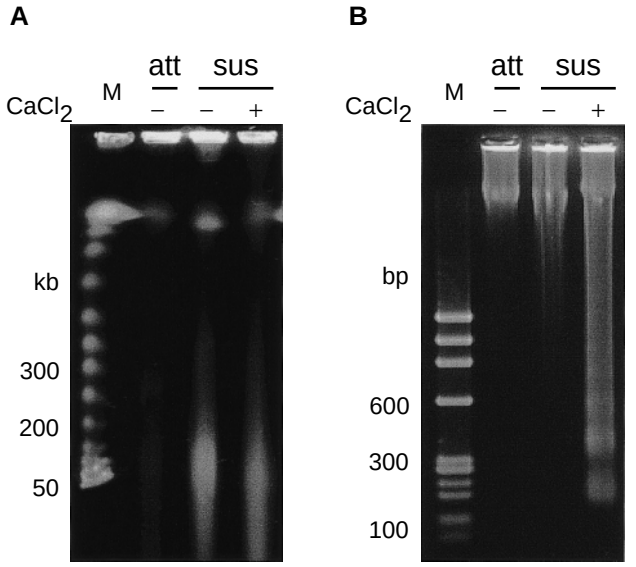

Fig. 6. DNA fragmentation assays of the immortalized cell line NCE16. (A) Fifty kb-scale DNA fragmentation analyzed by pulsed-field gel electrophoresis and (B) internucleosomal DNA fragmentation analyzed on 2% agarose gel. The cells were cultured on agarose-coated dishes with MCDB153 medium for 48 h, either with or without treatment with a final concentration of 2 m*M* $CaCl_2$ for 24 h.

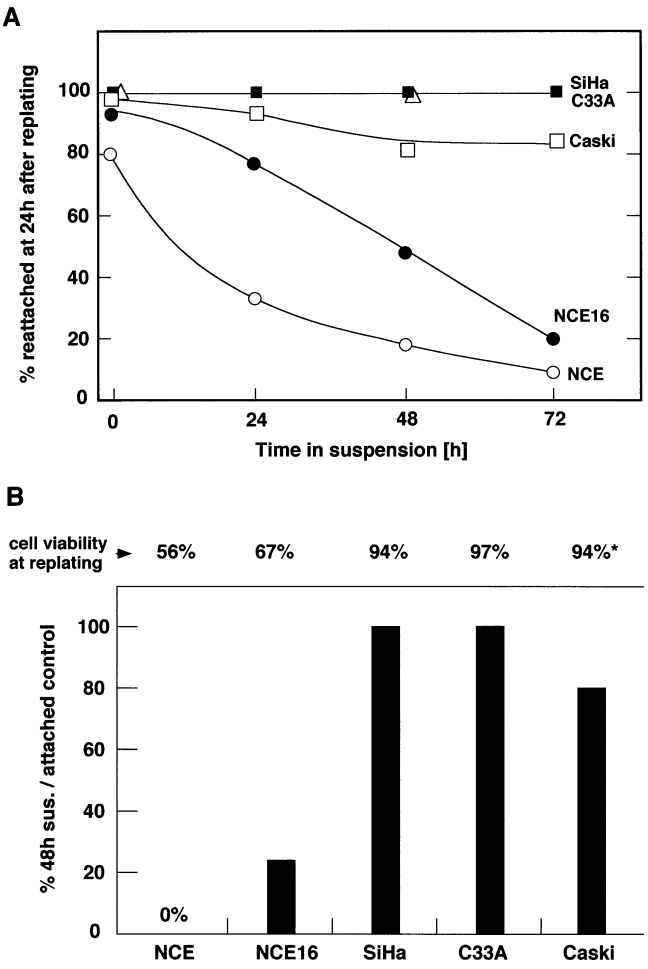

Fig. 7. (A) Time courses of the loss of cell adhesion in primary NCE (○), NCE16 (●), SiHa (■), C33A (△) and Caski (□) cells after plating in agarose-coated dishes. The cells were recovered from suspensions at the indicated times and 1×10^5 viable cells (determined by trypan blue dye exclusion) were replated in normal culture dishes. At 24 h later, both attached and unattached cells were counted and the percentages of reattached cells were determined. (B) Changes of growth capability of the various cell lines after 48 h in suspension. Viable cells (1×10^4) either cultured in suspension or cultured on plates (control) were replated in normal culture dishes. The viability of the cells at the time of replating as determined by trypan-blue dye exclusion is shown above the panel. Note that in the case of Caski cells (*), only cells that retained proper shape were counted, excluding cell debris. After 10 days (SiHa, C33A and Caski) or 14 days (NCE, NCE16), the cell numbers were counted. The ratios of cell numbers of the 48-h suspended cells to that of control cells are indicated, based on the latter as 100%.

apoptosis of the NCE16 cell line established by transfection of HPV16 DNA into NCE cells.[10] The NCE16 cells neither grow in soft agar nor form tumors in nude mice, so they are not malignantly transformed.[10] In suspended NCE16 cells, the 50 kb-scale DNA fragmentation was induced without addition of calcium (Fig. 6A) and internucleosomal DNA fragmentation was induced upon treatment of the cells with calcium for 24 h (Fig. 6B). Thus the NCE16 cells in suspension underwent apoptosis as the primary NCE cells did, implying that immortalization of the cells does not necessarily make the cells resistant to apoptosis induced by deprivation of cell anchorage.

Retention of cell adhesion and growth capability in suspended cervical cancer cells We next examined the changes of cell adhesion in the cervical cancer cell lines upon the deprivation of cell anchorage (Fig. 7A). The cells were kept in suspension for the indicated time periods then replated in culture dishes, and their reattachment ability was determined by counting reattached cells and unattached cells at 24 h after replating. While NCE cells gradually lost cell adhesion in suspension, the cancer-derived SiHa and C33A cells fully retained cell adhesion in suspension. As for Caski cells, since they underwent apoptosis in suspension, we examined the cell adhesion of viable cells as judged by their cell shapes and trypan-blue dye exclusion. While NCE cells judged as viable on the same criteria lost cell adhesion (Fig. 7, A and B), the surviving Caski cells retained the ability to reattach to culture

dishes. Thus, the three cancer cell lines showed a common ability to retain cell adhesion in suspension, unlike the NCE cells. This ability may be associated with malignant transformation, but not immortalization, of the cells because immortalized NCE16 cells lost cell adhesion as the NCE cells did, though they retained it longer than the NCE cells (Fig. 7A).

We then examined the growth of the reattached cells to determine whether they retain growth capability (Fig. 7B). Cells either suspended for 48 h or normally cultured were replated and cultured for 10 to 14 days, and the increases of cell numbers were compared. While NCE cells as well as immortalized NCE16 cells lost their growth capability after 48 h in suspension, SiHa and C33A cells completely retained their growth capability in suspension. The Caski cells, which did not show apoptosis induction in suspension also resumed cell growth after reattachment. The cell number at 10 days after replating was decreased by 20% (Fig. 7B). This decrease appears to coincide with the initial decrease (20%) in the number of the cells that had reattached (Fig. 7A), suggesting that essentially all of the Caski cells that had reattached could resume proliferation. These results suggest that retention of growth capability as well as cell adhesion in the absence of cell anchorage is more closely associated with cervical cancer cells than is resistance to apoptosis.

DISCUSSION

Apoptosis and the loss of cell adhesion of normal epithelial cells in response to the deprivation of cell anchorage *in vitro* are thought to be involved in the suppression of metastatic behavior of those cells, and disorders in these processes could be associated with malignant transformation of the cells. In this study, we examined the transformation of human ectocervical keratinocytes and found that resistance to apoptosis is not necessarily associated with cervical cancer cells, since the Caski cell line (derived from metastatic cervical carcinoma; ref. 12) was sensitive to apoptosis. Rather, the ability to retain cell adhesion in the absence of cell anchorage appears to be a more common feature of cervical cancer cells, including Caski cells.

We first examined whether the loss of cell adhesion in normal cells[3, 7] is independent of apoptosis or not, because execution of apoptosis may result in the loss of cell adhesion owing to cleavage of proteins involved in cell attachment.[17] We examined the effect of inhibition of apoptosis by an inhibitor of caspases on the loss of cell adhesion in normal human ectocervical keratinocytes (NCE cells) placed in suspension. While the inhibitor Z-Asp-CH_2-DCB effectively blocked induction of the apoptotic DNA fragmentation, it did not prevent the cells from losing adhesiveness. In addition, the result of TUNEL assay indicated that 6% of the cells suspended for 48 h were apoptotic and the percentage of apoptotic cells was increased to 30% of the population by addition of calcium. Because 80% of the cells lost adhesion at that time point, apoptosis could not fully account for the loss of cell adhesion in suspension. These results suggest that the loss of cell adhesion proceeds independently of apoptosis, though these two events proceed with similar time courses.[3] It has been reported that differentiation, as judged in terms of induction of involucrin, and apoptosis in suspended keratinocytes could proceed independently.[4] From this point of view, the loss of cell adhesion could be a part of the terminal differentiation process of keratinocytes.[18]

Among the three human cervical cancer cell lines studied here, C33A cells showed complete resistance to apoptosis in suspension. SiHa cells exhibited a weak but detectable level of sensitivity to apoptosis in suspension, and Caski cells were found to be highly susceptible to apoptosis. Although we cannot at present exclude the possibility that resistance to apoptosis is lost during prolonged *in vitro* culture, these results suggest that a malignant transformation of the epithelial cells could accompany induction of resistance to apoptosis by the deprivation of cell anchorage in some, but not all, cases. As for cell adhesion and growth capability, the cancer cell lines SiHa and C33A showed a complete retention of these capabilities in suspension. More importantly, Caski cells, which are susceptible to apoptosis induction, also retained cell adhesion and resumed cell growth upon the recovery of cell anchorage. The retention of cell adhesion and growth capability in suspension may be associated with malignant transformation, because they were lost in the immortalized NCE16 cell line deprived of cell anchorage, as in the primary NCE cells, though the time course was delayed. It remains to be elucidated how normal cells lose their cell adhesion, as well as how NCE16 cells tend to and cervical cancer cells do retain their abilities for cell adhesion in suspension cultures. In normal keratinocytes, reduction of the binding ability of β1 integrin has been reported,[7] although the mechanism is unclear.[18] In HPV-immortalized cell lines, the overexpression of p125 focal adhesion kinase, implicated in resistance to apoptosis,[9] has been reported.[19] Also, the interaction of the E6 protein of malignant types of HPV with paxillin has been reported.[20] These changes could be involved in the prolonged retention of cell adhesion in the NCE16 cell line carrying HPV16 DNA. However, because it was not as complete as that in the cancer cell lines, additional change(s) must be associated with the malignant conversion of the HPV-transformants to cancer cells.

In conclusion, the results presented here suggest that induction of apoptosis and the loss of cell adhesion in normal epithelial keratinocytes deprived of cell anchorage are independent processes. Resistance to apoptosis in suspen-

sion appears to be not necessarily a feature of cancer cell lines, although it tends to be present. Retention of cell adhesion in the absence of cell anchorage may be more tightly associated with cancer cells, although it remains to be determined whether ability to retain cell adhesion in suspension contributes to metastatic ability *in vivo*. Trials to restore the normal process(es) of cell adhesion in cancer cells will answer these questions and may provide a new strategy for prevention of cancer metastasis.

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