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Epidermal Growth Factor Induces Cell Cycle Arrest and Apoptosis of Squamous Carcinoma Cells Through Reduction of Cell Adhesion

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Abstract Most squamous epithelial cells are strictly anchorage-dependent cell types. We observed that epidermal growth factor (EGF) promoted the growth of A431 squamous carcinoma cells in suspension cultures but suppressed cell growth and induced apoptosis in monolayer cultures, suggesting that loss of adhesion is responsible for the effects observed in monolayer culture, before cell death. Consistent with this finding, we demonstrated that EGF reduced cell attachment, cell-cell interaction, and cell spreading. Treatment with EGF increased cell adhesion-regulated expression of p21 but suppressed expressions of cyclin A, D1, cdk2, and retinoblastoma protein (pRb), leading to cell cycle arrest and adhesion-regulated programmed cell death. To test directly whether promoting cell adhesion could reduce the effects of EGF, we grew cultures on plates coated with type II collagen. On these plates, cell adhesion was enhanced and EGF treatment had little effect on cell adhesion and apoptosis when cells were attached to the collagen. The collagen effects were dose dependent, and cell cycle and cell cycle-associated proteins were altered accordingly. Finally, when cultures were plated on bacterial Petri dishes, which completely disrupted cell attachment to substratum, the level of apoptosis was greatly higher and cell cycle was arrested as compared with monolayer cultures. Taken together, our results strongly suggest that the EGF-induced cell cycle arrest and apoptosis in monolayer cultures was the result of a decline in cell adhesion. J. Cell. Biochem. 77:569–583, 2000.

Key words: apoptosis; proliferation, adhesion; cell cycle; EGF

Most squamous epithelial cells, including their transformed counterparts, are strictly anchorage-dependent cell types. Adhesion to substratum is necessary for the cells to progress from G1 into S phase of the cell cycle. This phenomenon, referred as adhesion dependence, is mediated by attachment of cells to extracellular matrix (ECM) through integrin and cadherin, and subsequent signaling events via growth factor receptors [Ruoslahti and Reed, 1994; Meredith and Schwartz, 1997; Bottazzi and Assoian, 1997]. Fibroblasts detached from ECM stop growing, and epithelial and endothelial cells undergo adhesion-regulated programmed cell death [Meredith et al., 1993; Frisch and Francis, 1994; Re et al., 1994]. Detachment of cells from the ECM results in loss of integrin signaling and alteration of cell cycle patterns [Wary et al., 1996; Assoian, 1997; Assoian and Zhu, 1997].

Cell cycle progression is governed by specific activation and subsequent inactivation of cyclin-dependent kinases (cdks). Cdks are positively regulated by cyclins [Morgan, 1995] whose activity is regulated by cyclin binding, by phosphorylation or dephosphorylation and by associated inhibitory molecules. In particular, cell cycle progression through G1 into S phase is regulated by the activities of D-type cyclin, E-type cyclin, and A-type cyclin associated with cdk4/cdk6, and cdk2. The activity of cyclin/cdk complexes is negatively regulated by

Abbreviations used: EGF, epidermal growth factor; cdk, cyclin-dependent kinase; CKI, cdk-dependent kinase inhibitor; pRb, retinoblastoma protein; ECM, extracellular matrix.

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two families of cyclin-dependent kinase inhibitors (CKIs), which are known to bind to and inhibit the activity of cdks [Sherr and Roberts, 1995]. The INK4 family (p15, p16, p18, and p19) inhibits cdk4 and cdk6, while the Cip/Kip family (p21, p27, and p57) binds to and inactivates all cyclin-cdk complexes [El-Deiry et al., 1993; Xiong et al., 1993; Hengst et al., 1994; Polyak et al., 1994; Matsuoka et al., 1995]. Several studies have indicated that cell cycle progression requires signals provided by both growth factors and ECM. Adhesion to substratum is required for anchorage-dependent cell cycle progression. Cyclin A is strongly dependent on signals from ECM [Schulze et al., 1996], and cyclin D1 is the primary D type cyclin for several anchorage-dependent cell types [Zhu et al., 1996]. Cyclin E-cdk2 kinase activity is strongly growth factor-dependent and adhesion-dependent, while expression of p21 increases as cell adhesion decreases [Böhmer, et al., 1996; Zhu et al., 1996; Fang et al., 1996; Wu and Schönthal, 1997].

Integrin-mediated adhesion to ECM plays an important role in regulating cell survival and proliferation [Meredith et al., 1995]. This anchorage-dependent growth requires integrin-mediated signaling generated by cellular contact with ECM for regulation of cell cycling [Hansen et al., 1994; Giancotti, 1997; Assoian, 1997; Howe et al., 1998]. Cadherins are cell surface membrane proteins that mediate homotypic calcium-dependent cell-cell adhesion [Takeichi, 1991], mediate cell-cell contacts, and regulate cell growth and apoptosis [Kantak and Kramer, 1998]. Proliferation regulated by cadherin-mediated cell-cell contacts is linked to alteration in cyclin-dependent kinase activity [Croix, et al., 1996; Croix, et al., 1998]. Recent studies have shown that epidermal growth factor receptor (EGF-R) modulates E cadherin-dependent adhesion [Hazan and Norton, 1998].

EGF is a potent mitogen for most epithelial tissues [Rheinwald and Green, 1977]. It induces tyrosine phosphorylation of EGF-R, activates intracellular signal transduction and enhances transcription of growth-related genes [Ullrich and Schlessinger, 1990]. However, in squamous carcinoma A431 cells, which possess a high number of EGF-R [Gill and Lazar, 1981; Barnes, 1982; Kawamoto et al., 1983], low concentrations of EGF stimulates cell growth, while high concentrations inhibit proliferation, arrest cell cycle, alter cdk2 activity by p21, and induce cell apoptosis [Kawamoto et al., 1984; Buday and Downward, 1993; Stampfer et al., 1993; Fan et al., 1995; Chin et al., 1996; Jakus and Yeudall, 1996]. The mechanisms are unclear. It is known that Stat1 activation is required for EGF-induced growth inhibition [Chin et al., 1996; Bromberg et al., 1998] and that activation of EGF-R tyrosine kinase activity is involved in growth inhibition and apoptosis [Gulli et al., 1996].

To understand EGF-mediated cell cycle arrest and apoptosis, we investigated the mechanisms of cell cycle arrest and adhesion-regulated programmed cell death modulated by the growth factor. We demonstrated that EGF reduces cell adhesion, which leads to apoptosis and growth suppression through induction of p21 and reduction of cyclin A, D1, cdk2, and pRb expression.

MATERIALS AND METHODS

Cells and Reagents

The A431 human epithelioid squamous carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD). A431 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) at 37°C in an incubator containing 5% CO_2.

Antibodies to the $p21^{waf1/Cip1}$ (clone F-5, mouse monoclonal IgG2b), p27 (clone F-8, mouse monoclonal IgG1), p53 (clone DO-1, mouse monoclonal IgG2a), cdk2 (clone D-12, mouse monoclonal IgG1), cyclin A (clone H-432, rabbit affinity-purified polyclonal antibody), cyclin D1 (clone R-124, mouse monoclonal IgG1), cyclin E (clone HE12, mouse monoclonal IgG2b), retinoblastoma protein (pRb) (clone C-15, goat affinity-purified polyclonal antibody), and phosphotyrosine (clone PY99, mouse monoclonal IgG2b) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). EGF, type II collagen, propidium iodide (PI) and all chemicals were obtained from Sigma (St. Louis, MO). All tissue culture plates were purchased from Nalge Nunc International (Naperville, IL).

Cell Proliferation Assay

A431 cells were seeded on 6-well tissue culture plates (monolayer culture) and 1.5% aga-

rose gel-coated 6-well tissue culture plates (suspension culture) at a density of 2×10^5 cells/well in DMEM containing 10% FBS at 37°C in a tissue culture incubator containing 5% CO_2. The cells were treated with different concentrations of EGF (0, 0.01, 0.1, 1, 10, and 20 ng/ml) for 24 h. The cells were then harvested; a Coulter counter was used to count viable cells (as gauged by Trypan blue exclusion). In all studies described in the report, experiments were performed in triplicate, and the results were analyzed statistically using the *t*-test.

Cell Attachment Assay

A431 cells were seeded in 6-well tissue culture plates at a density of 2×10^5 cells/well as above. The cells were treated with varying concentrations of EGF (0, 0.01, 0.1, 1, 10, and 20 ng/ml). After incubation for 1 h, the plates were gently washed three times with PBS. Nonadherent cells were removed by aspiration, and adherent cells were counted with an inverted light microscope. Cell adhesion was normalized to the number of adherent cells under control conditions (no EGF). The cell number in each well was determined using a cytometer. Cell adhesion was expressed as follows:

$$\text{Cell adhesion } (\%) = \text{T/C}$$

where T is the number of cells attaching to the plates, and C is the total cell number.

To enhance cell attachment, cells were seeded as above in wells, which had been precoated with collagen (0, 0.1, 1, 10, 100, or 1,000 μg/ml) overnight at 4°C. The attachment of cells was examined as above.

Aggregation Assay

A431 cells were seeded onto 1.5% agarose gel-coated 6-well tissue culture plates (suspension culture), at a density of 2×10^5 cells/well as above. The cells were treated with different concentrations of EGF (0, 0.01, 0.1, 1, 10, or 20 ng/ml) for 24 h. Cell aggregation was visualized using an inverted light microscope. The conglomerates were removed by passing the cultures through cell strainers (40 μm). The individual cells were resuspended in culture medium, and cell number was determined using a cytometer. Cell aggregation was expressed as follows:

$$\text{Aggregation } (\%) = (\text{C} - \text{T})/\text{C}$$

where C is the total cell number, and T is the number of individual cells.

Cell Spreading Assay

Cells were seeded onto 6-well tissue culture plates at a density of 2×10^5 cells/well in DMEM containing 10% FBS at 37°C in the absence or presence of EGF (10 ng/ml). Cells were fixed at various time points with 2% paraformaldehyde in PBS for 30 min. Cell spreading was visualized using an inverted light microscope.

Cell Cycle and Apoptosis Assays

A431 cells were plated on 6-well tissue culture plates (monolayer culture) or 1.5% agarose gel-coated 6-well tissue culture plates (suspension culture), at a density of 2×10^5 cells/well as above. The cells were treated with different concentrations of EGF (0, 0.01, 0.1, 1, 10, and 20 ng/ml). After 24 h, levels of apoptosis and cell cycle were analyzed by DNA labeling with propidium iodide (PI) and flow cytometric analysis as described earlier [Nicoletti et al., 1991]. Briefly, the cells were collected with trypsin/EDTA, pelleted by centrifugation and resuspended in 1 ml hypotonic PI solution (50 μg/ml) dissolved in 0.1% sodium citrate plus 0.1% Triton X-100 (Sigma). The cells were measured using a FACScan (Becton Dickinson). To test the effect of cell adhesion on cell cycle patterns and cell apoptosis, 2×10^5 cells were seeded to 6-well tissue culture plates coated with type II collagen at concentrations of 0, 0.1, 1, 10, 100, and 1,000 μg/ml overnight at 4°C. Cell cycles and apoptosis were analyzed as above.

Western Blot Assay

Cells were lysed in ice-cold lysis buffer (0.1% NP-40, 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.02 mg/ml each of aprotinin, leupeptin, and pepstatin). The lysates were sonicated and cleared by centrifugation; the samples were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto a nitrocellulose membrane (Bio-Rad) in 1× TG buffer (Amresco) containing 20% methanol. The membrane was blocked in TBST (10 mM Tris-Cl, pH

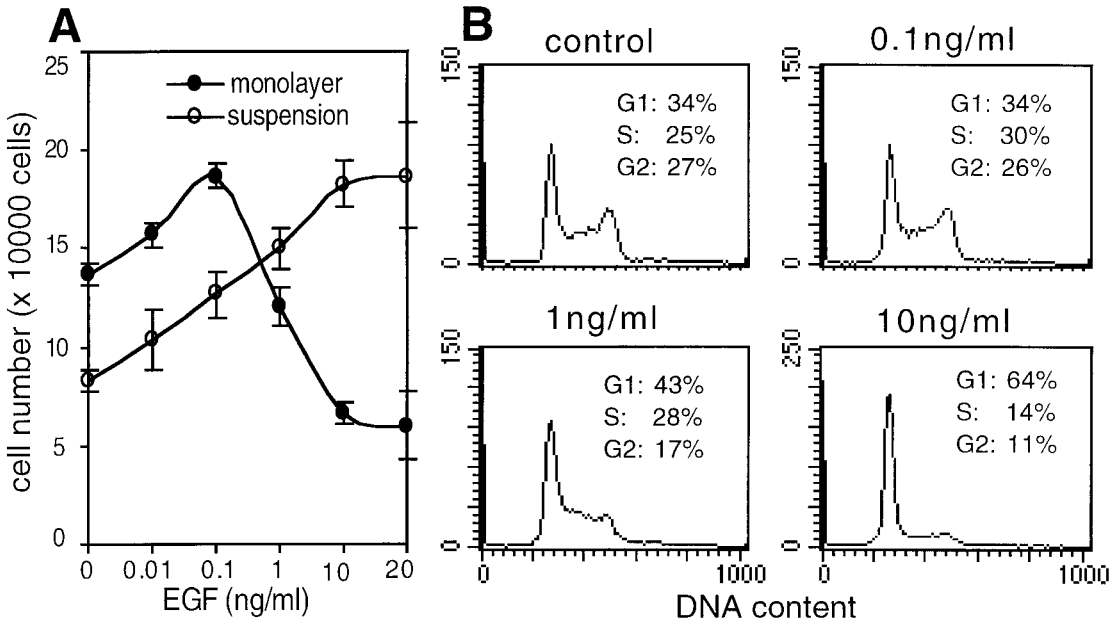

Fig. 1. Epidermal growth factor (EGF) enhanced proliferation of suspension cultures but inhibited proliferation of monolayer cultures at high concentrations (>1 ng/ml). **A:** A431 cells grown as monolayer or in suspension were treated with EGF for 24 h, then viable cells quantified by Trypan blue exclusion. **B:** After 36-h treatment with EGF, monolayer cells were stained with propidium iodide and analyzed for DNA content by flow cytometry.

8.0, 150 mM NaCl, 0.05% Tween 20) containing 10% nonfat dry milk powder (Carnation) (TBSTM) for 1 h at room temperature, and then incubated with primary antibodies at 4°C overnight. The membranes were washed with TBST (3 × 30 min) and then incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies in TBSTM for 1 h. After washing as above, the bound antibodies were visualized with an ECL kit according to the manufacturer's instructions (Amersham).

RESULTS

Opposing Effects of EGF on Cell Growth and Apoptosis in Monolayer and Suspension Cultures

Previous studies have reported that high concentrations of EGF caused growth inhibition, cell cycle arrest and apoptosis of A431 cells [Gill and Lazar, 1981; Barnes, 1982; Kawamoto et al., 1983; Fan et al., 1995; Jakus and Yeudall, 1996; Gulli et al., 1996], while low concentrations of EGF promoted cell proliferation [Kawamoto et al., 1984]. To investigate the mechanisms, by which EGF produces these effects, we treated monolayer and suspension culture with EGF at a range of concentrations to determine its effect on adhesion-dependent and -independent cultures. In suspension cultures, EGF enhanced cell proliferation in a dose-dependent manner at concentrations up to 20 ng/ml. In the monolayer cultures, high concentrations of EGF (≥1 ng/ml) inhibited cell growth, while low concentrations (0.01–0.1 ng/ml) of EGF promoted cell growth (Fig. 1A). Correspondingly, cell cycle was arrested in G1 phase by high concentrations of EGF in monolayer cultures (1 and 10 ng/ml of EGF) (Fig. 1B). In cell apoptosis assays, high concentrations of EGF (≥1 ng/ml) enhanced apoptosis of monolayer cells greatly while the apoptosis of suspension cultures decreased, perhaps due to the enhanced proliferation of the cultures (Fig. 2A). Monolayer cultures treated with 10 ng/ml EGF showed altered morphology compared with untreated cultures (Fig. 2B, top) and 24% of the cells underwent apoptosis (Fig. 2B, bottom).

The altered cell morphology suggested that EGF-induced apoptosis was associated with cell adhesion. To investigate this, we performed cell attachment assays. Cells were incubated with EGF (10 ng/ml) and the mixture was seeded to tissue culture plates. After 1 h, unattached cells were removed as described in

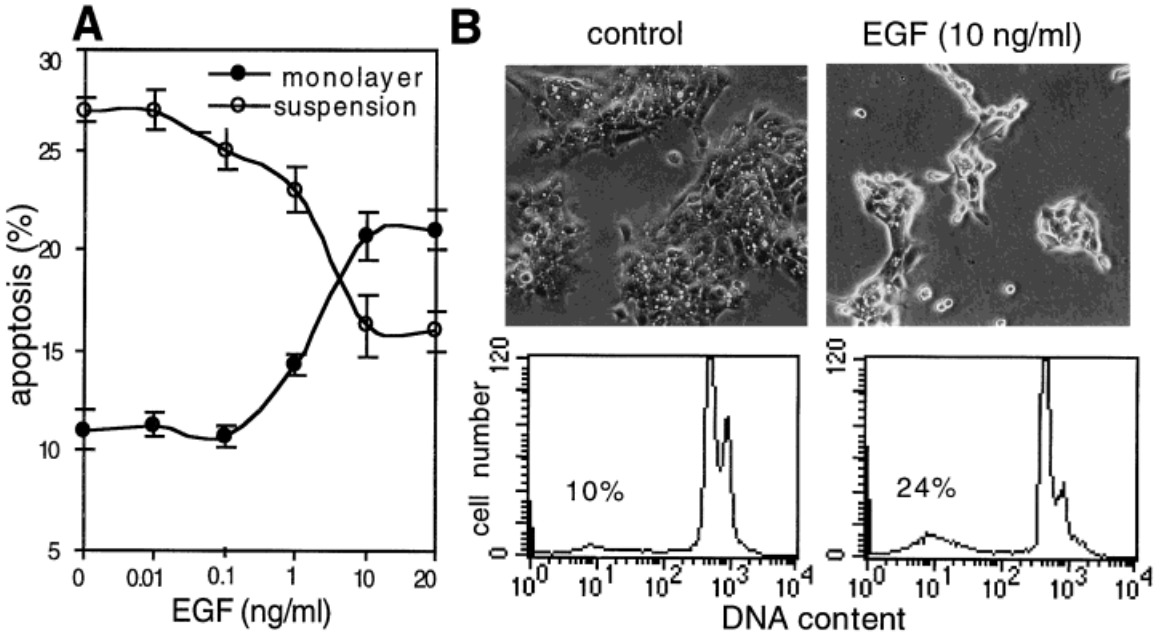

Fig. 2. Apoptosis induced in monolayer cultures and reduced in suspension cultures by epidermal growth factor (EGF). **A:** Monolayer cultures and suspension cultures, treated with different concentrations of EGF for 24 h, were analyzed with FACScan. In monolayer cultures treated with high concentrations of EGF, cell apoptosis was enhanced, but it was reduced in suspension cultures. **B:** Effects of 24-h EGF treatment on cell morphology (top) and on apoptosis (bottom). EGF (10 ng/ml) enhanced apoptosis and altered cell morphology.

the Methods section. This incubation inhibited cell attachment (Fig. 3A, top) and statistical analysis indicated that the effect of EGF (≥1ng/ml) was significant and dose dependent (Fig. 3B). In suspension cultures, EGF inhibited homotypic cell aggregation resulting in smaller and looser conglomerates (Fig. 3A, bottom). Interestingly, concentrations that inhibited cell attachment also significantly suppressed cell growth and enhanced cell apoptosis (≥1 ng/ml). We then examined the time course of the effects of EGF effects in cell spreading assays and found that 10 ng/ml EGF inhibited cell spreading and cell-cell interaction (Fig. 4A). Normally, A431 cells spread on tissue culture plates within 1 h of incubation, and cell-cell contact could be observed after 12 h. However, EGF treatment interfered with cell spreading and cell-cell contact at early time points, and apoptosis did not increase until 12 h had passed (Fig. 4B). These results implied that cell adhesion was affected well before apoptosis accelerated.

We then analyzed the effects of different EGF concentrations on cell cycle and the expression of cell cycle-associated proteins. In monolayer cultures, cell cycle was arrested in the G1 phase by high concentrations of EGF (≥1 ng/ml) (Fig. 5A). In suspension cultures, more cells stayed in S + G2 phases when incubated with high concentrations of EGF, while the number of cells in G1 phase decreased in a dose-dependent manner (Fig. 5B). Cell cycle transitions are governed by positive and negative regulators of cdks, and previous studies have indicated that EGF arrests cell cycle progression by increasing p21 activity and thereby inhibiting cdk2 activity [Fan et al., 1995; Chin et al., 1996; Jakus and Yeudall, 1996]. We further examined whether treatment with different concentrations of EGF could regulate expression of these proteins, and four types of results were observed: (1) the basal expression of p21 and p-Tyr (tyrosine kinase activity activated by EGFR) in monolayer culture of the A431 cells was not detected, but was strongly elevated by treatment with high concentrations of EGF (≥1 ng/ml); (2) the expression of cdk2 and cyclin A was greatly reduced; while (3) expression of cyclin D1 and pRb protein was weakly reduced in cultures incubated with high concentrations of EGF (≥1 ng/ml); and (4) the levels of p53 and cyclin E were not affected by EGF treatment (Fig. 6A). In the adhesion-

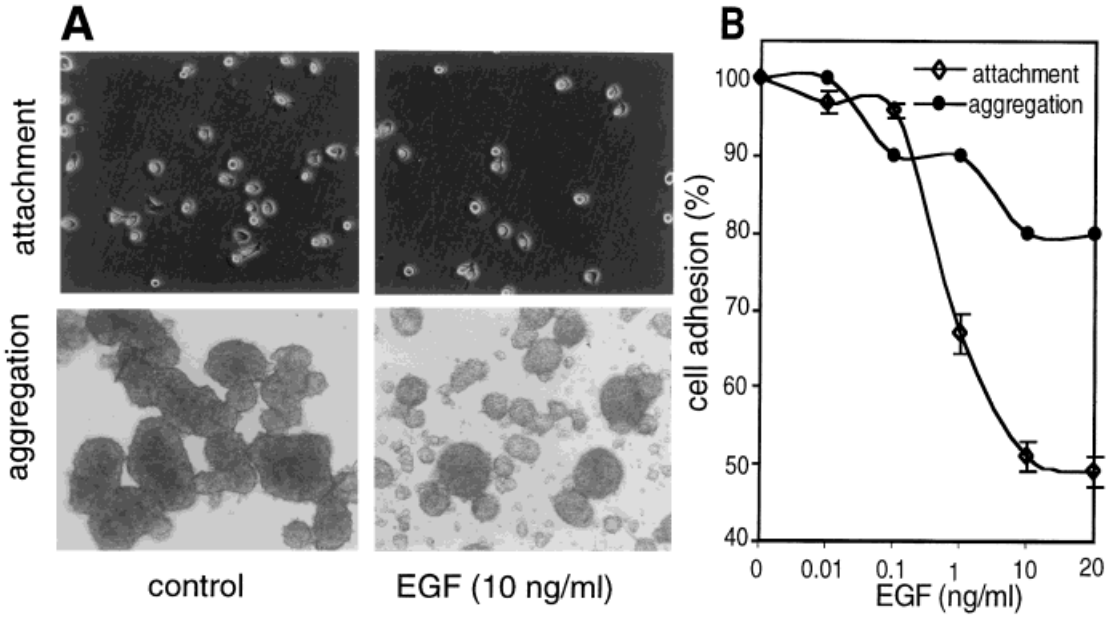

Fig. 3. Epidermal growth factor (EGF) inhibited cell attachment in monolayer cultures and cell aggregation in suspension cultures. **A:** A431 cells were maintained as monolayer cultures (top) and suspension cultures (bottom) as above. Cells were treated with or without EGF at a concentration of 10 ng/ml. Monolayer cells were incubated at 37°C for 1 h, then gently washed three times with PBS. Adherent cells were counted on an inverted light microscope. Treatment with EGF inhibited cell attachment. In suspension cultures treated at 37°C for 24 h, EGF inhibited cell aggregation. **B:** Effects of EGF on cell adhesion were analyzed statistically. At 1 ng/ml and higher, EGF inhibited cell attachment and cell aggregation significantly, compared with untreated control cells.

independent suspension culture, the effects of EGF on these proteins were analyzed. The result of one protein from each of the above group was shown in Figure 6B. The levels of p21 and cyclin A were not significantly altered after EGF treatment, but the levels of cyclin E were induced. Expression of cyclin D1 was not changed (Fig. 6B). As well, we did not observe any change of the other proteins except the p-Tyr, which was slightly induced (data not shown). Interestingly, the concentrations of EGF that arrested cell cycle and inhibited cell growth in monolayer cultures (as indicated by the expression of the appropriate regulatory proteins) were those concentrations, which reduced cell adhesion. For this reason, we sought to determine whether we could prevent apoptosis by blocking the loss of cell adhesion in the presence of EGF.

Effects of EGF on Cell Proliferation, Apoptosis, and Cell Cycle Arrest Are Modulated by Collagen

The findings that high concentrations of EGF induced apoptosis and arrested cell cycles in monolayer cultures but not in suspension cultures, and that inhibition of cell attachment and cell spreading by high concentrations of EGF, was an early event which occurred far before cells underwent apoptosis, strongly suggested that EGF induced apoptosis through reducing cell adhesion. To investigate how EGF exerted its effect, we developed a method to enhance cell attachment and cell spreading by culturing the A431 cells on collagen-coated plates. We found that attachment of A431 cells to collagen-coated plates was slightly higher in comparison to the uncoated plates, since A431 cells attach to untreated plates very well (Fig. 7). However, in the presence of 10 ng/ml EGF, cell attachment to untreated plates was significantly reduced, but attachment to collagen-coated plates was not affected (Fig. 7).

We further investigated whether or not increased cell adhesion suppressed EGF-induced apoptosis. We found that addition of EGF to cultures 4 h after cell inoculation (after cells had spread on the plates) had minimum effect on apoptosis of cells that had already spread on the collagen-coated plates (Fig. 8A, after). The addition of EGF before cell spreading (EGF mixed with cell inoculum) had a reduced effect

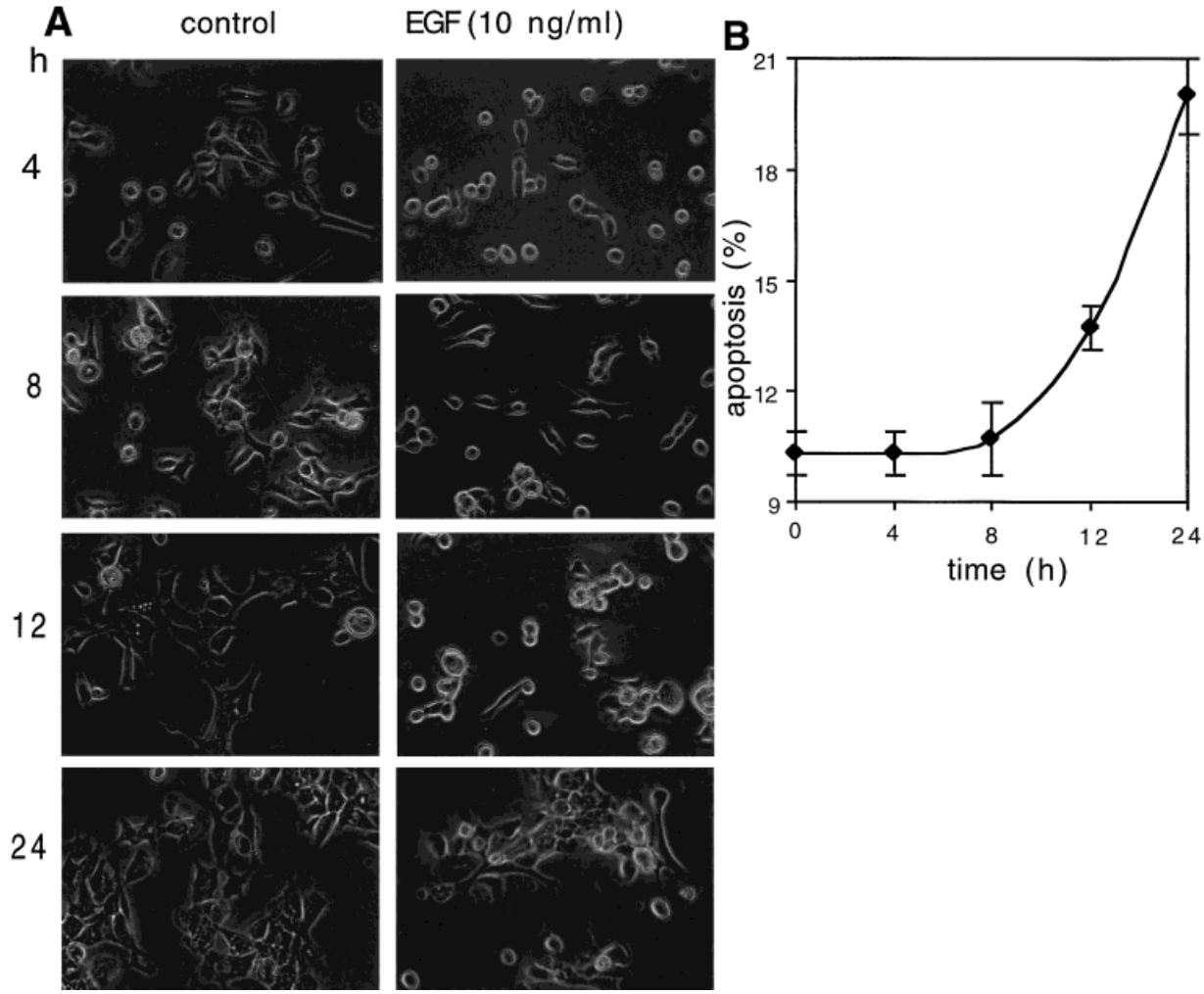

Fig. 4. Inhibition of cell spreading by epidermal growth factor (EGF). A431 cells were seeded to 6-well tissue culture plates at a density of 2×10^5 cells/well with or without EGF (10 ng/ml). **A:** Cells were fixed with 2% paraformaldehyde in PBS at the time points indicated. Cell spreading was visualized using an inverted light microscope. EGF inhibited cell spreading and reduced cell-cell interactions. **B:** Cell apoptosis was analyzed in the cultures with inhibited spreading. Cell apoptosis increased only after 12 h of treatment with EGF.

on apoptosis of cells inoculated on collagen-coated plates as compared with cells inoculated on normal tissue culture plates (Fig. 8A, before). The results indicated that the effect of EGF on cell apoptosis was strongly associated with cell adhesion. Statistical analysis showed that cell apoptosis was significantly lower in cultures seeded on collagen-coated plates than in cultures seeded on normal tissue culture plates (Fig. 8B).

To assay the dose dependence of the effect, collagen was coated on tissue culture plates at different concentrations, and cell attachment was tested. We confirmed that the adhesion of A431 cells decreased as the concentrations of collagen decreased (Fig. 9A, control). Importantly, the decrease in cell adhesion was amplified by EGF treatment (Fig. 9A, EGF), and loss of cell attachment was blocked by increased concentrations of collagen (Fig. 9A). In plates coated with collagen at a concentration of 1 mg/ml, EGF had no effect on cell adhesion as compared with the control cultures. As expected, apoptosis was suppressed by collagen in a dose-dependent fashion through enhanced cell adhesion (Fig. 9B). These results suggested that adhesion-regulated programmed cell death by EGF was the consequence of reduction of cell attachment, cell spreading and cell-cell contact.

We further investigated whether cell cycle arrest and alteration of the expression of cell

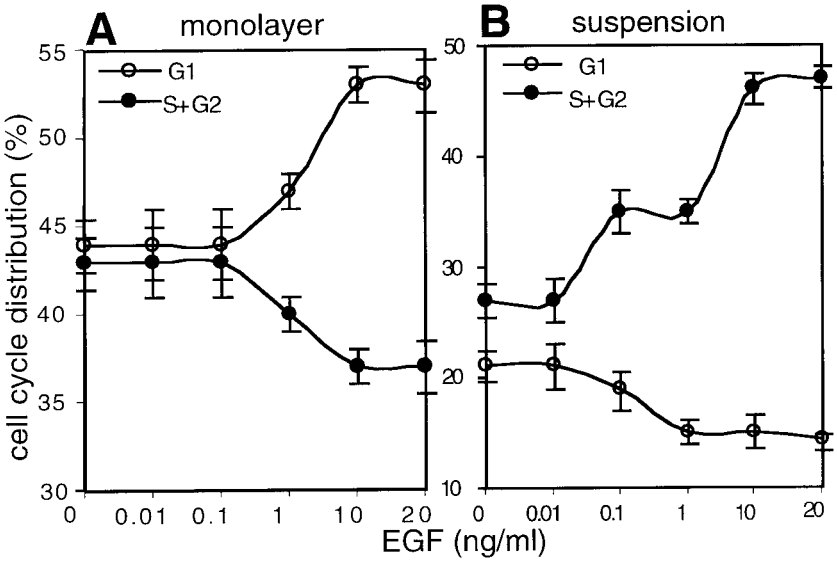

Fig. 5. Length of cell cycle altered by epidermal growth factor (EGF) in monolayer and suspension cultures. Monolayer (**A**) and suspension (**B**) cultures were incubated with EGF at 37°C for 24 h. The cells were harvested by trypsinization (monolayer cultures) or centrifugation (suspension cultures) and analyzed for DNA content using flow cytometry after staining with propidium iodide. Treatment of monolayer cultures with higher concentrations of EGF resulted in cell cycle arrest in G1 phase and reduced cell numbers in S and G2 phases (**A**). In the suspension cultures, EGF treatment reduced the number of cells in G1 phase and increased the number of cells in S and G2 phases (**B**).

cycle-associated proteins by EGF could be affected by changes in cell adhesion. We used the above system to maintain cultures in different adhesion states by coating the tissue culture plates with different concentrations of collagen. When collagen concentrations increased, EGF had less effect on cell adhesion and the patterns of cell cycles changed accordingly. These cell cycle effects were dose-dependent (Fig. 10A,B,C). At low collagen concentrations, treatment with EGF caused more cells to be arrested in G1, compared with cultures not exposed to EGF (Fig. 10A). In addition, fewer EGF-treated cells were found in S phase and G2 + M phases, compared with control (Fig. 10B,C). At high concentrations of collagen, EGF had a much smaller effect, and more treated and untreated cells were found in S and G2 + M phases. Altered expression of cell cycle-associated proteins by EGF treatment was reversed by increased concentrations of coating collagen (Fig. 11). Expression of p21 decreased in the presence of EGF (10 ng/ml) when the plates were coated with collagen at a concentration of 10 μg/ml, and it was undetectable at collagen concentrations of 100 and 1,000 μg/ml. Expression of cyclin A, cyclin D1, cdk2, and pRb increased slightly in EGF-treated cultures as the concentrations of collagen increased (≤1 mg/ml), reaching the same levels as the control cultures (without EGF). Interestingly, tyrosine phosphorylation of EGF receptor was not affected by EGF treatment on different concentrations of coating collagen (Fig. 11).

Direct demonstration of the importance of cell adhesion in cell apoptosis and cell cycle arrest was obtained in an extreme case where cell adhesion was completely disrupted. A431 cells were seeded at a low density of 2×10^5 cells/plate to 100-mm tissue culture plates (monolayer culture) or bacterial Petri dishes (suspension culture, similar to agarose-coated plates), which lack a hydrophilic treatment. Each bacterial Petri dish has a surface area of 59.4 cm^2, and each well of the 6-well tissue culture plates has a surface area of 9.6 cm^2. Thus, the cell density in bacterial Petri dishes was 6.2-fold lower than that in the 6-well plates (Fig. 1). In the bacterial Petri dish cultures, cell attachment to plates was abolished and, due to low cell densities, cell-cell interaction was minimized. Analysis of apoptosis indicated that 21% of the cells in suspension underwent apoptosis compared with 12% apoptosis observed in normal tissue culture plates after 24 h of incubation (Fig. 12, top). Arrest of the suspension culture in G1 phase was not as obvious but the number of cells in S and G2 phase was much less as compared with the monolayer cultures (Fig. 12, bottom). The results suggested that reduced cell adhesion might induce cell cycle arrest and apoptosis, and this is consistent with the hypothesis that EGF induces cell cycle arrest and apoptosis by interfering with cell adhesion.

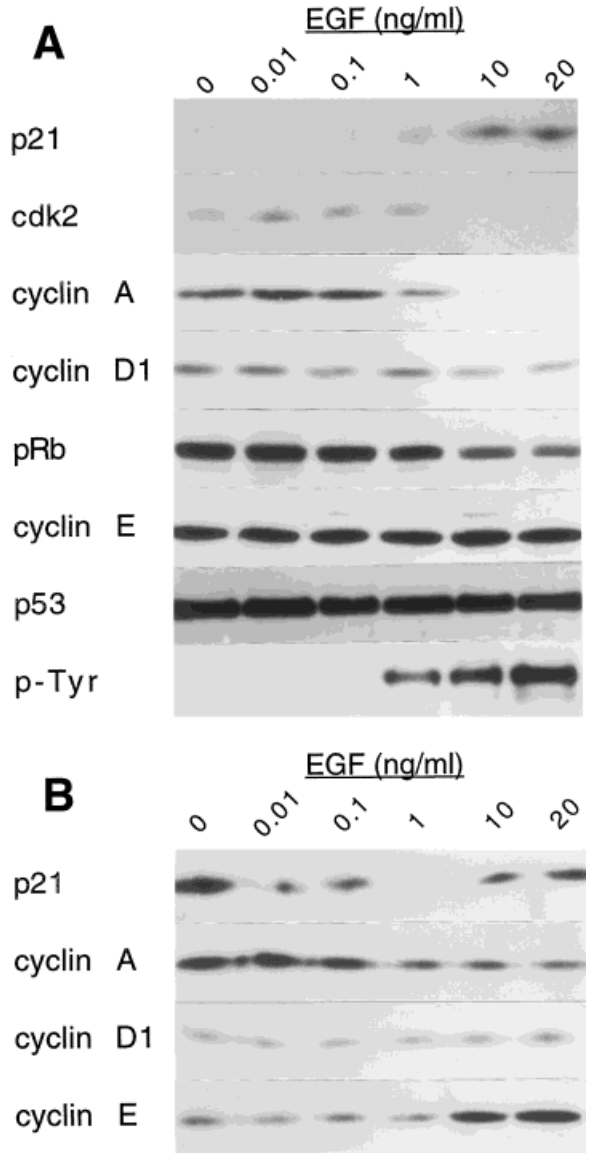

Fig. 6. Changes in expression of cell cycle proteins in monolayer and suspension cultures after epidermal growth factor (EGF) treatment. A431 cells were plated on 6-well tissue culture plates (**A**, monolayer cultures) or 1.5% agarose gel coated 6-well tissue culture plates (**B**, suspension cultures), at a density of 2×10^5 cells/well. Cell lysates were analyzed by immunoblot after treatment with EGF for 24 h. In monolayer cultures, EGF stimulated $p21^{waf1/Cip1}$ and p-Tyr expression, but suppressed expression of cdk2, cyclin A , cyclin D1, and retinoblastoma protein. In suspension cultures, EGF increased cyclin E expression, reduced the expression of p21 and cyclin A slightly, but had no effect on the expression of D1.

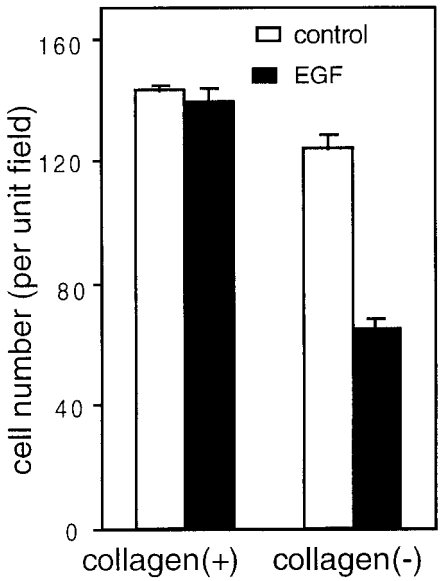

Fig. 7. Epidermal growth factor (EGF)-induced loss of cell adhesion was prevented in monolayer cultures grown on collagen II-coated plates. 2×10^5 A431 cells were seeded on 6-well tissue culture plates (collagen −) and plates coated with type II collagen (1 mg/ml, 4°C, overnight, collagen +). Cultures in the plates were incubated with or without EGF (10 ng/ml) at 37°C for 1 h, and cell adhesion was analyzed. EGF had little effect on cell adhesion on collagen-coated plates compared with normal tissue culture plates.

DISCUSSION

The A431 cell line is a unique model system to investigate the molecular mechanisms associated with EGF-induced growth inhibition, cell cycle arrest and apoptosis although such EGF effects have not yet been detected in vivo. As a research model, A431 cells have attracted much attention and led to insight into the mechanisms by which EGF signaling occurs. Previous studies have indicated that EGF inhibits the proliferation of A431 cells, which possess high numbers of EGF receptors on their cell surface [Gill and Lazar, 1981; Barnes, 1982; Kawamoto et al., 1983]. In addition, EGF arrests cell cycle progression by inhibiting cdk2 activity via p21 [Fan et al., 1995; Chin et al., 1996; Jakus and Yeudall, 1996], and activates EGF-R mediated tyrosine kinase activity leading to growth inhibition and apoptosis [Gulli et al., 1996]. Our data provide new insights into the mechanism by which EGF induces growth inhibition, cell cycle arrest and apoptosis. In this study, we used monolayer and suspension culture systems to elucidate the mechanism of EGF-mediated growth inhibition, cell cycle arrest, and apoptosis on A431 cells. In the adhesion-dependent monolayer culture, high concentrations of EGF (≥1 ng/ml) inhibited cell proliferation and arrested cell cycle progression, while low concentrations of EGF induced cell proliferation. The results are in agreement

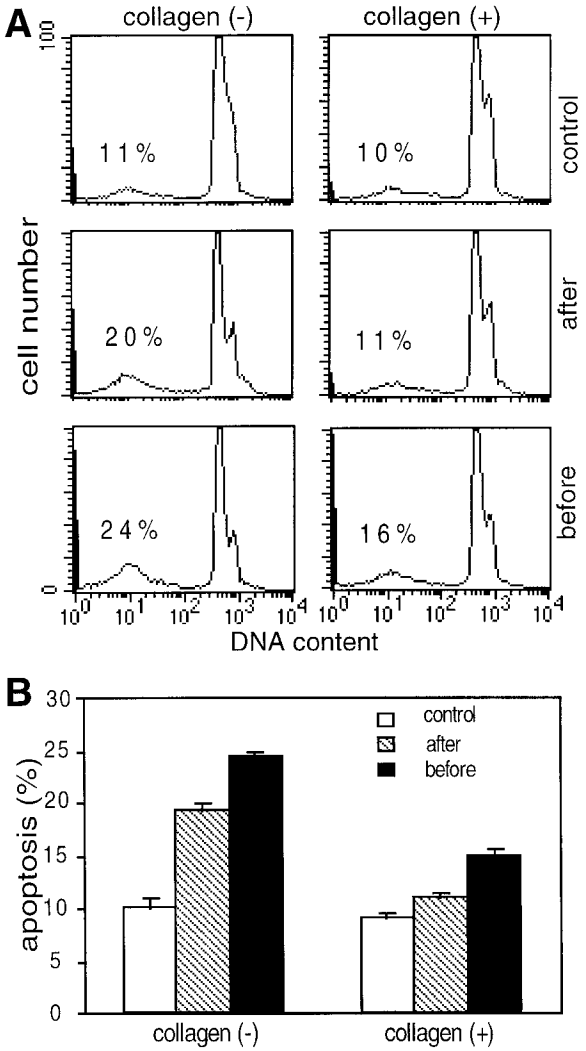

Fig. 8. Collagen protects against epidermal growth factor (EGF)-induced apoptosis of monolayer cultures. A431 cells were seeded on 6-well tissue culture plates (collagen −) and plates precoated with collagen II plates (1 mg/ml, 4°C, overnight, collagen +) at a density of 2×10^5 cells/ml. EGF (10 ng/ml) was added either after cells had attached to plates (4 h after incubation, "after") or immediately before cells were plated ("before") and incubated for 24 h. **A:** Typical set of results from one trial. Coating with collagen had little effect on cell apoptosis in cultures lacking EGF. Addition of EGF after cell spreading enhanced cell apoptosis on tissue culture plates but had no effect on cell spreading on type II collagen. Addition of EGF before cell spreading induced increased apoptosis on tissue culture plates but the effect was less pronounced in cells plated on collagen-coated plates. **B:** The assays were repeated three times and analyzed statistically. Type II collagen had a significant effect on EGF-induced cell apoptosis ($n = 4$, $p < 0.01$).

with those reported previously [Gill and Lazar, 1981; Barnes, 1982; Kawamoto et al., 1983, 1984]. However, in adhesion-independent suspension cultures, EGF enhanced cell proliferation, and this effect was dose-dependent at all concentrations used (≤20 ng/ml). In the monolayer culture, high concentrations of EGF (≥10 ng/ml) induced apoptosis, but EGF suppressed apoptosis in suspension culture.

On the basis of our data, we hypothesized that reduction of proliferation and induction of apoptosis by high concentrations of EGF were associated with changes in cell adhesion since apoptosis in suspension cultures was not enhanced by high concentrations of EGF. To test this, we investigated the effect of different concentrations of EGF on cell attachment, spreading and cell-cell interaction. We found that high concentrations of EGF (10 ng/ml), which stimulated apoptosis in monolayer cultures, also reduced cell attachment, cell spreading and cell-cell interactions. However, low concentrations of EGF, which enhanced cell proliferation and had no effect on cell apoptosis, attachment, spreading, and cell-cell interaction. It should be noted that, when culture medium obtained from the apoptotic monolayer cells was applied to suspension cultures, it did not induce apoptosis in the latter (data not shown) indicating that it is unlikely that EGF functions by stimulating cells to release toxic apoptotic products in cultures. Our hypothesis and results are strongly supported by recent studies using MDA-MB-468 breast cancer cells: Kottke et al. [1999] reported that reduction of adhesion is associated with EGF-induced apoptosis of these cells.

To determine whether reduction of adhesion led to changes in cell cycle regulation, we manipulated cell adhesion by coating tissue culture plates with different concentrations of collagen. Cell attachment was enhanced on collagen II-coated plates. Treatment with EGF reduced cell attachment to normal tissue culture plates, but had little effect on cell attachment and spreading on collagen II-coated plates. Increased cell adhesion suppressed EGF-induced apoptosis and decreased cell adhesion enhanced apoptosis. It was recently reported that collagen interacts with integrin and enhances cell proliferation [Pozzi et al., 1998], and it is possible that this effect may counteract the effects of EGF on apoptotic and anti-proliferative, accounting in part for the results seen in the experiments using collagen-coated plates.

It has long been known that cell survival and proliferation are modulated by cell adhesion [Ruoslahti, 1997; Chen et al., 1997]. Integrins

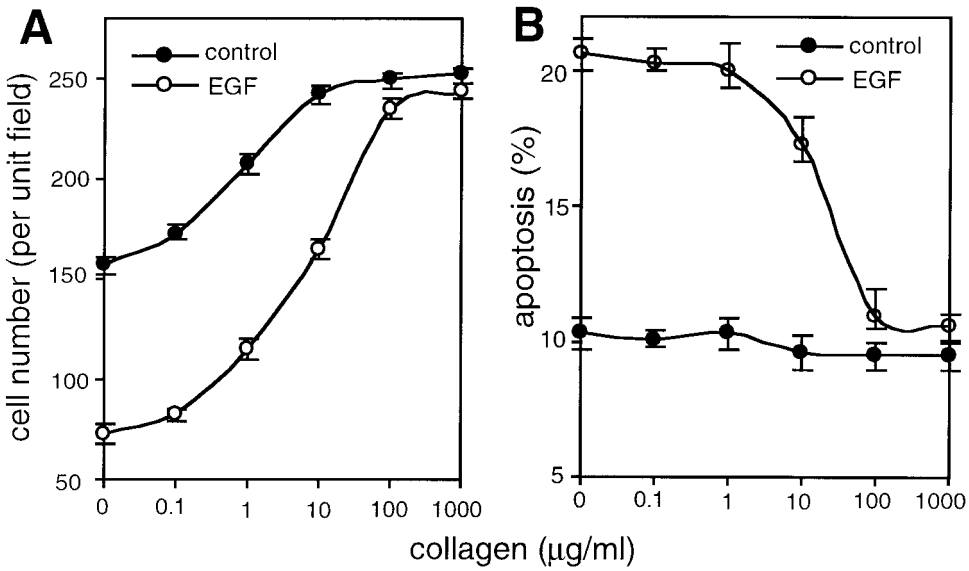

Fig. 9. Concentration-dependent effects of collagen coating on epidermal growth factor (EGF)-mediated adhesion and apoptosis. **A:** A431 cells were seeded at 2×10^5 cells/well in 6-well tissue culture plates coated with type II collagen at the concentrations indicated. The cultures were incubated with or without 10 ng/ml EGF at 37°C for 1 h. Cell adhesion was reduced at low collagen concentrations (control), and this effect was amplified when the cultures were incubated with 10 ng/ml EGF. As the concentration of collagen increased, the effects of EGF were abolished. **B:** Cells were harvested after 24 h of incubation and analyzed with FACScan. Collagen coating of tissue culture plates had little effect on apoptosis of cells under control conditions. However, apoptosis was greatly enhanced at low collagen concentrations. As concentrations increased, the effects of EGF were completely suppressed.

are cell surface receptors which anchor cells to matrix and suppress cell apoptosis and support cell proliferation. Loss of matrix adhesion triggers cell apoptosis [Ruoslahti and Reed, 1994; McGill et al., 1997]. Cadherins are cell surface membrane proteins which mediate homotypic calcium-dependent cell interactions and regulate cell growth and apoptosis [Takeichi, 1991, Kantak and Kramer, 1998]. They are also linked to cyclin-dependent kinases [Croix, et al., 1996, 1998]. In A431 cells, EGF interacts with EGF receptor (EGFR) inducing rapid rounding and morphological change of the cells (Chinkers et al., 1979, 1981]. Activation of the EGF receptor tyrosine kinase induces tyrosine phosphorylation of β-catenin and plakoglobin, and correlates with decreased cell-cell adhesion and cell migration [Hoschuetzky et al., 1994; Shibamoto et al., 1994]. A recent study has shown that EGF-R directly modulates E cadherin-dependent adhesion [Hazan and Norton, 1998].

Recent reports suggest that the cdk inhibitor p21 accumulates in the EGF-treated A431 cells [Fan et al., 1995; Chin et al., 1996; Jakus and Yeudall, 1996]. In our study, we found that not only did EGF induce p21, but it also reduced cdk2, cyclin A and D1. However, cyclin E, p53 and p27 were not altered by EGF treatment. Interestingly, in adhesion-independent cultures, p21 was not altered, but expression of cyclin E was enhanced. These data are consistent with EGF inhibiting growth and arresting cell cycle in the adhesion-dependent monolayer culture, but not in the adhesion-independent suspension culture. They also suggested that expression of p21 may be affected by cell adhesion. Further support came from our studies on plates coated with different concentrations of collagen, to which EGF was added at a concentration of 10 ng/ml. We demonstrated that expression of p21 decreased when the collagen concentrations increased. Our results implied that expression of p21 was negatively correlated with cell adhesion. As cell adhesion increased (on plates coated with high concentrations of collagen), expression of p21 decreased. On the other hand, tyrosine phosphorylation of EGF receptor was not affected by changing the concentrations of collagen. These results confirmed that tyrosine phosphorylation of EGF receptor was a product of EGF treatment and not an effect of cell adhesion. The other cell cycle associated proteins, such as cyclin A, cyclin D1, cdk2, and pRb, weakly increased as the

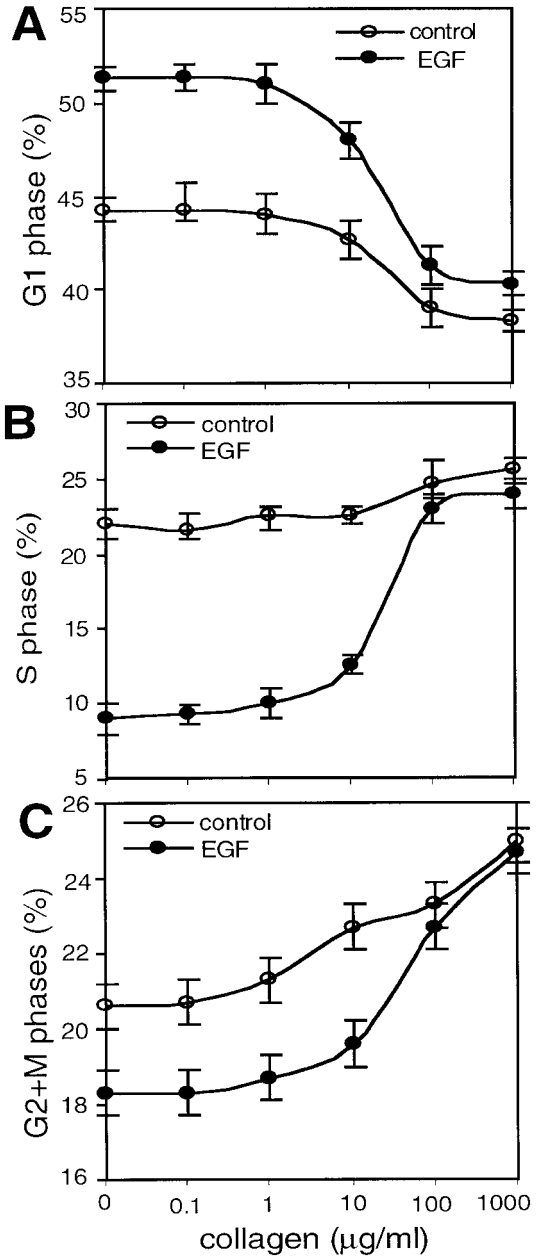

Fig. 10. Alteration of cell cycles by epidermal growth factor (EGF) on collagen-coated plates. A431 cells were seeded on plates coated with different concentrations of collagen and incubated with or without EGF (10 ng/ml) at 37°C for 24 h. The cells were analyzed by flow cytometry after staining with propidium iodide (PI). At low collagen concentrations, EGF arrested a higher proportion of cells in G1 (**A**). Fewer cells were found in S phase (**B**) and G2 + M (C) phases. As the concentrations of collagen increased, the effects of EGF were reduced and eventually abolished.

concentrations of coating collagen increased. Increased expression of these proteins appeared to a result of cell adhesion.

Several hypotheses have been proposed to explain the mechanisms by which EGF inhibits

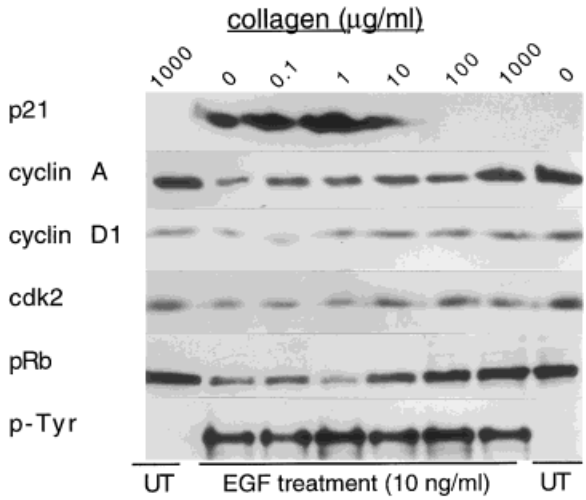

Fig. 11. Effect of epidermal growth factor (EGF) on cell cycle proteins in the presence of collagen. A431 cells were seeded on plates coated with type II collagen as indicated, and incubated with or without 10 ng/ml EGF at 37°C for 24 h. Lysed cell samples were subjected to immunoblot. At low collagen concentrations, EGF treatment enhanced expression of $p21^{waf1/Cip1}$, but reduced expression of cdk2, cyclin D1, cyclin A, and pRb. The levels of phosphotyrosine were not affected by the concentrations of collagen. However, at higher collagen concentrations, the effects of EGF on the expression of different cell cycle proteins were abolished.

cell proliferation, arrests cell cycle, and induces apoptosis. It has been reported that EGF arrests cell cycle progression through inhibiting cdk2 activity by p21 [Fan et al., 1995; Chin, et al., 1996; Jakus and Yeudall, 1996], and Stat1 activation is required for EGF-induced growth inhibition [Chin et al., 1996; Bromberg et al., 1998]. Activation of EGF-R tyrosine kinase activity leads to growth inhibition and apoptosis [Gulli et al., 1996]. To elucidate the mechanisms, in this study, we investigated the relationship of cell cycle arrest and adhesion-regulated programmed cell death. Our results suggested that EGF suppression of cell proliferation was, perhaps, due to apoptosis of the cultures and the apoptosis occurred as cell cycle arrested.

We concluded that reduced cell adhesion modulated cell proliferation, cell cycle arrest and apoptosis, but not vice versa, on the basis of the following observations:

1. EGF suppressed proliferation and induced apoptosis only in monolayer cultures but not in suspension cultures. This finding implied that reduction of cell adhesion was not an effect of EGF inhibition of cell growth or induction of cell apoptosis, but perhaps the

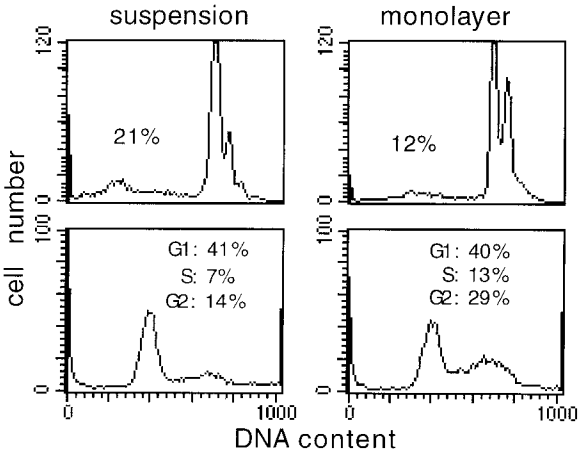

Fig. 12. Disruption of cell attachment-induced cell cycle arrest and adhesion-regulated programmed cell death. A431 cells were seeded in 100-mm tissue culture plates (monolayer culture) and bacterial Petri dishes (suspension culture), at a low density of 2×10^5 cells/plate. The cultures were maintained at 37°C for 24 h. The cells were harvested by trypsinization or centrifugation of the cultures. Cell apoptosis and cell cycle were analyzed with FACScan. In cultures maintained in bacterial Petri dishes, apoptosis was enhanced compared with cultures maintained in tissue culture plates (top). The number of cells in S and G2 phases was lower in suspension cultures than in monolayer cultures (bottom).

loss of cell adhesion caused the other phenomena. In fact, EGF promoted A431 cell growth and reduced cell apoptosis in suspension cultures.

2. Even though EGF promoted cell growth and reduced cell apoptosis, it still inhibited cell aggregation resulting in forming smaller and looser conglomerates. However, as the suspension cultures are not adhesion dependent, reduced cell aggregation did not cause cell death.
3. EGF inhibited cell adhesion within 1 h of its application, but its effects on cell growth and cell apoptosis were observed only after overnight incubation.
4. EGF inhibited cell spreading almost immediately but the increase in apoptosis was not observed until after 8 h of incubation.
5. When cell adhesion increased, the effects of EGF on cell growth and cell apoptosis were abolished. As the cell adhesion decreased, the effects of EGF became augmented.
6. The level of cell apoptosis was much higher when EGF was added to cultures that were not yet dispersed on the plates. After cell spreading, the effect of EGF on apoptosis was reduced. For the reasons stated above, we believe that, in this case, the higher adhesion levels protected the cells from EGF-induced apoptosis, although signaling through collagen-integrin interactions may have contributed to the observed effect.
7. Finally, in an extreme case, reduced cell adhesion on bacterial Petri dishes without EGF treatment also induced cell cycle arrest and apoptosis.

Taken together, we believe this constitutes strong evidence that EGF suppressed cell proliferation, induced cell apoptosis and altered cell cycle patterns by reducing cell adhesion. Nevertheless, the effects of EGF on cell adhesion are only observed in the presence of high EGF concentrations and only in cells expressing high levels of EGF receptor such as A431 and some breast tumor cell lines (i.e., MDA-MB-468). Low concentrations promote cell proliferation but cannot reduce cell adhesion. In cells expressing low levels of EGF receptors, such as NIH3T3 fibroblasts, chicken fibroblasts and chicken chondrocytes, EGF has no effect on cell adhesion. Thus, in cells containing high levels of EGF receptors, this points to the existence of two distinct pathways, one mediating cell proliferation (triggered by low concentrations of EGF) and one mediating cell adhesion (at high concentrations of EGF). These cells thus provide a unique model to study the mechanisms by which EGF signals.

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