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Suppressed Transformation and Induced Differentiation of *HER-2/neu*-overexpressing Breast Cancer Cells by Emodin[1]

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ABSTRACT

The amplification and overexpression of the *HER-2/neu* proto-oncogene, which encodes the tyrosine kinase receptor p185^{neu}, have been observed frequently in tumors from human breast cancer patients and are correlated with poor prognosis. To explore the potential of chemotherapy directed at the tyrosine kinase of p185^{neu}, we have found that emodin (3-methyl-1,6,8-trihydroxyanthraquinone), a tyrosine kinase inhibitor, suppresses autophosphorylation and transphosphorylation activities of *HER-2/neu* tyrosine kinase, resulting in tyrosine hypophosphorylation of p185^{neu} in *HER-2/neu*-overexpressing breast cancer cells. Emodin, at a 40-μM concentration, which repressed tyrosine kinase of p185^{neu}, efficiently inhibited both anchorage-dependent and anchorage-independent growth of *HER-2/neu*-overexpressing breast cancer cells. However, the inhibition was much less effective for those cells expressing basal levels of p185^{neu} under the same conditions. Emodin also induced differentiation of *HER-2/neu*-overexpressing breast cancer cells by exhibiting a morphological maturation property of large lacy nuclei surrounded by sizable flat cytoplasm and by showing a measurable production of large lipid droplets, which is a marker of mature breast cells. Therefore, our results indicate that emodin inhibits *HER-2/neu* tyrosine kinase activity and preferentially suppresses growth and induces differentiation of *HER-2/neu*-overexpressing cancer cells. These results may have chemotherapeutic implications for using emodin to target *HER-2/neu*-overexpressing cancer cells.

INTRODUCTION

The proto-oncogene *HER-2/neu* (also known as c-*erb*B-2) encodes a 185-kilodalton (p185^{neu}) transmembrane tyrosine kinase growth factor receptor, which is related to but distinct from the EGFR[3] (1–5). Amplification and overexpression of the *HER-2/neu* proto-oncogene occur in as many as 30% of breast cancers and have been found to be correlated with both poor prognosis (6–9) and decreased survival in breast cancer patients (6, 7). Furthermore, the results from cellular and animal experiments show that enhancement of *HER-2/neu* tyrosine kinase activity increases malignant phenotypes (10–17). These results suggested that the tyrosine kinase activity enhanced by *HER-2/neu* overexpression may play a critical role in the development of human tumors. Therefore, an inhibitor of the *HER-2/neu* tyrosine kinase activity may be able to repress cell transformation through repression of tyrosine kinase activity. To explore this possibility, we examined human breast cancer cells that overexpress the *HER-2/neu* oncogene and found that emodin (3-methyl-1,6,8-trihydroxyanthraquinone), an inhibitor for the protein tyrosine kinase p56^{lck} (18) isolated from *Polygonum cuspidatum*, inhibited activity of p185^{neu} tyrosine kinase, preferentially blocked the growth of the *HER-2/neu*-overexpressing human breast cancer cells, and induced differentiation of these cells.

MATERIALS AND METHODS

Cell Culture. Human breast cancer cell lines MDA-MB453, BT-483, MDA-MB231, and MCF-7 and immortalized breast cell line HBL-100 were obtained from the American Type Culture Collection (Rockville, MD). AU-565 cells were obtained from the Naval Bioscience Laboratory (Oakland, CA). MDA-MB453, BT483, and AU-565 cells overexpress *HER-2/neu*, whereas MDA-MB231, MCF-7, and HBL-100 cells express basal levels of *HER-2/neu*. All cells were grown in DMEM/Ham's F12 (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum and gentamicin (50 μg/ml). Cells were grown in a humidified incubator at 37°C under 5% CO_2 in air.

Immunoprecipitation and Western Blot Analysis. As described previously (19, 20), cells were washed three times with PBS and then lysed in lysis buffer [20 mM Na_2PO_4 (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 100 mM NaF, and 2 mM Na_3VO_4]. Protein content was determined against a standardized control using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). A total of 500 μg of protein were immunoprecipitated with an anti-p185^{neu} mAb [c-*neu* (Ab-3); Oncogene Science, Inc., Uniondale, NY] on protein A-Sepharose. The immune complexes were washed, separated by 6% SDS-PAGE, and transferred to nitrocellulose filter paper (Schleicher & Schuell, Inc., Keene, NH). Nonspecific binding on the nitrocellulose filter paper was minimized with a blocking buffer containing nonfat dry milk (5%) and Tween 20 (0.1%, v/v) in PBS. The treated filter paper was incubated with primary antibodies [anti-p185^{neu} antibody c-*neu* (Ab-3) for detection of p185^{neu} or anti-PY antibody (Upstate Biotechnology, Inc., Lake Placid, NY) for detection of PY], followed by incubation with horseradish peroxidase-conjugated goat antimouse antibody (1:1000 dilution; Boehringer Mannheim Corp., Indianapolis, IN), and bands were visualized with the enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL).

Immunocomplex Kinase Assay. The immunocomplex kinase assay was modified from those described previously (21). In brief, cells were treated with or without 40 μM emodin for 24 h and then washed three times with PBS. Cells were then collected and lysed in lysis buffer. Cell lysates (500 μg) were incubated with anti-p185^{neu} mAb c-*neu* (Ab-3) for 1 h at 4°C, precipitated with 50 μl of protein A-conjugated agarose (Boehringer Mannheim) for 30 min at 4°C, and washed three times with 50 mM Tris-HCl buffer containing 0.5 M LiCl (pH 7.5) and once in assay buffer [50 mM Tris-HCl (pH 7.5) and 10 mM $MnCl_2$]. To 40 μl of beads (protein A-conjugated agarose), 10 μCi of [γ-^{32}P]ATP (Amersham) and 10 μl of enolase (Sigma Chemical Co., St. Louis, MO) were added and incubated for 20 min at room temperature. The reactants were separated by 7.5% SDS-PAGE. The gel was dried and visualized by autoradiography.

Proliferation Assay. Cells were detached by trypsinization, seeded at 2×10^4 cells/ml in a 96-well microtiter plate overnight, treated with various concentrations of test samples, and incubated for an additional 72 h. The effect of emodin on cell growth was examined by MTT assay (22, 23). In brief, 20 μl of MTT solution (5 mg/ml; Sigma) were added to each well and incubated for 4 h at 37°C. The supernatant was aspirated, and the MTT formazan formed by metabolically viable cells was dissolved in 150 μl of DMSO then monitored by a microplate reader (Dynatech MR 5000 fluorescence; Dynatech Corp., Burlington, MA) at a wavelength of 590 nm.

Colony Formation in Soft Agarose. As described previously (14), cells (1×10^3 cells/well) were seeded in 24-well plates in culture medium containing 0.35% agarose (FMC Corp., Rockland, ME) over a 0.7% agarose layer and incubated for 3 weeks at 37°C. Colonies were then stained with *p*-iodonitrotet-

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[3] The abbreviations used are: EGFR, epidermal growth factor receptor; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PY, phosphotyrosine.

razolium violet (1 mg/ml), and colonies larger than 100 μM were counted. Each determination was performed four times.

Lipid Visualization. As described previously (24), a modified Oil Red O in propylene glycol method was used to visualize neutral lipids (25, 26).

RESULTS

Effect of Emodin on Tyrosine Phosphorylation in Breast Cancer Cells That Overexpress *HER-2/neu*. To test whether emodin, a tyrosine kinase inhibitor for the protein tyrosine kinase p56^{lck} (18), also inhibits *HER-2/neu* tyrosine kinase, MDA-MB453 human breast cancer cells that overexpress p185^{neu} were used to test the effect of emodin on tyrosine phosphorylation of p185^{neu}. Cells were treated with varying concentrations of emodin at 37°C for 24 h then analyzed for the protein level of p185^{neu} and its tyrosine phosphorylation. The p185^{neu} was first immunoprecipitated by anti-p185^{neu} antibody, and the immunoprecipitates were then blotted with anti-PY antibody for detection of PY or anti-p185^{neu} antibody for detection of p185^{neu}. Emodin, at a 40-μM concentration, induced a significant reduction in the level of tyrosine phosphorylation (Fig. 1*A*) but had no obvious effect on p185^{neu} protein level. The reduced tyrosine phosphorylation of p185^{neu} could be detected readily after 12 h (Fig. 1*B*). To confirm further that the reduced tyrosine phosphorylation by emodin is a general phenomenon for p185^{neu}, other *HER-2/neu*-overexpressing breast cancer cell lines were also examined, and similar results were obtained (Fig. 2). These cell lines include BT-483, AU-565 (Fig. 2), SKBr-3, and MDA-MB361 (data not shown). When MCF-7 cells that express basal levels of p185^{neu} protein were examined similarly, the PY level of p185^{neu} was almost undetectable under our experimental condition. Therefore, it is insignificant to compare the effect of emodin on PY level on p185^{neu} in MCF-7 cells (data not shown).

Repression of Autophosphorylation and Transphosphorylation by Emodin *in Vitro*. The results shown above indicated that PY level of p185^{neu} could be repressed by emodin. To examine whether the reduced tyrosine phosphorylation could affect the tyrosine kinase activity of p185^{neu}, we carried out the immunocomplex kinase assay. The autophosphorylation ability of p185^{neu} from MDA-MB453 cells treated with emodin for 24 h was inhibited, and the transphosphorylation ability of p185^{neu} for enolase, an exogenous substrate for tyrosine kinase, also was significantly decreased compared with that of untreated cells (Fig. 3*A*). These data (Figs. 1 and 3) indicated that emodin-treated cells result in reduced PY levels in p185^{neu}, which, in turn, exhibits lower tyrosine kinase activity. To address further whether emodin can directly inhibit the intrinsic tyrosine kinase activity of p185^{neu}, p185^{neu} was immunoprecipitated from the untreated MDA-MB453 cells; the precipitates were then treated with varying concentrations of emodin, and the kinase activity was measured. The tyrosine kinase activity for both autophosphorylation and transphosphorylation of p185^{neu} is inhibited by emodin in a dose-dependent manner (Fig. 3*B*). These results show conclusively that emodin represses the intrinsic tyrosine kinase activity of p185^{neu}. Therefore, the reduced PY level in p185^{neu} treated with emodin is most likely caused by inhibition of p185^{neu} tyrosine kinase activity.

Effect of Emodin on the Proliferation of Human Breast Cancer Cells. Because emodin effectively inhibits the tyrosine kinase activity of p185^{neu}, which is critical for cell growth, it is of interest to investigate whether emodin may inhibit cell proliferation preferentially for the breast cancer cells that overexpress p185^{neu}. To address this issue, we chose six cell lines for additional study. MDA-MB453, BT-483, and AU-565 are *HER-2/neu*-overexpressing breast cancer cell lines, as mentioned above, and MCF-7 and MDA-MB231 are two human breast cancer cell lines that express basal levels of p185^{neu} (27). The HBL-100 cell line is derived from a normal human breast tissue transformed by SV40 large T antigen (43) and expresses a basal level of p185^{neu}. As shown in Fig. 4, the growth of these cells was inhibited by emodin in a dose-dependent manner but to varying extents. At a 40-μM concentration, which effectively inhibited tyrosine kinase activity of p185^{neu} (Figs. 1, 2, and 3), emodin blocked 68, 72, and 84% of growth in MDA-MB453, BT-483, and AU-565 cells, respectively. However, under the same conditions, it inhibited only 37 and 23% of growth in MCF-7 and MDA-MB 231 cells, respectively. Emodin had little effect on HBL-100 breast cells even up to an 80-μM concentration. The differential growth effect was not obvious when the cells were treated with 10 μM emodin, which did not affect tyrosine kinase activity of p185^{neu}. These results indicate that emodin preferentially suppresses growth of the *HER-2/neu*-overexpressing

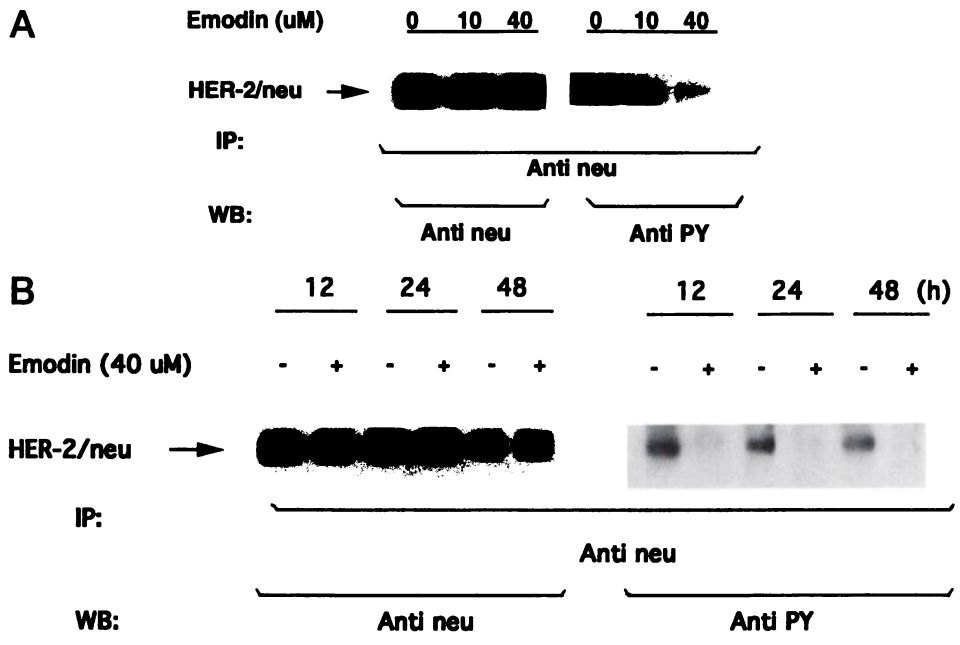

Fig. 1. Effect of dose dependence and time course of emodin treatment on tyrosine phosphorylation and expression of *HER-2/neu* in MDA-MB453 cells. *A*, cells in the serum-free medium were incubated without (0) or with emodin (10 or 40 μM) at 37°C for 24 h. *B*, cells were incubated without (−) treatment or with (+) emodin (40 μM) at 37°C for varying times. Cell extracts were immunoprecipitated by anti-p185^{neu} antibody (*Anti neu*) and then Western blotted with either anti-PY antibody (*Anti PY*) or anti-p185^{neu} antibody, as described in "Materials and Methods."

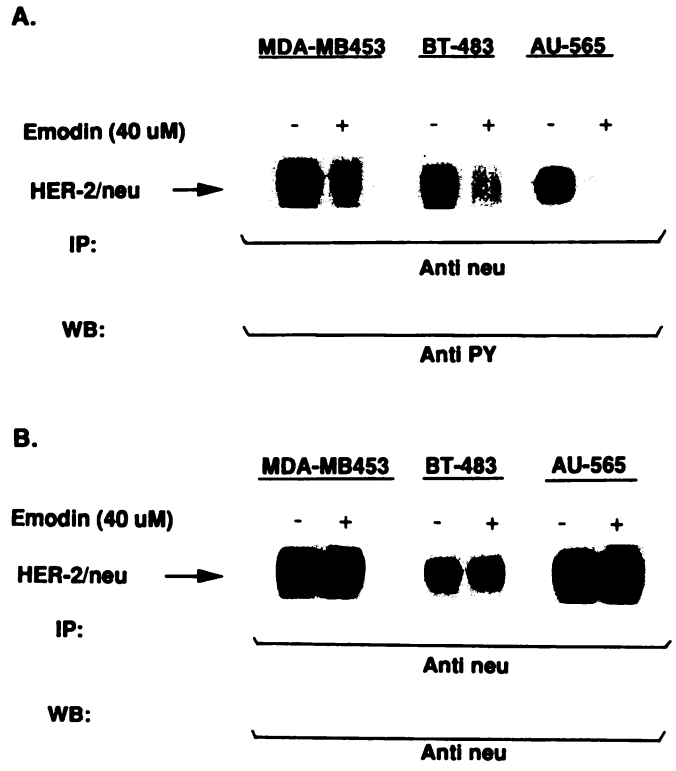

Fig. 2. Effect of emodin on tyrosine phosphorylation and expression of *HER-2/neu* in human *HER-2/neu*-overexpressing breast cancer cells. Cells in the serum-free medium were incubated without (−) or with (+) 40 μM emodin at 37°C for 24 h, and then cell lysates were immunoprecipitated by anti-p185^{neu} antibody and blotted with anti-PY antibody (*A*) or anti-p185^{neu} antibody (*B*), as described in "Materials and Methods."

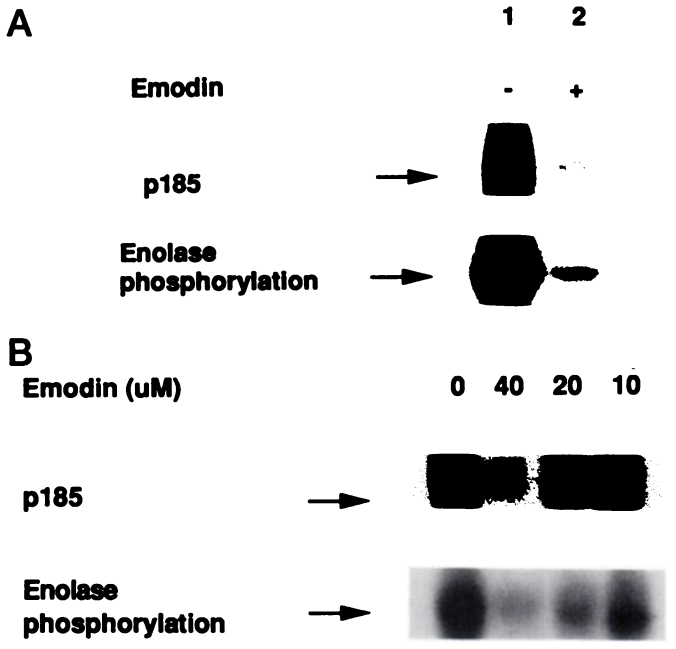

Fig. 3. Effect of emodin on autophosphorylation and transphosphorylation of P185^{neu} in MDA-MB453 breast cancer cells. *A*, cells were incubated without (*Lane 1*) or with (*Lane 2*) emodin (40 μM) at 37°C for 24 h, then cell lysates (500 μg) were immunoprecipitated, and kinase activities were measured by incubation with [γ-^{32}P]ATP and enolase. *B*, cell lysates from untreated cells were immunoprecipitated then incubated with [γ-^{32}P]ATP, enolase, and varying concentrations of emodin for 20 min at room temperature. Reactants were resolved on 7.5% SDS-PAGE. The phosphorylation products were dried and visualized by autoradiography, as described in "Materials and Methods."

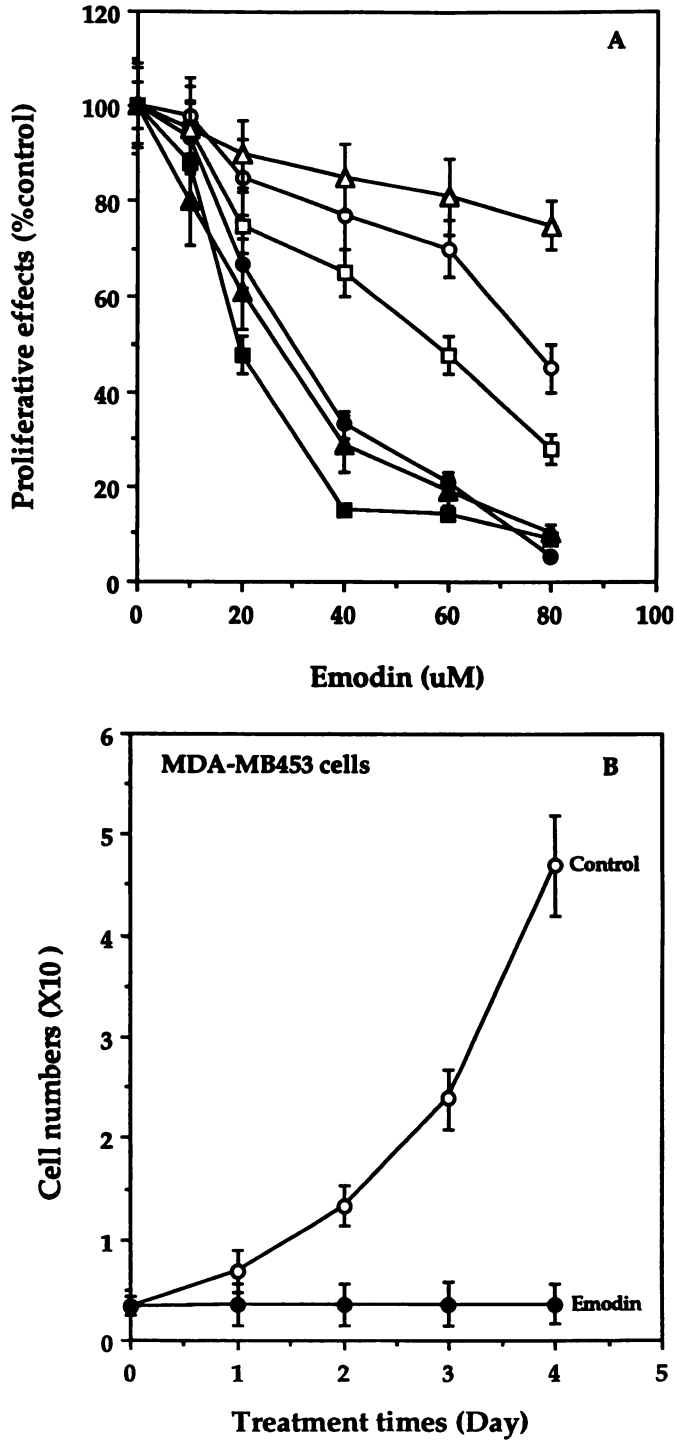

Fig. 4. Effect of emodin on the proliferation of human breast cancer cells expressing different levels of *HER-2/neu*. *A*, MDA-MB453 (●), AU-565 (▲), and BT-483 cells (■), which overexpress *HER-2/neu*, and MCF-7 (□), MDA-MB231 (○) and HBL-100 cells (△), which express normal levels of *HER-2/neu*, were incubated without or with different concentrations of emodin at 37°C for 72 h. The effect on cell growth was examined by MTT assay, and the percentage of cell proliferation was calculated by defining the absorption of cells without treatment of emodin as 100%. *B*, MDA-MB453 cells were incubated without (control) or with emodin (40 μM) at 37°C for different times, and cells were washed and counted by trypan blue exclusion with hemacytometer. All determinations were made in triplicate. *Bars*, SD.

breast cancer cells and suggest that the differential suppression effect is likely to occur through repression of p185^{neu} tyrosine kinase.

Because emodin at a 40-μM concentration inhibited p185^{neu} tyrosine kinase activity (Figs. 1, 2, and 3) and significantly suppressed

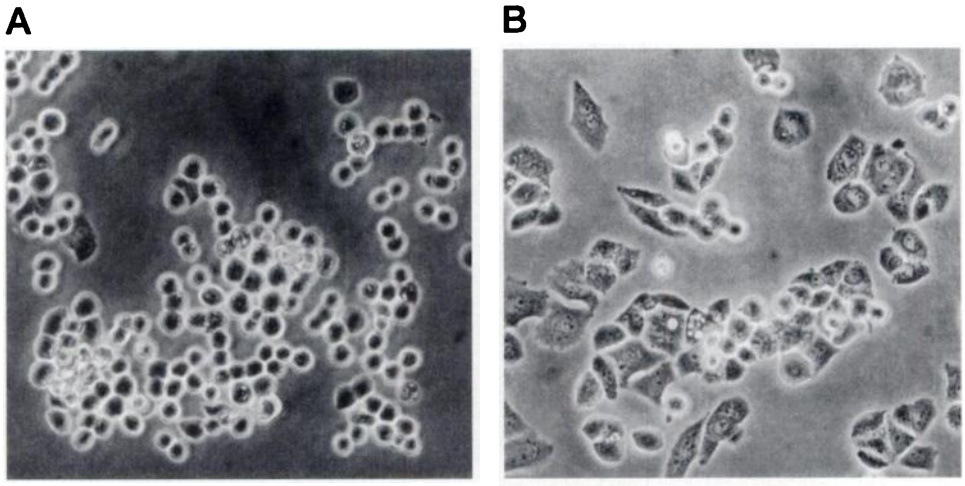

Fig. 5. Effect of emodin on morphology of MDA-MB453 human breast cancer cells. Cells were incubated without (*A*) or with 40 μM emodin (*B*) for 24 h. The change in cell morphology was examined and photographed under an inverted microscope.

growth of breast cancer cells that overexpress p185^{neu} (Fig. 4*A*), we examined further the effect of time course on proliferation of MDA-MB453 cells by emodin at a 40-μM concentration. Emodin inhibited cancer cell growth completely, and, when the viability of cells was measured by trypan blue assay, more than 90% of cells were found to be alive (Fig. 4*B*). The results suggest that the mechanism that causes inhibition of cell growth is primarily a result of repression of proliferation and not induction of cell death under this condition.

Emodin Induces Differentiation of Breast Cancer Cells. Because emodin inhibited breast cancer cell growth, and no significant cell deaths were observed (Fig. 4*B*), it was of interest to investigate whether emodin would induce differentiation of breast cancer cells. When MDA-MB453 cells were treated with emodin (40 μM) for 24 h), cells displayed a flat morphology with larger nuclei and increased cytoplasm, which were shown to be characteristic for differentiation (28; Fig. 5*B*), compared with untreated cells, which are moderately adherent and have a rounded morphology (Fig. 5*A*). A similar morphological change was also observed in AU-565 cells (data not shown). In contrast, no significant morphological change could be observed in the MCF-7 cells treated by emodin compared with untreated cells (data not shown).

Because the maturation of breast cells is characterized by the presence of lipid droplets that are milk components, we examined further whether the morphological change induced by emodin was accompanied by the production of lipid droplets. Large droplets containing neutral lipid were readily detectable in emodin-treated cells (Fig. 6, *B* and *D*); in contrast, no obvious large lipid droplets could be observed in the untreated cells (Fig. 6, *A* and *C*). When the lipid-producing cells were counted, more than 90% of the emodin-treated cells produced large lipid droplets, but only 2 to 5% of the untreated cells contained lipid drops of a much smaller size. In contrast, no obvious large lipid droplets could be observed in the MCF-7 cells treated with 40 μM emodin (data not shown). These results indicate that emodin preferentially induces differentiation of *HER-2/neu*-overexpressing breast cancer cells, suggesting that the enhanced tyrosine kinase activity of p185^{neu} may prevent breast cancer cells from differentiation.

Effect of Emodin on Transformation of Breast Cancer Cells. One hallmark of the transformed state is the ability of cells to exhibit anchorage-independent growth. To determine whether emodin may affect this property in breast cancer cells, cells were seeded into soft agarose and monitored for colony formation (Fig. 7). The colony-forming activity of *HER-2/neu*-overexpressing breast cancer cells MDA-MB453, BT483, and AU565 in soft agarose containing 40 μM emodin was suppressed dramatically. However, under the same condition, the cells that express basal levels of p185^{neu}, namely MCF-7, MDA-MB231, and HBL-100, still formed a significant number of colonies. The decreased ability to grow in soft agarose of the *HER-2/neu*-overexpressing breast cancer cells could not simply reflect the slower proliferation rate shown in Fig. 4, because 3 additional weeks of incubation did not increase the number of colonies. Furthermore, no significant change in colony-forming activity could be observed when the cells grew in soft agarose containing 10 μM emodin (data not shown), which did not inhibit tyrosine kinase activity of p185^{neu} (Figs. 1 and 3*B*). Taken together, the results indicate that emodin preferentially suppresses the transformation ability of *HER-2/neu*-overexpressing cancer cells and suggest that the transformation suppression by emodin may be mediated through its ability to inhibit tyrosine kinase activity of p185^{neu}.

DISCUSSION

Emodin has been reported to be a tyrosine kinase inhibitor that restricts the activity of p56^{lck} kinase by preventing the binding of ATP *in vitro* (18) and that has the ability to inhibit the growth of cancer cells, including lymphocytic leukemia (29), HL-60 human leukemia cells (30), and *ras*-transformed human bronchial epithelial cells (31), by an unknown mechanism. In this study, we demonstrated that emodin suppressed tyrosine kinase activity of *HER-2/neu*, inhibited cell growth and the transformation phenotype *in vitro* for human breast cancer cells that overexpress the *HER-2/neu* oncogene, and induced differentiation of these cells.

Tyrosine kinase receptors play a vital role in the cascade of signals leading to cell growth and differentiation (32). It has been shown previously that several agents, including a specific mAb against p185^{neu} (26), mycophenolic acid, and phorbol ester (25), can induce differentiation of breast cancer cells through down-regulation or translocation of p185^{neu} to cytoplasmic and nuclear sites. In this study, we report that emodin, which suppresses tyrosine kinase activity of p185^{neu} without changing the p185^{neu} protein level, can inhibit both anchorage-dependent and anchorage-independent cell growth and induce the mature phenotype. These results suggest further that the tyrosine kinase activity of p185^{neu} is critical for cell growth and *HER-2/neu*-induced transformation, and that repression of the kinase activity may also induce differentiation.

The p185^{neu} transmembrane glycoprotein with intrinsic tyrosine kinase activity is homologous to EGFR (2, 4, 5). To examine whether the inhibitor of EGFR tyrosine kinase affects the tyrosine kinase activity of p185^{neu}, we also tested the effect of genistein, a specific inhibitor of the EGFR tyrosine kinase (33), on tyrosine phosphoryl-

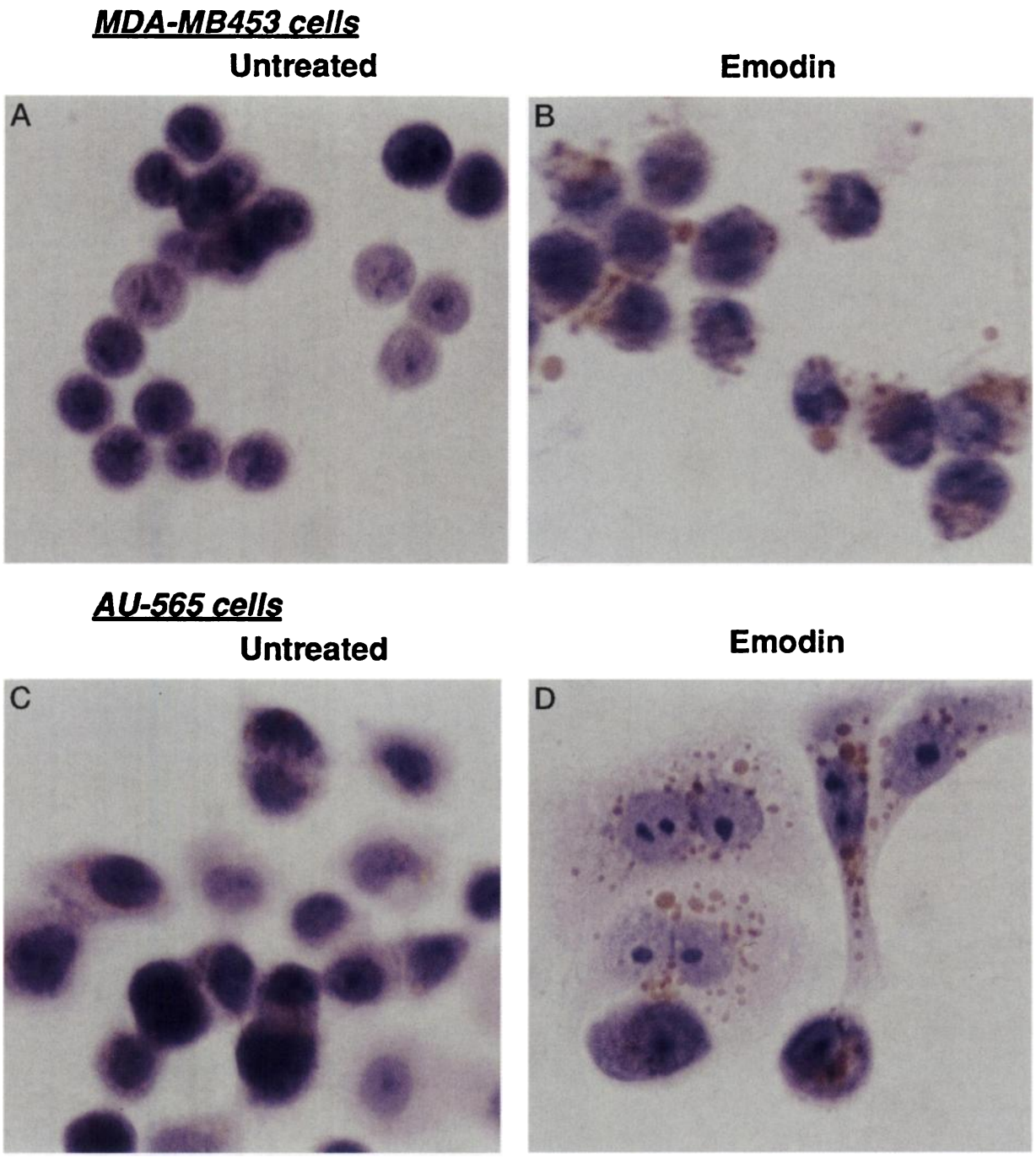

Fig. 6. Emodin-induced appearance of neutral lipid droplets in MDA-MB453 and AU-565 cells. Cells were treated without (*A* and *C*) or with (*B* and *D*) emodin (40 μM) for 5 days then processed for visualization of neutral lipids as described in "Materials and Methods."

ation of $p185^{neu}$ in MDA-MB453 cells that express high levels of $p185^{neu}$ and basal levels of EGFR (27, 34). Genistein, at a concentration (6 μg/ml) known to inhibit tyrosine kinase activity of EGFR (33), had no obvious effect on tyrosine phosphorylation of $p185^{neu}$ (data not shown). Although additional study is needed, these results suggest that different tyrosine kinase receptors may respond differently to different tyrosine kinase inhibitors. To prevent complications from the interaction between EGFR and $p185^{neu}$, the cell lines used in this study express basal levels of EGFR only (34).

Another interesting issue for the emodin-induced effect on *HER-2/neu*-overexpressing cells is whether the effect is reversible. MDA-MB453 cells were treated with 40 μM emodin for 1 day in the absence of serum, emodin was removed, and the cells were incubated in the presence of serum with or without emodin for 2 more days; we found that, in the absence of emodin for two more days, both the PY level of $p185^{neu}$ and the growth rate of the cells were significantly higher than those in the presence of emodin (data not shown). The results suggest that the suppression effect of emodin on cell proliferation and PY level of $p185^{neu}$ may not be irreversible under the conditions described above.

The autophosphorylation of tyrosine kinase receptors is believed to be the first step in signal transduction and a prerequisite for the phosphorylation of downstream targets by the receptors (35–37). The $p185^{neu}$ protein has been shown previously to be associated with and to phosphorylate phospholipase C-γ, the GTPase-activating protein GAP, phosphatidylinositol 3′-kinase, and Shc (38–42). Activation of

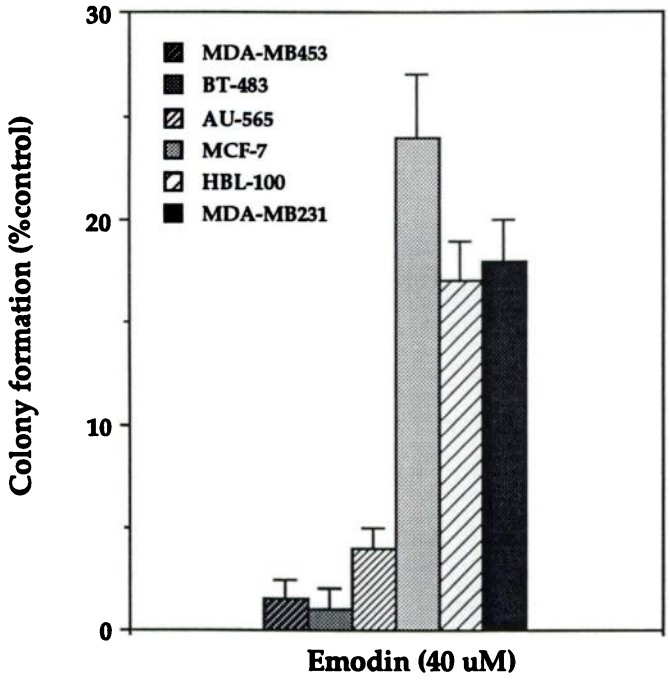

Fig. 7. The effect of emodin on human breast cancer cell colony growth in soft agarose. Cells (1×10^3 cells/well) were seeded into 24-well plates in culture medium containing 0.35% agarose over a 0.7% agarose layer with or without 40 μM emodin and incubated for 3 weeks at 37°C. Colonies were stained with *p*-iodonitrotetrazolium violet and counted, and the percentage of colony formation was calculated by defining the number of colonies in the absence of emodin as 100%. All determinations were made four times. *Bars*, SD.

these downstream targets by phosphorylation requires tyrosine kinase activity of p185^{neu}. Because intrinsic tyrosine kinase activity of p185^{neu}, which triggers both autophosphorylation and transphosphorylation, is effectively repressed by emodin (Fig. 3), these downstream targets are not likely to be activated. It is known that *ras* plays a role in one of the downstream targets (38–42). Because emodin was shown to inhibit growth of *ras*-transformed cells (31), inactivation of the *ras* pathway might contribute to the growth suppression of *HER-2/neu*-overexpressing breast cancer cells. However, as shown in this study, emodin can affect multiple biological properties of *HER-2/neu*-overexpressing breast cancer cells, including suppression of anchorage-dependent and anchorage-independent growths and induction of differentiation. It will be of interest to investigate further how the different downstream targets of p185^{neu} may trigger cell growth and differentiation. In addition, because emodin preferentially inhibits growth of cancer cells with *HER-2/neu*-overexpression, it may have therapeutic implications for cancers with *HER-2/neu* overexpression.

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