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Growth Inhibition by Anchorage-deficiency Is Associated with Increased Level but Reduced Phosphorylation of Mutant p53

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Human breast carcinoma MCF-7 cells seeded on type I collagen-coated dishes were provided with an anchor via the collagen receptor, integrin, and grew as actively as those in plastic tissue culture dishes. In contrast, cells seeded on a layer of soft agar became anchorage-deficient and their growth was significantly inhibited, although the cell viability and the cell cycle distribution were unaffected. Immunoprecipitation analysis revealed that mutant p53 was phosphorylated at tyrosine in the anchorage-provided cells. In contrast, the p53 in the anchorage-deficient cells was present in 2-fold greater amount, but was phosphorylated to a lesser extent. Addition of a potent protein-tyrosine kinase inhibitor, herbimycin A, to the anchorage-provided cells caused an elevated level of p53, and inhibitions of cell proliferation and p53 phosphorylation, without interfering with the cell adhesion to the substratum. These results demonstrated that the growth inhibition by anchorage-deficiency or by herbimycin A is associated with an elevated p53 level and reduced p53 phosphorylation at tyrosine.

Key words: Mutant p53 level — Tyrosine phosphorylation — Growth inhibition — Herbimycin

Loss of heterozygosity of markers on the short arm of choromosome 17 occurs at high frequency in many different human tumors including breast cancer,[1] lung cancer,[2] colon cancer,[3] and astrocytomas.[4] By using multiple probes on 17p, a region that is commonly lost in colon cancers was defined as that containing the site of the p53 gene.[3] The p53 gene was initially reported to be a dominant transforming oncogene as a result of co-transfection assays of plasmids encoding activated *ras* and p53 in rodent cells.[5-7] However, subsequent work on murine erythroleukemia cells, in which the p53 expression is often undetectable, led to the proposal that p53 may also be a tumor suppressor gene.[8] Whereas some tumors are completely lacking in p53, both cDNA sequencing and immunocytochemical analyses have shown that many different human tumors, including those of breast, colon, liver, and lung, commonly contain p53 mutants.[9-17] The accumulating data on p53 genetic alteration show that it is the most frequently involved gene in human carcinogenesis. Cotransfection assays demonstrated that these mutants had lost the ability to function as tumor suppressors, whereas some of them can act as dominant oncogenes by cooperating with *ras*.[18-20]

With respect of the participation of p53 in growth control, there is good evidence that wild-type p53 causes growth arrest of cells in G1[21-26] and microinjection of an anti-p53 monoclonal antibody blocks serum-induced entry into S phase of the cell cycle.[27] Wild-type p53 as an anti-proliferative protein is involved in the control of initiation of DNA synthesis; wild-type murine[28-31] and human[32] p53 protein strongly blocks SV40 large T antigen replication functions. It has recently been demonstrated that both human and murine wild-type p53 proteins bind specifically to sequences adjacent to the late border of the SV40 origin of replication.[33] These reports imply that for cells to enter S phase of the cell cycle, wild-type p53 has to be inactivated. The activity of the protein appears to be potentially controlled both by phosphorylation[34] and by regulation of the protein level.

Contrary to wild-type p53, however, involvement of p53 mutant in the regulation of cell proliferation in transformed or cancerous cells remains unclear. With the exception of blood cells, various cell types can grow anchorage-dependently in monolayer culture. Human breast cancer MCF-7 cells cultivated under conditions that included collagen anchorage grew actively, while cells seeded on soft agar were anchorage-deficient and hardly grew. The anchorage-provided cells have been found to contain p53 mutant which was immunoprecipitated with the monoclonal antibody to various p53 mutants[35] and was phosphorylated exclusively at tyrosine, not serine or threonine. In contrast to this, the p53 in the anchorage-deficient cells on soft agar was elevated in its level but phosphorylated to a lesser degree.

MATERIALS AND METHODS

Cell growth assay MCF-7 cells were maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS, GIBCO). Exponentially growing cells were

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harvested with 0.125% (w/v) trypsin and 0.05% (w/v) EDTA (GIBCO), and seeded on type I collagen-coated plastic dishes (Iwaki Glass) or on a layer of 0.5% (w/v) soft agar (Agar noble; Difco). Cell viability of cultures on soft agar was examined by dye exclusion and colony formation. In some experiments, a potent tyrosine kinase inhibitor, herbimycin A,[36-38] was added to cultures on collagen-coated dishes 24 h after the beginning of culture at the indicated doses. The concentration of herbimycin A giving half-maximal inhibition of cell survival was 2.8 μg/ml. Normal human breast epithelial cells (Kurabo) were maintained in a serum-free medium as previously described.[39]

Inhibition of cell adhesion To identify the anchorage receptor, antibody raised against the putative collagen receptor, integrin $\alpha 2$[40] (Oncogene Science), was used. The cells were seeded at 1×10^6 cells per 60-mm collagen-coated culture dish with the antibody at the final concentrations indicated. FBS was heat-inactivated at 56°C for 30 min prior to use. After incubation of the cells for 1 h at 37°C, the cells adhering to the surface of the dish were harvested with trypsin and EDTA as described above and the number of cells was scored with a hemocytometer.

Flow cytometer Cells were trypsinized, permeabilized with 0.1% Triton X-100, and stained with 50 μg/ml propidium iodide prior to flow cytometric analysis in a FACScan (Becton-Dickinson), using the Polynominal model.

Phosphorylation of p53 Cells cultivated for 4 days on collagen or soft agar were labeled with 0.25 mCi/ml [^{32}P]orthophosphate (ICN) in phosphate-free medium for 3 h. The ^{32}P-labeled cells were lysed in RIPA buffer (10 m*M* Tris-HCl, pH 7.4, 0.15 *M* NaCl, 1% Na-deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1 m*M* phenylmethylsulfonyl fluoride) and the monoclonal antibody PAb 240[35] or PAb 1801 (Oncogene Science) was added to the cell lysate followed by incubation for 1 h at room temperature. In some experiments, the monoclonal antibody 1G2[41] (Oncogene Science) to phosphotyrosyl protein was used. A Protein A-Sepharose slurry (Pharmacia LKB) was added and the incubation was continued for 1 h with tumbling at room temperature. Finally, the Sepharose beads were washed 4 times with 20 m*M* phosphate buffer, pH 8.6, 0.1% NP-40, and 0.5% (w/v) SDS by centrifugation. The labeled proteins were separated by SDS-polyactrylamide gel electrophoresis[42] or two-dimensional gel electrophoresis[43] using a carrier ampholite having a pH range of 3–10 (Bio-Rad). Prior to one-dimensional gel electrophoresis, the protein content of loaded samples was normalized. After electrophoresis, the gels were stained with Coomassie blue or silver,[44] dried under vacuum, and autoradiographed at −70°C using an intensifying screen. For measurement of the net amount of p53, an image analyzer (Olympus) was applied to the p53 band on silver-stained gels, using bovine serum albumin as a standard.

Phosphoamino acid analysis The ^{32}P-labeled proteins were separated by electrophoresis and visualized by autoradiography as described above. The p53 band was cut from the dried gel and the protein was extracted from the gel slice in 50 m*M* ammonium bicarbonate, 0.1% (w/v) SDS, 1 m*M* EDTA, and 40 μl/ml 2-mercaptoethanol,[45] then acid-hydrolyzed for 1 h at 110°C in 6 *N* constant-boiling HCl (Pierce). After being dried with a vacuum concentrator, the residue was separated by single-dimensional thin-layer electrophoresis on a cellulose plate in a mixture of pyridine-acetic acid-water (5:45:945) at 500 V for 45 min on ice. Phosphoamino acid standards consisting of phosphoserine, phosphothreonine, and phosphotyrosine (Sigma) were visualized with ninhydrin and the plate was then autoradiographed using an intensifying screen at −70°C.

RESULTS

Proliferation of anchorage-provided or anchorage-deficient cells To identify the type of anchorage, tissue culture dishes coated with type I collagen, one of the extracellular matrices, were used in the present work. MCF-7 cells seeded on collagen-coated dishes spread with a morphology characteristic of epithelial-like cells in uncoated plastic dishes (Fig. 1a). Approximately 86% of the cells adhered to collagen within 1 h after seeding and became anchored (Fig. 2). Addition of an antibody raised against the putative collagen receptor, integrin $\alpha 2$, at seeding, resulted in a significant inhibition of cell adhesion to collagen in a dose-dependent manner (Fig. 2). This implies that the cells seeded on collagen-coated plastic dishes anchored by means of the interaction between collagen and its cellular receptor, integrin. In contrast, cells seeded on soft agar failed to adhere to it and remained as single cells or formed small aggregates which floated in the medium (Fig. 1b). After culture for 4 days on soft agar, the cells were harvested and the cell viability was examined by dye exclusion and colony formation. The viability of cells on soft agar was 95.6% by dye exclusion or 91.0% by colony formation. These values are similar to those of cells cultivated on collagen, i.e., 96.0% and 94.3% by dye exclusion and by colony formation, respectively. This indicated that the cells cultivated on soft agar for 4 days were fully viable.

The cells anchored with collagen actively grew and the cell number increased to 5 times (2.5×10^5) that seeded after 4 days of culture. In contrast, the anchorage-deficient cells grew slowly and the number of cells after 4 days in culture was 1.5×10^5, being significantly smaller

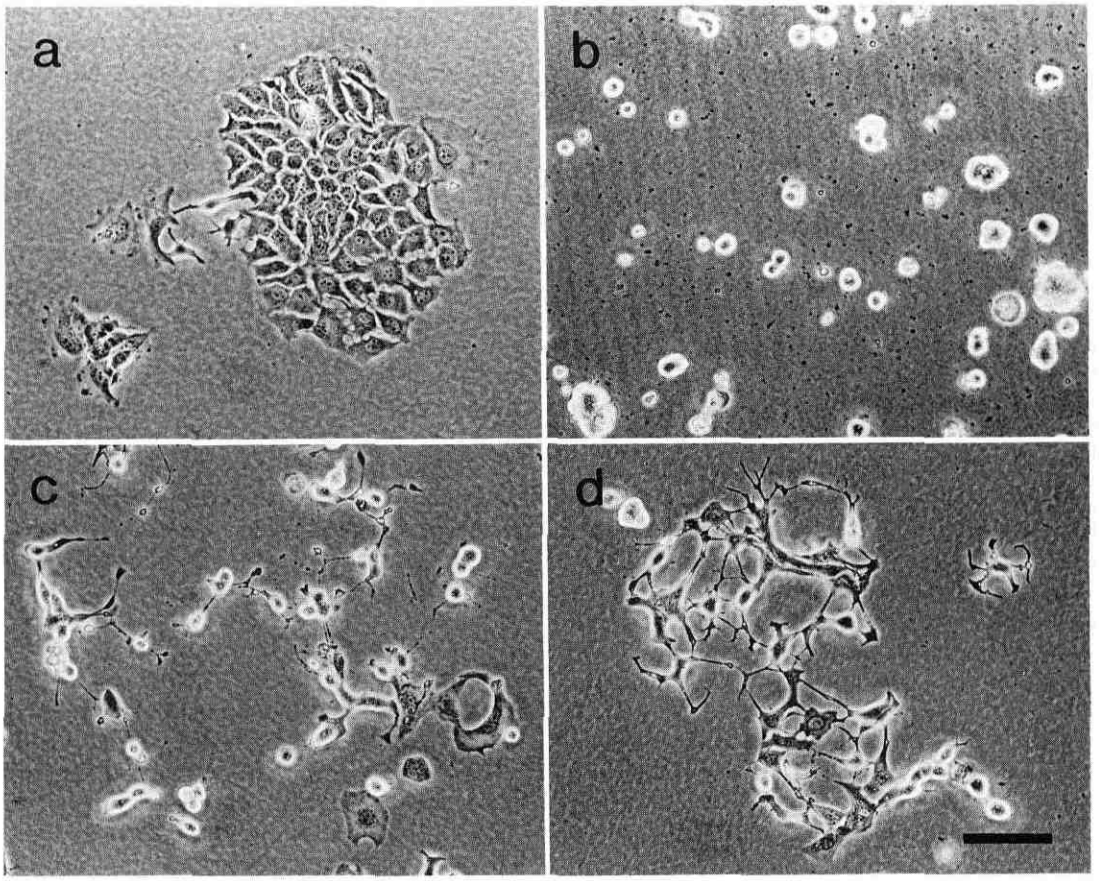

Fig. 1. Effects of culture substrata and herbimycin A on the morphology of MCF-7 cells. Culture of the cells for 4 days on type I collagen-coated plastic dishes (a) and 0.5% (w/v) soft agar (b). Herbimycin A was added to cultures on collagen 24 h after seeding to give at 0.5 μg/ml (c) and 1.0 μg/ml (d). Bar, 100 μm.

($P<0.01$, Student's t test) than that of the cells grown on collagen. Cell proliferation was inhibited by 40% in the absence of anchorage. Flow cytometry revealed that this growth inhibition of cells on soft agar was not accompanied by a significant alteration in the cell cycle distribution when compared with that of the anchorage-provided cells with collagen (not shown).

Net amount and phosphorylation of p53 Immunoprecipitation analysis revealed that the net amount of p53 protein was 4.9 ng per 1×10^5 anchorage-provided cells. This p53 was found to be heavily phosphorylated per protein (Fig. 3). Image analysis of triplicate cultures revealed that the mean value plus or minus SD (arbitrary unit) for the phosphorylation per 1 ng of p53 protein was $14,185\pm642$. The same amount of p53 from the cells on soft agar was phosphorylated to a much lesser degree ($2,615\pm109$; $P<0.01$, Student's t test) during the same labeling period. In the anchorage-deficient cells, the net amount of the protein was 10.3 ng per 1×10^5 cells, i.e., twice as much as that of the anchorage-provided cells. Phosphoamino acid analysis showed that the phosphorylated p53 released exclusively phosphotyrosine and no phosphoserine or phosphothreonine. The putative wild-type p53 which was immunoprecipitated from normal human breast epithelial cells with a monoclonal antibody PAb1801 was phosphorylated at serine as well as at tyrosine (Fig. 4).

Two-dimensional gel electrophoresis revealed that the p53 had an electrophoretic mobility identical to that of a protein which was immunoprecipitated with a monoclonal antibody to phosphotyrosyl protein, with an apparent molecular size of 53 kD (Fig. 5). When the p53 and 53-kD protein were mixed and electrophoresed, they comigrated completely and no other spot could be observed in a two-dimensional gel. This indicated that the p53 contained phosphotyrosyl residues and was the predominant phosphotyrosine-containing protein in the cells.

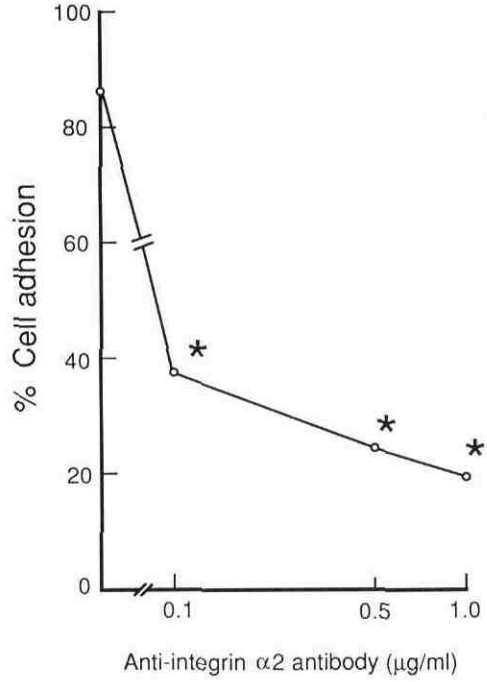

Fig. 2. Inhibition of cell adhesion to collagen by antibody to integrin $\alpha 2$. MCF-7 cells were seeded in collagen-coated dishes with or without anti-integrin $\alpha 2$ antibody at the concentrations indicated. After incubation for 1 h at 37°C, the number of cells that adhered to the dish was scored for each triplicate culture and the mean value is given; asterisks indicate that control and experimental groups are significantly different, $P < 0.01$.

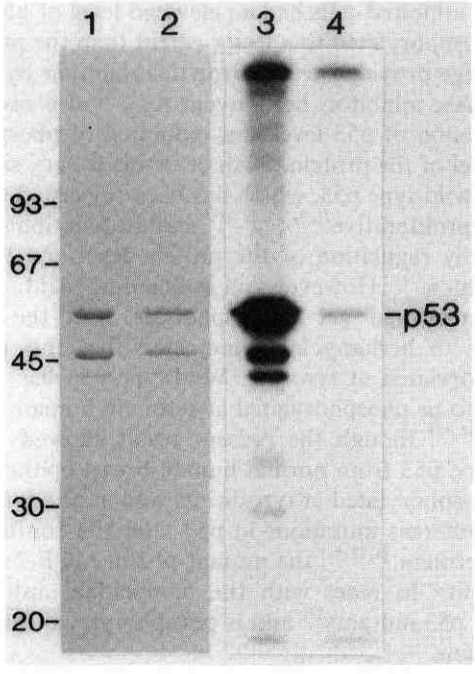

Fig. 3. Phosphorylation of p53 in MCF-7 cells growing on collagen or on soft agar. The same amount of the ^{32}P-labeled protein, that was immunoprecipitated with the monoclonal antibody PAb240 from cells on collagen (lanes 1 and 3) or on soft agar (lanes 2 and 4), was loaded. Coomassie blue staining (lanes 1 and 2) and autoradiography of the gels (lanes 3 and 4) are shown.

Effects of herbimycin A on anchorage-provided cells To determine the relationship between the cell adhesion to substratum and the phosphorylation of mutant p53, the effects of a tyrosine kinase inhibitor, herbimycin A, were examined in the anchored cells. Addition of herbimycin A to the cells 1 day after the beginning of culture did not affect the cell adhesion to collagen but altered the cell morphology (Figs. 1c and d). The mean cell number of the control culture not exposed to the antibiotic was 5.9×10^5 after a 2-day culture, while that of culture with herbimycin A at 0.5 μg/ml or 1.0 μg/ml was 2.1×10^5 or 1.6×10^5, respectively. The inhibition of cell proliferation was 63% ($P < 0.01$) and 73% ($P < 0.01$) by herbimycin A at the respective doses. In the growth-inhibited cells, the phosphorylation level of p53 was reduced as the dose of herbimycin A increased (Fig. 6). However, the net

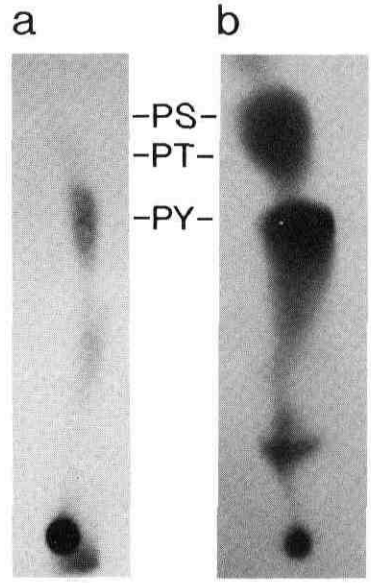

Fig. 4. Phosphoamino acid analysis of the ^{32}P-labeled p53. After autoradiography, the p53 band was excised from the gel, hydrolyzed, and the resulting residues were separated by single-dimensional thin-layer electrophoresis on a cellulose plate. p53 from MCF-7 cells (a) and from normal human breast epithelial cells (b). The positions of phosphoserine, phosphothreonine, and phosphotyrosine are indicated by PS, PT, and PY, respectively.

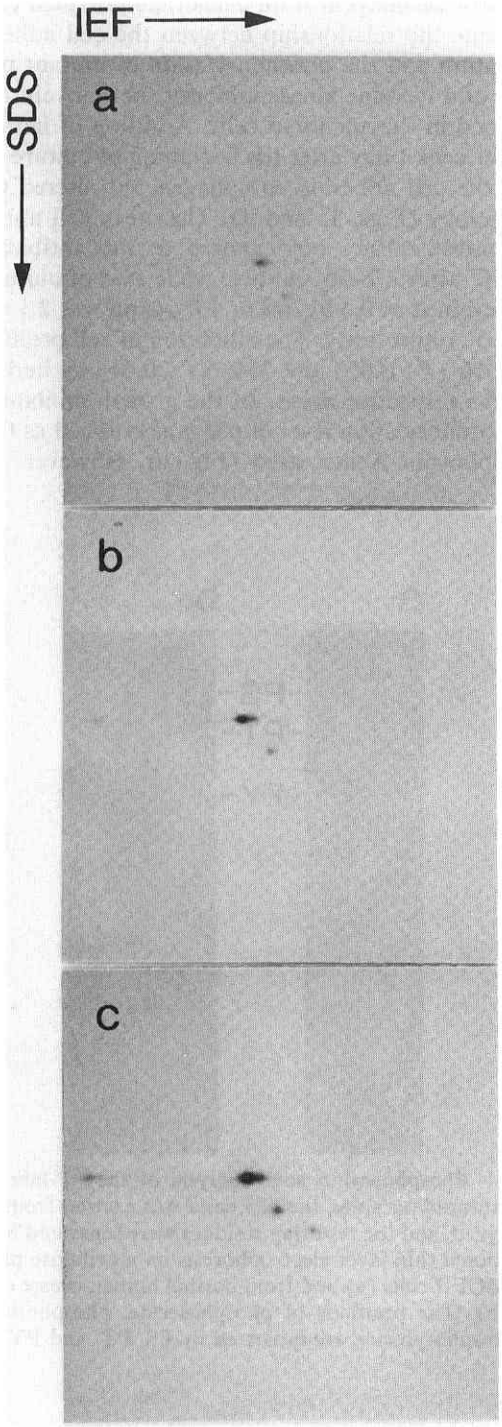

Fig. 5. Two-dimensional gel electrophoresis of ^{32}P-labeled p53. The [^{32}P]orthophosphate-labeled proteins were immunoprecipitated with the monoclonal antibody PAb240 to p53 mutants (a), or the monoclonal antibody 1G2 to phosphotyrosine-containing protein (b). A mixture of the samples in (a) and (b) is shown in (c). The directions of isoelectric focusing and SDS-polyacrylamide gel electrophoresis are indicated by arrows labeled IEF and SDS, respectively.

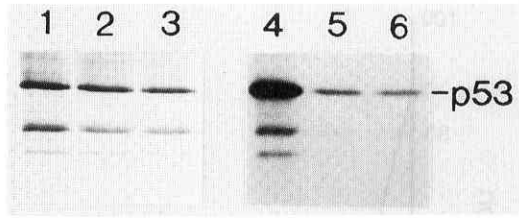

Fig. 6. Inhibition of p53 phosphorylation in MCF-7 cells growing on collagen by herbimycin A. Cells were cultivated and labeled with [^{32}P]orthophosphate in the absence (lanes 1 and 4) or the presence of herbimycin A at 0.5 μg/ml (lanes 2 and 5) and 1.0 μg/ml (lanes 3 and 6). The same amount of the p53 protein was loaded on one-dimensional gels. Silver staining (lanes 1–3) and autoradiography (lanes 4–6).

amount of the protein increased from 5.1 ng per 1×10^5 cells in the control culture to 12.0 ng and 16.5 ng per 1×10^5 cells in the presence of the antibiotic at 0.5 μg/ml and 1.0 μg/ml, respectively.

DISCUSSION

MCF-7 cells adhering to type I collagen-coated dishes thrived and had phosphorylated p53 mutant that was immunoprecipitated with a monoclonal antibody to various p53 mutants.[35] In contrast, cells seeded on soft agar became anchorage-deficient and hardly grew. This growth inhibition of cells on soft agar was not due to reduced cell viability and was not accompanied by a significant alteration in the cell cycle distribution as compared with that of the cells growing on collagen. The growth-inhibited cells had an elevated level of p53 which was phosphorylated to a lesser extent than the protein in anchorage-provided cells. Growth inhibition by a tyrosine kinase inhibitor, herbimycin A,[36-38] also resulted in an elevation of p53 level and reduction of phosphorylation level of the protein. Such behavior is very similar to that of wild-type p53, which has been suggested to act as an antiproliferative[21, 22, 24, 27, 32] and is functionally controlled by regulation of the protein level and by phosphorylation.[34] However, phosphoamino acid analysis, two-dimensional gel electrophoresis, and the responsiveness to herbimycin A indicated that this p53 was phosphorylated at tyrosine. Wild-type p53 has been reported to be phosphorylated at serine in human[34] and in mouse,[46-48] though the present result showed that the wild-type p53 from normal human breast epithelial cells was phosphorylated at tyrosine as well as at serine. Since many different mutations in p53 alter the conformation of the protein,[35, 49-51] the mutant p53 in MCF-7 cells has the ability to react with the monoclonal antibody to various p53 mutants[35] and is phosphorylated exclusively at tyrosine.

Anchored cells were suggested to adhere to collagen via the putative collagen receptor, integrin,[40] since antibody to the extracellular subunit integrin $\alpha 2$ interfered with cell adhesion to collagen. The integrin receptors, a family of transmembrane glycoproteins consisting of non-covalent heterodimers,[52] participate in cell-to-substratum and cell-to-cell adhesion and are localized in the adherens junction.[53] The relationship between the adherens junctions and tyrosine phosphorylation was demonstrated by the fact that the undercoat of adherens junction of Rous sarcoma virus-transformed cells contained the *src* gene product, $pp60^{v\text{-}src}$, which is a tyrosine kinase.[54] Further investigations have shown that tyrosine kinases such as v-*abl*,[55] c-*yes* and c-*src* associated in the junctions where the level of tyrosine phosphorylation was elevated.[56] The p53 phosphorylation was separable from the integrin-mediated cell adhesion, since herbimycin A significantly inhibited the p53 phosphorylation without interfering with the cell adhesion. This suggests that the integrin-mediated cell adhesion may indirectly activate the tyrosine kinase. Thus, although the precise mechanism involved in the activation of tyrosine kinase remains to be elucidated, the present results demonstrate that growth stimulation by providing an anchorage was associated with an elevated level of p53 phosphorylation in the cells.

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