The New Sulindac Derivative IND 12 Reverses Ras-induced Cell Transformation

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ABSTRACT

The nonsteroidal anti-inflammatory drug Sulindac has chemopreventive and antitumorigenic properties. Its metabolites induce apoptosis and inhibit signaling pathways critical for malignant transformation, including the Ras pathway. Here we show that the new Sulindac derivative IND 12 reverses the phenotype of Ras-transformed MDCK-f3 cells and restores an untransformed epitheloid morphology characterized by growth in monolayers with regular cell-cell adhesions. Moreover, IND 12 treatment induces the expression at membranes of the cell adhesion protein E-cadherin and increases the level of the E-cadherin-bound β-catenin. As a consequence, IND 12-treated MDCK-D cells lose their invasion capacity and regain the ability to aggregate. In the presence of IND 12, MDCK-f3 cells show regenerated expression and activity ratios of the small GTPases Rac and Rho normally found in untransformed MDCK cells. Strikingly, IND 12 treatment decreases the levels of phosphorylated mitogen-activated protein kinases, which are downstream substrates of the Ras-effector Raf kinase (12). These results offer an explanation for the finding that Sulindac inhibits the growth of tumors harboring Ras gene mutations more effectively than the growth of tumors with the wild-type Ras gene (17).

INTRODUCTION

Sulindac and other nonsteroidal anti-inflammatory drugs reduce the overall risk to develop cancer and can inhibit the growth of tumors (1–3). Nonsteroidal anti-inflammatory drugs inhibit the key enzymes of the eicosanoid metabolism, the COX1 and 2, and thereby decrease the levels of proliferation-activating eicosanoids (4). The COX inhibitor effect of Sulindac is mediated by its main physiological metabolite Sulindac sulfide, whereas Sulindac itself and its sulfone metabolite do not affect the COX pathway (5). Nevertheless, Sulindac sulfone, which is ineffective on COX, can induce apoptosis and is able to inhibit tumor growth (6). Additional findings that Sulindac metabolites cause apoptosis in epithelial tissue and in cultured cancer cells independently from the eicosanoid metabolism indicated that Sulindac might exert its antitumorigenic effects independently from the eicosanoid pathway (7–9). Sulindac is used in the therapy and the prevention of tumors in patients with the cancer predisposition familial adenomatous polyposis caused by inherited mutations in the APC gene (7, 10). Experiments performed with the Min mouse, the animal model for familial adenomatous polyposis, also demonstrated that the antitumor effect of Sulindac is independent of prostaglandin biosynthesis (11).

MATERIALS AND METHODS

Overall Experimental Strategy. On the first level of analysis, we screened for drug-induced alterations of cell morphology, cell growth, cell aggregation, and cell invasion behavior. We visualized these parameters by microscopy or in cell biological assays. On the next level, we asked for the molecular background of the detected drug-induced effects. To address this question, we analyzed the levels and the activities of proteins, which are known to play a pivotal role in the regulation of cell adhesion and cytoskeletal arrangement. At this point, our results indicated that the drug directly inhibits the Ras-signaling pathway. Therefore, we looked for the activity of the Ras pathway under drug influence by measuring the levels of Ras-activated effector proteins and Ras-induced gene expression.

The Drug IND 12 and the Cell Lines. The synthesis and the chemical properties of IND 12 have been described (18). The drug was dissolved in DMSO as a 1000 × stock solution. In all experiments, DMSO controls showed no significant effects. The linear correlation between drug concentration and UV absorption at the wavelength of the characteristic absorption maximum revealed no hints of insolubility or micelle formation in buffered aqueous solutions at the applied concentrations.

Epithelial MDCK cells and MDCK-f3 cells were kindly provided by John G. Collard (Amsterdam). The MDCK-f3 cell line is a subclone of the MDCK-ras-f cell line, which is a virally Ras-transformed derivative of the MDCK cell line (20). MDCK-f3 cells are cytoskeleton positive and show a fibroblast-like phenotype (19). Rabbit kidney epithelial-like RK13 cells were purchased from American Type Culture Collection. Cells were cultured under standard conditions.

Fluorescence Microscopy and SEM. The cellular morphology was analyzed by light microscopic or confocal microscopic analysis of cells immuno-
labeled with a monoclonal mouse rhodamine-coupled antibody directed against F-actin or polyclonal rat antibodies directed against E-cadherin (Sigma Chemical Co.). Binding sites of the primary rat antibodies were detected using a secondary antirat dichlorotriazinylaminofluorescein-coupled antibody (Transduction Laboratories). For SEM analysis, cells were grown on thermoxan slides and fixed by incubation in 4% paraformaldehyde and 1% glutaraldehyde in 200 mM HEPES (pH 7.4) for 45 min. Fixed cells were dehydrated in an ascending ethanol series and then critical point dried using methanol as intermediate and CO2 as drying medium. To avoid charging artifacts, the dried cells were covered with gold by sputter coating. Covered cells were studied with a Hitachi S-800 SEM equipped with a field emission gun. Cell surface morphology was imaged by the signal of secondary electrons.

RESULTS

IND 12 Restores Epithelial Morphology in Malignantly Transformed MDCK-f3 Cells, Reactivates Cell Aggregation, and Inhibits Cell Invasion. MDCK cells display the typical cellular and cell surface morphology of polarized transporting epithelial cells as revealed by scanning electron and light microscopy (Figs. 2 and 3). MDCK cells are characterized by numerous cell surface microvilli and well-organized cell-cell contacts and grow in an epithelium-like monolayer. In contrast, oncogenically transformed MDCK-f3 cells show the spindle-shaped form of functionally dedifferentiated tumor cells only sparsely covered with microvilli, strong membrane ruffling, and a reduced number of cell-cell contacts. They grow in single cell clones and partly overlap each other indicative for the loss of contact inhibition (Figs. 1–3). To test the effective concentration and the time-dependent effects of IND 12 on MDCK-f3 cells, we monitored morphological changes after IND 12 incubation. We observed that the morphology of MDCK-f3 cells changed in an IND 12 concentration-dependent manner toward the epithelioid morphology that is characteristic for untransformed MDCK cells (Fig. 1). The maximum effect was reached at 100 μM IND 12. Further increase of the IND 12 concentration showed no additional effect on cellular morphology. Therefore, we chose 100 μM as incubation concentration in the additional assays. IND 12-treated MDCK-f3 cells also resembled untransformed MDCK cells in their growing manner and number of cell contacts (Fig. 2). The treatment of untransformed MDCK cells had no effect. The morphological reversion of MDCK-f3 cells from the fibroblastoid to the epithelioid phenotype was confirmed by electron microscopy to be dependent on the drug-incubation time (Fig. 3). The fibroblastoid MDCK-f3 cells changed to cells with smooth, flattened cell surface with less filopodia compared with untreated MDCK-f3 cells. The extent of this reversion increased time dependently up to an incubation time of 8 h. Longer incubation had no additional effects. Even after 24 h, the IND 12-induced reversion of the transformed into the untransformed phenotype was not complete, e.g., IND 12-treated MDCK-f3 cells did not show as many microvilli as MDCK cells (Fig. 3).

In contrast to MDCK-f3 cells, MDCK cells form cell aggregates when seeded on soft agar (Fig. 4). When MDCK-f3 cells were grown in the presence of IND 12, they regained this ability and formed cell aggregates typical for untransformed MDCK cells. Although the aggregation phenotype of IND 12-treated MDCK-f3 cells is somewhat different from MDCK cells, our results show that IND 12 treatment restores the general ability of MDCK-f3 cells for aggregation and that IND 12 initiates the reversal to the aggregation phenotype of nontransformed cells. Moreover, IND 12 also inhibited the invasion of MDCK-f3 cells into a collagen matrix. The invasion index of IND 12-treated MDCK-f3 cells was decreased by >60% compared with untreated cells (Fig. 5). These results demonstrate that IND 12 is able to restore typical features of an epithelioid phenotype in Ras-transformed cells as contact inhibition, aggregation ability, and the absence of invasive behavior. This was also confirmed by the observation that IND 12-treated MDCK-f3 on the top of the collagen matrix showed very similar phenotype as MDCK cells (data not shown).

IND 12-treated MDCK-f3 Cells Regain the Levels of the E-cadherin/β-catenin Complex and the Relative Levels of Active Rac and Rho. Whereas the cell adhesion membrane protein E-cadherin is barely detectable in the Ras-transformed MDCK-f3 cells,
the treatment of MDCK-f3 cells with IND 12 led to the increase of the level of membrane-bound E-cadherin (Fig. 2). IND 12 treatment also led to the re-expression of the E-cadherin/β-catenin complex to levels normally found in untransformed MDCK cells (Fig. 6). In contrast, the level of total β-catenin remained unchanged in MDCK-f3 cells after IND 12 treatment, indicating that the observed increase of the E-cadherin/β-catenin complex is because of an increase in the level of membrane-bound E-cadherin that recruits cytosolic β-catenin (Figs. 2 and 6).

To gain further insight into the molecular mechanisms that are responsible for the observed morphological alterations, we analyzed the levels of the active GTPases Rac and Rho.
Fig. 4. IND 12 reactivates the aggregation ability of MDCK-f3 cells in a cell aggregation assay. MDCK (left), MDCK-f3 (middle), or MDCK-f3 cells in the presence of 100 μM IND 12 (right) were grown on soft agar and visualized by light microscopy at ×250 magnification. MDCK cells were adhesive and grew attached to each other within large aggregations, in contrast to MDCK-f3 cells, which formed only very small aggregations of cells. In the presence of IND 12, MDCK-f3 cells formed large aggregations of cells similar to untransformed MDCK cells.

Fig. 5. IND 12 reduces the invasive potential of MDCK-f3 cells in a cell invasion assay. MDCK cells, MDCK-f3 cells, or MDCK-f3 cells in the presence of 100 μM IND 12 were assayed for invasion through a matrigel-coated membrane. The invasion index was calculated as the percentage of invasive cells among the total number of cells. In contrast to untransformed MDCK cells, MDCK-f3 cells are highly invasive. In the presence of IND 12 at 50 or 100 μM, the invasion index of MDCK-f3 cells was drastically reduced.

MDCK cells
MDCK-f3 cells
IND 12

Fig. 6. IND 12 restores the level of the cell adhesion complex between E-cadherin and β-catenin, but the levels of immunoprecipitated E-cadherin and β-catenin remain unchanged by IND 12. Western blot analysis (WB) of E-cadherin and β-catenin in anti-E-cadherin and anti-β-catenin immunoprecipitates (IP; middle row) from MDCK, MDCK-f3, and MDCK-f3 cells after treatment with 100 μM IND 12. In MDCK-f3 cells, the level of the E-cadherin-bound β-catenin was reduced in comparison with the level in untransformed MDCK cells (middle row). IND 12 treatment restored the level of the E-cadherin-bound β-catenin to levels found in untreated MDCK cells. This restoration is not attributable to an increase of the level of the complexed E-cadherin (top row) and not to an increase of the level of total β-catenin in the cell lysates (bottom row), as shown by WB using anti-E-cadherin or anti-β-catenin antibodies, respectively.

DISCUSSION

In a recent study, we found that the sulfide metabolite of Sulindac inhibits the Ras pathway at micromolar concentrations (12). To identify new drugs that can inhibit the Ras pathway more efficiently and at lower concentrations, we synthesized new compounds based on Sulindac as a lead structure (18). One of these, IND 12, showed antiproliferative effects on cultured tumor cells and a lower unspecific antiproliferative effects on cultured tumor cells and a lower unspecific...
As a first sign for the reversion of transformation, IND 12 changed the fibroblastoid phenotype of MDCK-f3 cells toward the epithelioid morphology of the untransformed, normal MDCK cells. Cell-cell contacts are formed by membrane proteins of the cadherin superfamily, which are the starting point of the molecular cascade that leads to the formation of junctional desmosomes and tight junctions (31). On the cytoplasmic side, cadherins bind to the catenins, which is a prerequisite for the link between cell-cell contacts and the cytoskeleton and, thus, for the stability of cell-cell adhesion. Ras-transformed MDCK-f3 cells are highly invasive as a result of reduced E-cadherin-mediated cell-cell adhesion (19, 20). A reversion of MDCK cell transformation is accompanied by the translocation of cytosolic β-catenin to the membrane and the increase of E-cadherin/β-catenin complex (26). The reversal of the transformed phenotype by IND 12 correlated with a partial restoration of the levels of membrane-bound E-cadherin and of E-cadherin-bound β-catenin. This suggests that the observed morphological changes induced by IND 12 in these cells are based on a reorganization of both molecules at the membrane or in complexes, respectively. These results also show that IND 12 can initiate restoration of membrane-bound E-cadherin, retranslocation of β-catenin to the cell membrane, and the reformation of complexes between these molecules. Thus, it is conceivable that IND 12-treated cells regain the ability for contact inhibition and lose their invasive potential. The exact molecular mechanism by which IND 12 can exert this reversal of phenotype and the restoration of the levels of the membrane-bound E-cadherin and of the E-cadherin/β-catenin complex remains to be determined. However, the morphological differences between MDCK-f3 and MDCK cells have been shown to be a consequence of Ras-induced transformation of MDCK-f3 cells (19, 20). In this model, sustained Ras activation leads to the down-regulation of the relative level of active Rac in MDCK-f3 cells (25). Rac plays a role as a suppressor of invasion of epithelial cells and regulates cytoskeletal rearrangement and epithelial morphology and, thus, plays a role in the control of cell morphology and migration (26, 32). A result of down-regulated Rac activity, the level of the active GTPase Rho increases. We show here that in the presence of IND 12, the ratio of active Rac and Rho levels relative to the total levels of the two proteins is altered in MDCK-f3 cells and resembles the ratios normally found in untransformed MDCK cells. These findings sug-
gest that the observed effects of IND 12 on the transformed MDCK-f3 cells are associated with the expression or activity of these small G-proteins. Because the alteration of the levels of active Rac and Rho in MDCK-f3 cells is because of the sustained activation of the Ras pathway, an inhibition of the Ras pathway by IND 12 would offer an interesting explanation for the restoration of the Rac and Rho levels and the observed effects of IND 12 on MDCK-f3 cells. A number of other findings suggest that IND 12 exerts its effect by directly interfering with Ras-initiated signal transmission: (a) the levels of the phosphorylated active downstream effectors p44-MAPK and p42-MAPK decreased in the presence of IND 12; (b) IND 12 lowered the gene expression activated by oncogenic Ras but not the gene expression activated by the Ras downstream effector MAP-KK(EE); and (c) in a recent study, we showed that IND 12 inhibits the interaction between the proto-oncoprotein p21ras and the Raf kinase in vitro (18). Altogether, our findings make IND 12 a promising candidate with high potential in the all-important search for new antitumorigenic drugs with effects on the Ras pathway. Future studies have to demonstrate the effects of this drug on tumors in vivo.

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