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Effects of down-regulating the *Id* genes in human colorectal cancer cells on early steps of haematogenous metastasis

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ARTICLE INFO

Article history:
Received 9 June 2005
Received in revised form
8 November 2005
Accepted 15 November 2005
Available online 19 January 2006

Keywords:
Id gene
Colon cancer
Cell proliferation
Cell migration
Integrin α6
Lung metastasis
Cell adhesion
Laminin

ABSTRACT

Id genes (inhibitor of DNA binding/differentiation) play important roles in tumour growth. We have previously described crucial roles of *Id* gene over-expression in endothelial cells for tumour angiogenesis. Here, we have evaluated direct effects of *Id* gene down-regulation on tumour cells, namely on cell proliferation, motility, and adhesion to lung microvasculature during haematogenous metastasis. For this purpose, *Id* genes were stably down-regulated by RNA interference in human colorectal cancer cells. These cells showed delayed proliferation, inhibited motility and decreased expression of integrin α6 and consequently reduced adhesion to lung microvasculature in mice. Static adhesion assays and laminar flow assays revealed decreased laminin binding capacity of these cells, and blocking experiments confirmed that it could be attributed to decreased expression of integrin α6. The present results indicate important roles of *Id* genes in tumour cells during early steps of haematogenous metastasis and suggest dual effects from their therapeutic inhibition.

1. Introduction

Id (inhibitor of DNA binding/differentiation) genes stimulate cellular proliferation by interfering with cell cycle regulators, and are over-expressed in developing tissues and cancer, whereas only minimally detected in normal adult tissues.[1–3] We and others have previously described crucial role of *Id* gene over-expression in endothelial cells for tumour angiogenesis and growth.[4–6] However, knowledge is still lacking about the roles of *Id* genes in tumour cells. Recently, *Id* genes were reported to be over-expressed in colorectal cancer cells [7] and only minimally detected in normal adult colons.[8] Consequently it can be hypothesized that a therapeutic down-regulation of *Id* genes could have dual effects on colorectal cancer, i.e., not only on tumour endothelium but also on tumour cells. The aim of this study was to test, in a human colorectal cancer model, if down-regulation of *Id* genes can have direct effects on the invasive properties of tumour cells.

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0959-8049/$ - see front matter
doi:10.1016/j.ejca.2005.11.016

2. Materials and Methods

2.1. Tumour cells and Id gene down-regulation

Human colorectal cancer cell line HT29 was purchased from the American Type Culture Collection (Manassas, VA), and cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS) and 1% antibiotic/antimycotic (i.e., 100 U/ml penicillin G, 100 μg/ml streptomycin sulphate, 250 ng/ml amphotericin B; Life Technologies, Grand Island, NY). *Id* genes *Id1* and *Id3* were stably down-regulated by using RNA interference [9] as described previously.[5] Tumour cells transfected with vectors containing *Id1*/*Id3* shRNAs (small hairpin RNAs) or control vectors were selected for by resistance to G418 and named '*Id1*/*Id3*-kd HT29' or 'control HT29', respectively.

2.2. Flow cytometry and immunoprecipitation of Id proteins

Tumour cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Tween-20 and blocked with 0.2% BSA overnight at 4 °C. The cells were then washed twice with PBS, incubated with 2 μg/ml antibodies against Id1 or Id3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 30 min at 4 °C, washed twice, incubated with FITC-labelled secondary antibodies for another 30 min at 4 °C, and after another two washes, analyzed in the flow cytometer (FACSCalibur, Becton-Dickinson, San Jose, CA). For negative control, rabbit IgG was used as an isotype control antibody.

In immunoprecipitation experiments, 500 μl whole tumour cell lysates of equal protein concentrations were pre-cleared with normal rabbit serum and protein A – sepharose (Pharmacia Biotech AB, Uppsala, Sweden), and incubated with 5 μl antibodies against Id1 or Id3 for 1 h at 4 °C, and with 30 μl protein A – sepharose for another 1 h. Immunoprecipitates were centrifuged, washed four times, subjected to 12.5% SDS-PAGE, and stained with Silver Stain Plus Kit (Bio-Rad Laboratories, Hercules, CA). Optical density of proteins in gels was determined by OptiQuant Software Ver. 03.00 (Packard Instrument Co., Inc., Meriden, CT).

2.3. Cell proliferation assay

Tumour cells were washed, suspended in culture media, placed 1×10^3/100 μl/well in quadruplicates in 96-well plates and cultured for 6 days. Viable cells were detected by MTS assay (Promega, Madison, WI) at days 0, 1, 3 and 6. Proliferation rates were calculated as the ratios to values measured at day 0 that were considered 100%.

2.4. Wound closure assay

Migration experiments were performed as described previously.[10,11] Briefly, confluent monolayers of tumour cells were denuded first with a sterile razor blade and then with a plastic tip, washed and cultured in medium containing 100 nM phorbol 12-myristate 13-acetate (PMA) and 5 μg/ml mitomycin C (MMC; Sigma Chemical Co., St. Louis, MO) for 4 days. The extent of wound closure was calculated as the ratio between wound widths measured in five different areas at day 4 and day 0. Wound width at day 0 was considered 100%.

2.5. Flow cytometry of adhesion molecules

Tumour cells were washed twice with PBS and incubated with 2 μg/ml antibodies against integrins α1 (Becton-Dickinson), α2 (Beckman Coulter, Fullerton, CA), α3 (Dako, Glostrup, Denmark), α6 (GoH3; Santa Cruz Biotechnology), αV and αM (Immunotech, Marseille, France); carbohydrates Sialyl Lewis X or Sialyl Lewis A (Chemicon, Temecula, CA); and 67 kDa laminin receptor (MLuC5; Abcam, Cambridge, UK) for 30 min at 4 °C. Then, the cells were washed twice and incubated with FITC-labelled secondary antibody for another 30 min at 4 °C. After another two washes, the cells were analyzed in the flow cytometer. For negative control, mouse IgG was used as an isotype control antibody.

2.6. In vivo model of metastatic tumour cell adhesion

For in vivo models, six-week old female Balb/cA Jcl-nu/nu mice were purchased from Saitama Experimental Animal Supply Co., Ltd (Sugitomachi, Japan). Experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Tokyo. Tumour cells were labelled with sodium chromate (4×10^7 cells/150 μl ^{51}Cr in PBS and 20 μl FCS) for 1 h at 37 °C, washed three times with PBS, suspended in Hanks' Balanced Salt Solution and injected into the tail veins of mice (2×10^6 cells/0.2 ml/mouse). Fifteen hours later, mice were sacrificed, and lungs removed. Tumour cells adhered to lung microvasculature were quantified in a γ-counter and whole lungs were subjected to autoradiography (Cyclone™ Storage Phosphor System, OptiQuant Software Ver. 03.00, Packard Instrument Co., Inc.).

2.7. Static adhesion assays

Laminin, fibronectin or collagen type I were coated on 96-well immunoplates at 10 μg/ml for overnight at 4 °C, and blocked for non-specific binding with 0.2% BSA for 30 min at 37 °C. Tumour cells suspended in medium were placed at 1×10^4/100 μl/well in quadruplicates. After 10 min, non-adhered cells were gently washed out, and firmly adhered cells quantified by MTS assay. Adhesion rate was calculated as a ratio to the starting number of placed cells that was considered 100%. For negative control, uncoated wells were used.

2.8. Laminar flow assay

Flow experiments in a laminar flow chamber were performed as we described previously.[12] Briefly, tumour cells were washed twice with PBS, incubated with antibody against integrin α6 or isotype control antibody at 25 μg/ml for 1 h at 4 °C, suspended in medium at a concentration of 1×10^6 cells/ml and stored on ice. Cells were warmed to 37 °C and perfused on laminin for 2 min (1×10^6 cells/ml at wall shear stress 1.0 dyn/cm^2). Weakly adhered cells were washed out by medium perfused for 15 min, and firmly adhered cells counted in six different fields.

2.9. Statistical analysis

The results were analyzed by ANOVA in combination with Scheffe's post-hoc procedure, and expressed as mean ± standard deviation. Differences were considered statistically significant at $P < 0.01$ or $P < 0.05$.

3. Results

3.1. Down-regulation of Id genes in tumour cells

Id genes were stably down-regulated in human colorectal cancer cell line HT29. Flow cytometry (Fig. 1(a)) and immunoprecipitation (Fig. 1(b)) confirmed decreased Id1 (67% of control HT29) and Id3 (66% of control HT29) in *Id1/Id3*-kd HT29 cultured for two months, suggesting that their down-regulation was stable. When compared with parental cells or cells treated with a control vector, *Id1/Id3*-kd HT29 showed identical morphology and colony formation.

3.2. Delayed proliferation

Next, cell proliferation was evaluated during the period of 6 days (Fig. 2(a)). At day 6, there were 538 ± 15% parental HT29, 602 ± 17% ($P = 0.93$) control HT29, but only 354 ± 11% ($P < 0.01$) *Id1/Id3*-kd HT29 measured in cell cultures, suggesting delayed proliferation of *Id1/Id3*-kd HT29.

3.3. Inhibited motility

Tumour cell motility was evaluated in a standard wound closure assay (Fig. 2(b)). At day 4, the wounds were closed to 41 ± 9% by parental HT29, 45 ± 6% ($P = 0.66$) by control HT29, but still remained open at 95 ± 7% ($P < 0.01$) by *Id1/Id3*-kd HT29, suggesting inhibited motility of these cells.

3.4. Decreased expression of integrin α6

Then, expression of adhesion molecules in tumour cells was measured. There was markedly decreased expression of integrin α6 in *Id1/Id3*-kd HT29 (40% of control HT29; Fig. 3(a)); other integrin levels remained unchanged (α1, α2, α3, αM and αV) as did carbohydrates Sialyl Lewis X and Sialyl Lewis A; while the 67 kDa laminin receptor was absent in parental, control and *Id1/Id3*-kd HT29 (not shown).

3.5. Reduced ability to adhere to lung microvasculature in mice

For in vivo observation of adhesive interactions between circulating tumour cells and lung microcirculation, ^{51}Cr-labelled single-cell suspensions were injected into tail veins of nude mice. Fifteen hours later, the lungs were removed and their radioactivity measured in a γ-counter. As a result, 1160 ± 40 cpm was measured in parental HT29, 1209 ± 26 cpm ($P = 0.21$) in control HT29, but only 545 ± 17 cpm ($P < 0.01$) in *Id1/Id3*-kd HT29 (Fig. 3(b)), suggesting decreased numbers of these cells adhered to lung microvasculature.

3.6. Decreased laminin binding capacity

Finally, adhesion of tumour cells to various extracellular matrix (ECM) proteins was evaluated in static adhesion assays and laminar flow assays. Under static conditions, *Id1/Id3*-kd HT29 showed decreased binding capacity for laminin, given that the adhesion rates were 73 ± 0% in parental HT29, 73 ± 2% ($P < 0.01$) in control HT29, but only 54 ± 3% ($P < 0.01$) in *Id1/Id3*-kd HT29. In contrast, binding capacity for fibronectin, collagen type I or uncoated wells remained unchanged (Fig. 3(c)). Under dynamic conditions of laminar flow, the final

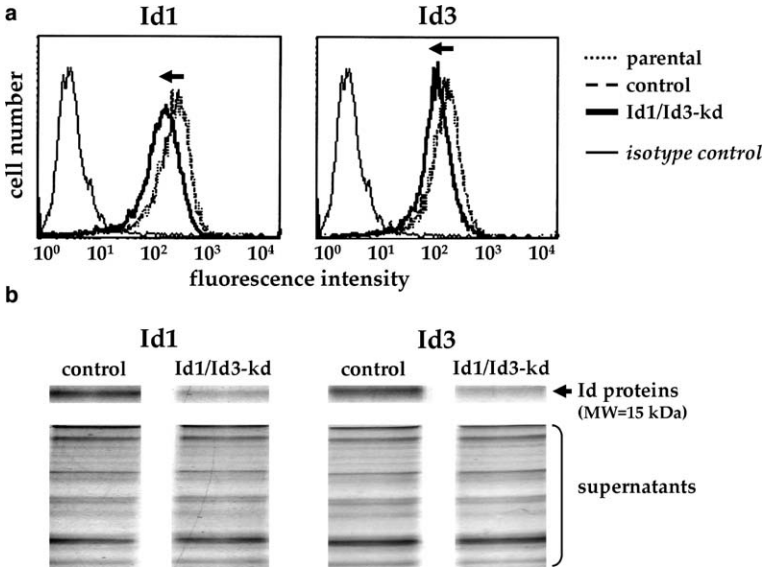

Fig. 1 – Down-regulation of *Id* genes. *Id* gene expression in HT29 was determined by (a) flow cytometry and (b) immunoprecipitation with similar results. When compared to parental HT29, Id1 and Id3 protein levels were unchanged in control HT29 but reduced in *Id1/Id3*-kd HT29 (67% and 66% of control HT29 for the respective Id). Supernatants (b) are shown to demonstrate equivalent protein concentrations of samples subjected to immunoprecipitation.

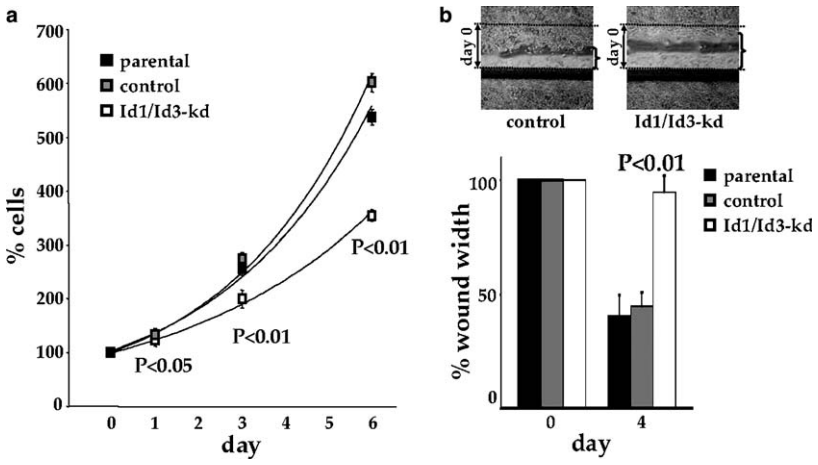

Fig. 2 – Proliferation and motility: (a) tumour cells were cultured for 6 days and their proliferation rates determined by MTS assay. Graph shows significantly delayed proliferation of *Id1/Id3*-kd HT29; (b) cell motility was examined in a wound closure assay. Graph shows relative wound widths at day 4 and day 0. Representative wounds at day 4 are shown (dotted lines show wound width at day 0).

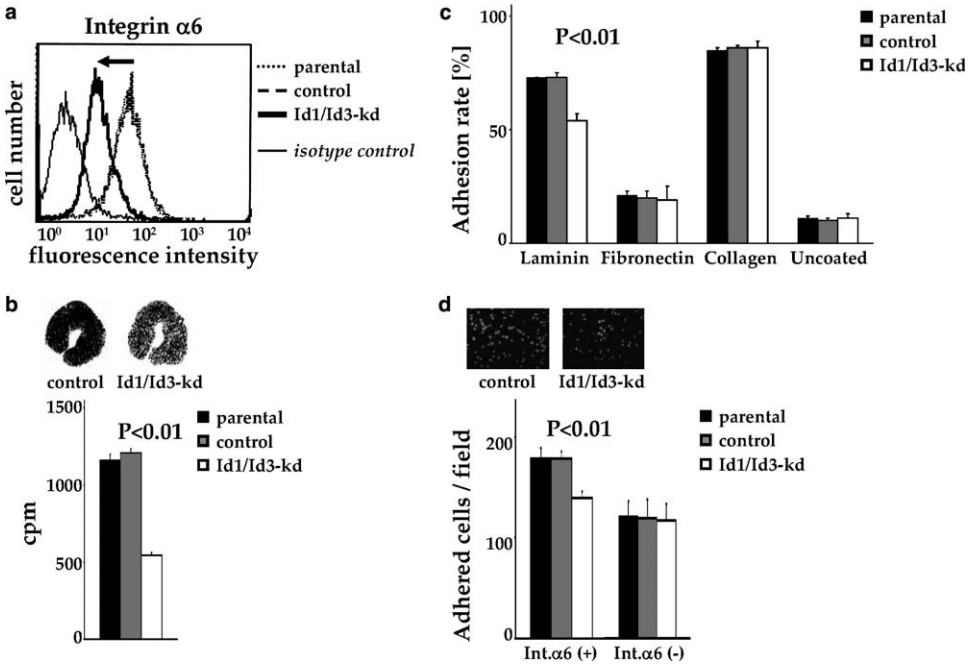

Fig. 3 – Integrin α6 expression and its consequences in vivo and in vitro: (a) integrin α6 expression in HT29 as determined by flow cytometry. Unchanged expression levels of integrin α6 in control HT29 but markedly decreased in *Id1/Id3*-kd HT29 are shown; (b) in a mouse model of metastatic tumour cell adhesion, *Id1/Id3*-kd HT29 showed significantly decreased adhesion to lung microvasculature. Graph shows radioactivity of lungs measured 15 h after intravenous injection of ^{51}Cr-labelled single tumour cell suspension. Autoradiographs of representative whole lungs are shown; (c) static adhesion assays revealed significantly decreased binding capacity of *Id1/Id3*-kd HT29 for laminin whereas unchanged for fibronectin, collagen type I and uncoated wells; (d) similar results for laminin binding were obtained also under conditions of laminar flow. Graph demonstrates that in contrast with unblocked cells (Int. α6(+)), the cells that were pre-blocked with a monoclonal antibody against integrin α6 (Int. α6(–)) had identical laminin binding capacities. Photographs show representative adhered cells with unblocked integrin α6.

numbers of adhered cells/field were 179 ± 6 in parental HT29, 179 ± 11 ($P = 1.00$) in control HT29 but only 140 ± 6 ($P < 0.01$) in *Id1/Id3*-kd HT29 (Fig. 3(d)), supporting the results of static adhesion assays. Moreover, pre-blocking of cells with anti-integrin α6 monoclonal antibody resulted in identical laminin binding capacities of parental (122 ± 16 cells/field), control (121 ± 19 cells/field; $P = 0.99$) and *Id1/Id3*-kd HT29 (119 ± 16 cells/field; $P = 0.95$) under flow conditions, suggesting a major role of integrin α6 deregulation in decreased laminin binding by *Id1/Id3*-kd HT29.

4. Discussion

In this study, *Id* genes *Id1* and *Id3* were stably inhibited in human colorectal cancer cell line HT29 to test if it can have direct effects on invasive properties of tumour cells. Among the four members of *Id* gene family, *Id1* and *Id3* genes are up-regulated not only in colorectal cancer but also in cancers of many other tissue origins,[8,13] and therefore, their down-regulation could have important therapeutic implications. Interestingly, *Id1* and *Id3* are evolutionally closely related and can functionally substitute each other.[2,14] Consequently, in the cells expressing both *Id1* and *Id3*, down-regulation of single *Id* is not sufficient for effective inhibition of processes controlled by these genes, and simultaneous inhibition of both *Id1* and *Id3* is necessary.[5,6]

Id genes are known to stimulate cell proliferation but their other roles are still unclear. In the present study, we observed not only delayed proliferation, but also inhibited motility and decreased expression of integrin α6 in *Id1*/*Id3*-kd HT29, suggesting multiple regulatory roles of *Id* genes in cellular processes. The latter finding was supported by others, showing a positive correlation between the expression levels of *Id* genes and integrin α6 in endothelial cells.[15]

Integrin α6 is one of the major receptors for laminin,[12,16] a molecule present not only in extracellular matrix or basement membranes but also on the luminal surface of lung microvasculature.[17] Therefore, it can be hypothesized that decreased expression of integrin α6 in tumour cells due to *Id* gene down-regulation might decrease their adhesion to lung microvasculature, and so impair early steps of haematogenous metastasis. To test this hypothesis, we injected ^{51}Cr-labelled single tumour cell suspensions intravenously in mice. We found that *Id1*/*Id3*-kd HT29 had significantly reduced ability to adhere to lung microvasculature. To address the question whether decrease of only integrin α6 or also other adhesion molecules was responsible for this effect, we examined expression patterns of other integrins and carbohydrates, including Sialyl Lewis A, a receptor for endothelial selectin,[18] and determined binding capacity of tumour cells for various extracellular matrix proteins. *Id1*/*Id3*-kd HT29 showed unchanged expression levels of examined integrins (except for integrin α6) and carbohydrates. In addition, *Id1*/*Id3*-kd HT29 showed unchanged binding capacities for fibronectin and collagen type I that are additional ligands of many laminin receptors except for integrin α6. Moreover, pre-blocking of tumour cells with anti-integrin α6 monoclonal antibody resulted in identical laminin binding capacities in control and knocked-down cells under flow conditions. From these results we concluded that down-regulation of integrin α6 played a major role in decreased laminin binding capacity of *Id1*/*Id3*-kd HT29.

To our knowledge, there is still just one report in the literature on direct effects of *Id* gene down-regulation on tumour cells.[19] In this report, the authors showed that *Id* gene down-regulation in breast cancer cells impaired lung metastasis through inhibition of their MT-MMP1 activity. However, our present observations in colorectal cancer cells demonstrated that earlier step of haematogenous metastasis, i.e., adhesion of tumour cells to the vascular bed of target organs could be involved in therapeutic effect of *Id* gene down-regulation. We suggest that further studies concerning the roles of *Id* genes in tumour cells will help to provide a solid basis for a neoadjuvant therapy of cancer with dual effects on both endothelial and tumour cells.

Conflict of interest statement

None declared.

Acknowledgements

The authors thank Dr. Aki Kamijo, Ms. Mitsuko Miyamoto, Ms. Mika Matsuhashi and Ms. Chieko Uchikawa for their kind advisory and technical assistance. This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by the Ministry of Health, Labor and Welfare of Japan, and by a grant from the Sankyo Foundation of Life Science.

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