Up-regulation of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Activity in Left-sided Human Colon Cancer

MARIA NOTARNICOLA[1], CATERINA MESSA[1], MARIA PRICCI[1], VITO GUERRA[2], DONATO FRANCESCO ALTOMARE[3], SEVERINO MONTEMURRO[4] and MARIA GABRIELLA CARUSO[1]

[1]Laboratory of Biochemistry,
[2]Laboratory of Epidemiology and Biostatistic, National Institute of Digestive Disease, Castellana Grotte, Bari;
[3]Division of Surgery, DETO, University of Bari;
[4]Division of Surgery, National Institute of Oncology, Bari, Italy

Abstract. *Background. We evaluated the HMGCoA reductase activity and LDL receptor levels in human colon cancer as well as the effects of simvastatin on in vitro cell growth and apoptosis of DLD-1 and Caco2 cell lines. Materials and Methods: HMGCoA reductase activity and LDL receptor were measured by radiochemical assay and ELISA method, respectively. Cell growth and apoptosis were evaluated by MTT-test and DNA fragmentation analysis, respectively. Results: Higher HMGCoA reductase activity and LDL receptor levels were detected in cancer than in normal mucosa. An up-regulation of HMGCoA reductase activity was detected in left-sided tumors. Simvastatin treatment produced marked anti-proliferative and pro-aptotic effects in DLD-1. Cell growth inhibition, but no apoptosis, was also evident in Caco2 cells. Conclusion: The cholesterol pathway is involved in colon malignant transformation. Therapeutic strategies using HMGCoA reductase inhibitors as anti-cancer compounds should also take into consideration the biological and clinical differences detected inside colon tracts.*

3-Hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase is a microsomal enzyme that catalyses the conversion of HMGCoA to mevalonate, the rate-limiting step in cholesterol biosynthesis (1). Cholesterol is essential in maintaining cellular membrane structure and integrity, as well as serving as a precursor for the synthesis of steroid hormones and bile acid (2). In addition to cholesterol, the mevalonate pathway produces various end products that are important for many different cellular functions. It appears that one of the potential mechanisms that supports colorectal carcinogenesis is the up-regulation of mevalonate-mediated cell growth. Cancer cells seem to require an increased concentration of cholesterol and cholesterol precursors and this requirement may be fulfilled by increased HMGCoA reductase activity or enhanced Low Density Lipoprotein (LDL) receptor activity, or both (3). Alterations in the biosynthetic processes of the mevalonate-pathway and in the levels of enzyme products participating in this biochemical system may contribute to the cell growth advantage acquired during the carcinogenic process and to the development of malignancy (4). In malignancy, high levels of mevalonate-derived metabolites, such as isoprenoid compounds, have been demonstrated (4-7). Several HMGCoA metabolites, such as farnesyl pyrophosphate and geranyl pyrophosphate, are implicated in oncogene activation and tumorigenesis (8). High HMGCoA reductase activity have been found in human hepatocellular carcinoma (9), hematological malignancies (10,11) and in brain tumors (12).

The levels of HMGCoA reductase activity have also been evaluated in gastric tumor (13), where an increased enzymatic activity was detected in neoplastic tissue compared to normal gastric mucosa. Moreover, in a previous study, we showed that high HMGCoA reductase activity levels were present in human colon cancers not expressing the LDL receptor (14). We have also demonstrated that colorectal cancer patients without LDL receptors have shorter survival that those with LDL receptors (15). The absence of the LDL receptor induces tumors to depend on endogenous cholesterol synthesis for their proliferation, resulting in an increase of HMGCoA reductase activity inside the cells.

Several studies have shown that prevention of tumor cell growth can be achieved by restricting either cholesterol availability or cholesterol synthesis (16). A number of highly

selective HMGCoA reductase inhibitors have been developed, with the aim of decreasing elevated plasma levels of cholesterol. These drugs have been considered as potential anti-tumor agents because of their ability to inhibit cellular growth and proliferation dependent on cholesterol synthesis (17).

Simvastatin, as an inhibitor of the HMGCoA reductase, leads to depletion of mevalonate and its downstream products and, thus, may have a significant influence on many critical cellular functions.

In this study, we evaluated the HMGCoA reductase activity levels and LDL receptor content in normal colorectal mucosa and cancer from patients operated for colorectal carcinoma, as well as the effects of simvastatin on *in vitro* cell growth and apoptosis of two human colon cancer cell lines (DLD-1 and Caco2 cells).

Materials and Methods

Patients. Seventy colorectal cancer patients (37 males and 33 females, mean age 65.3±10.2 years) were enrolled in the study. Colorectal normal mucosa and cancer were obtained from each of them. Specimens were taken immediately after the surgical procedure and stored at -80˚C until assayed.

For each patient, information on clinical characteristics and histopathological features was recorded.

Microsomal HMGCoA reductase activity assay. HMGCoA reductase activity was measured as [^{14}C]-mevalonolactone formed in resuspended microsomal pellets, by radiochemical assay using DL-3-hydroxy-3-methyl-[3-^{14}C]-glutaryl-coenzyme A (^{14}C-HMGCoA) as substrate. The colon microsomes were prepared as previously described (14). Microsomal HMGCoA reductase activity was assayed in two sequential steps: a) a pre-incubation period, during which microsomes were incubated in the presence of *Escherichia coli* alkaline phosphatase, in order to prime the enzyme, and b) a subsequent incubation period, during which the HMGCoA reductase activity was measured. Both phases have previously been extensively described (13). HMGCoA reductase activity was expressed as picomoles of [^{14}C]-mevalonate formed per minute per milligram of microsomal proteins (pmol/min/mg prot). Normal liver tissue was used as the positive control in each experiment.

LDL receptor. LDL receptor protein content was assessed in normal colorectal mucosa and cancer using an enzyme-linked immunosorbent assay (ELISA), as described elsewhere (18). Briefly, the solubilized membrane proteins (1 μg per well) were absorbed by overnight incubation at 37˚C on a solid-phase (96-well plate). The first antibody used was monoclonal anti-human LDL receptor mouse IgG C8 (Calbiochem-Novabiochem, La Jolla, CA, USA). The optimal amount of IgG anti-LDL receptor was 0.2 μg per well. The second immunological reagent was peroxidase-conjugated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) (0.004 mg per well), and *o*-phenylenediamine chromogenic substrate (OPD) was used with the enzyme-coupled antibody. The absorbance of the samples was recorded at 492 nm on a reader (Tirtek Multiskan; MCC, Helsinki, Finland). The values were expressed as nanograms of bound anti-LDL receptor monoclonal antibody per milligram of membrane proteins (ngAb/mg prot) derived from a standard curve.

Cell culture conditions. The human colon adenocarcinoma-derived cell lines, DLD-1 and Caco2, were obtained from the ICLC (IST, Genova, Italy). DLD-1 cells were grown in RPMI 1640 and Caco2 cells in MEM supplemented with 10% fetal bovine serum (FBS), 1% non essential amino acids (NEAA), 2mM glutamine, 100U/ml penicillin, 100 mg/ml streptomycin, in monolayer cultures, and incubated at 37˚C in a humidified atmosphere containing 5% CO_2 in air. All cell culture components were purchased from Sigma-Aldrich, Milan, Italy. Simvastatin was kindly provided by Sigma-Tau.

Simvastatin treatment. Cell growth: In the experiments investigating the effect exerted by simvastatin on cellular growth, the cells were plated at a density of 2 x 10^5 cells/5ml of phenol red-free RPMI 1640 or MEM containing 10% FBS in 60-mm tissue culture dishes (Corning Costar, Cambridge, MA, USA). After 24 hours, to allow for attachment, the medium was removed and fresh medium containing 3% Lipoprotein Deficient Serum (LPDS) was added to the cells. The cells were incubated for a further 24 hours, reaching approximately 60% confluence, then the medium was removed. Simvastatin dissolved in DMSO was added to fresh LPDS medium at increasing concentrations of 1, 2.5, 5, 10 and 20 μM for 48 hours.

Each experiment included an untreated control. Triplicate cultures were set up for each simvastatin concentration and for controls, and each experiment was repeated six times. Cell viability, determined using the trypan blue exclusion method, always exceeded 90%.

Assessement of cell proliferation: After DLD-1 and Caco2 cells had been cultured for 48 hours with different concentrations of simvastatin (1-20 μM), the proliferative response was estimated by colorimetric MTT-test (Sigma-Aldrich) (19).

Apoptotic death assay: To study the effects of simvastatin on apoptosis of DLD-1 and Caco2 cells, the same incubation procedure as in the assessment of cell proliferation was used. Analyses were carried out directly in 24-well plates in which the cells were seeded at a density of 20-25x10^{3}cells/cm^2.

After 48 hours from inhibitor administration, the apoptotic response to the different drug concentrations was estimated by DNA fragmentation analysis.

Cytosolic DNA-histone complexes generated during apoptotic DNA fragmentation were evaluated using the cell death detection ELISA kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol.

For the ELISA test, after drug treatment, the cells were washed once with PBS and 0.5 ml lysis buffer was added. After a 30-minute incubation, the supernatant was recovered and assayed for DNA fragments. Each experiment was performed in triplicate. Additional plates treated as above were analyzed for cell number with the use of the MTT test. The A_{405} obtained from the DNA fragmentation assay was then normalized for cell number.

Statistical analysis. The differences in HMGCoA reductase activity and LDL receptor protein levels between normal mucosa and colon cancer were analysed by Wilcoxon signed-rank test. Multiple Linear Regression analysis was used to determined which, if any, of the parameters such as age, sex of patient, location of tumor and the LDL receptor protein levels was related with HMGCoA reductase activity in normal and neoplastic samples. Variables were kept in the model only if their interaction terms improved the fit of the model. Age and LDL receptor were considered as continuous variables, while sex (male, female) and tumor site

Table I. *Clinico-histopathological features of colorectal cancer patients.*

	Cases (n=70)
Age (mean±SD)	65.3±10.2
Sex	
male	37
female	33
Tumor side[a]	
rectum	34
left	19
right	17
Tumor stage[b]	
stage I	4
stage II	28
stage III	26
stage IV	12
Histological grading	
well-differentiated	10
moderately-differentiated	36
poorly-differentiated	18
mucinous	6

[a] Left side: descending colon and sigmoid. Right side: hepatic flexure, cecum and ascending colon.
[b] Clinical staging performed by using UICC system.

Table II. *Multiple linear regression between HMGCoA reductase activity in cancer and sex, age, left side, right side and LDL receptor.*

	Coefficient	St. Error	*P* value	95% C.I.
Sex	-1.99	1.73	0.25	-5.46 - 1.4
Age	0.017	0.05	0.77	-0.1 - 0.135
Left side	3.73	1.92	0.04	-0.14 - 7.6
Right side	-1.15	2.08	0.58	-5.3 - 3.03
LDL receptor	-0.005	0.008	0.52	-0.02 - 0.01

(rectum, left and right colon) were scored as categorical variables.

Statistical analysis was performed with statistical software package STATA 6. Analysis of variance and Dunnett's Multiple Comparison Test were used to evaluate the *in vitro* effects exerted by simvastatin on cell growth and apoptosis.

Results

The clinical and histopathological features of all patients studied are given in Table I. Higher HMGCoA reductase activity levels were detected in cancer than in normal mucosa [5.07 (0.9-28.7) *vs* 3.82 (0.6-6.8), median values and range expressed as pmol/min/mg prot., $p=0.001$]. LDL receptor content was significantly enhanced in cancer with respect to normal mucosa [103.5 (0-384.6) *vs* 57.4 (0-238.7), median values and range expressed as ngAb/mg prot., $p=0.004$].

To investigate whether HMGCoA reductase activity was associated with clinicopathological variables, we performed a multivariate regression analysis with HMGCoA reductase activity as the dependent variable. In normal tissue, the HMGCoA reductase activity levels are not associated with the examined variables (data not shown). Table II shows the model used to evaluate, in neoplastic tissue, the

associations among variables. The final model included sex, age, tumor site and LDL receptor. Enhanced activity of HMGCoA reductase in neoplastic tissue was associated with the location of tumors. The tumors located on the left side showed a HMGCoA reductase activity three times higher than the enzyme activity detected in the tumors located on the rectum.

Exposure of DLD-1 and Caco2 cells to increasing concentrations of simvastatin (ranging from 1 to 20 μM) determined a reduction of cellular HMGCoA reductase activity and mRNA expression in a dose-dependent manner (data not shown). Drug treatment produced an evident anti-proliferative action in the DLD-1 cell line in a dose-dependent manner (Figure 1). The inhibitory effect of simvastatin on cell growth was only detected at 20 μM in the Caco2 cell line (Figure 2). The exposure of DLD-1 cells to increasing concentrations of simvastatin also gave rise to a marked pro-apoptotic effect. A significant increase in DNA fragmentation compared to the control was observed at a concentration from 1 μM ($p<0.05$), whereas no apoptosis was detected in Caco2 cells (Figure 2). These effects of drug on cellular growth and apoptosis were reversed by addition of 1 mM mevalonate, as shown in Figures 1 and 2.

Discussion

An important feature of malignant transformation is the loss of the cholesterol feedback inhibition mechanism that regulates cholesterol synthesis. The main cholesterol feedback defect in malignant cells has been located at the HMGCoA reductase step. In the present study, we found a significantly higher HMGCoA reductase activity and LDL receptor levels in cancer than in normal mucosa. In addition, colon tumors on the left side showed higher HMGCoA reductase activity than tumors located on the other colon segments (rectum and right). Several studies have indicated that there are differences in the aetiology, clinical behavior, pathological features and genetic abnormalities in cancer of the right colon *versus* the left colorectum (20). This evidence supports the theory that the oncogenesis of the left-, right-
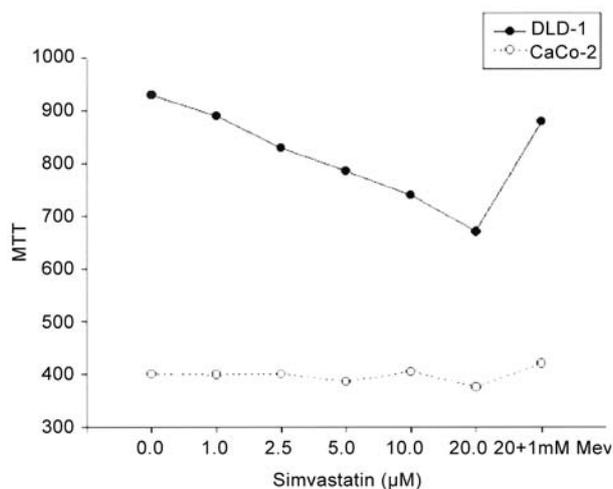
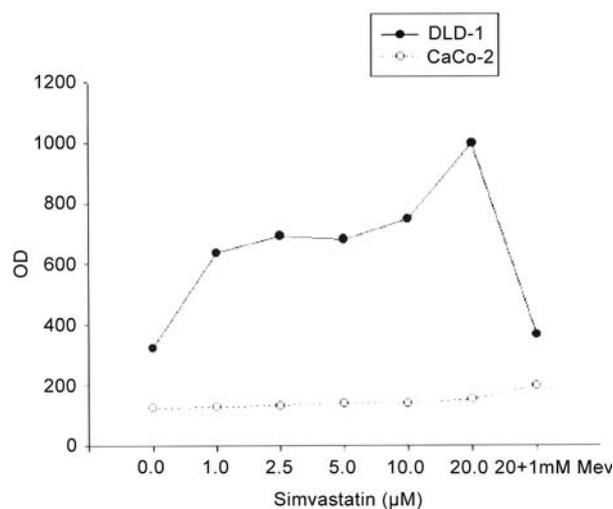Figure 1. *Simvastatin effects on cell proliferation measured by MTT assay in DLD-1 and Caco2 cell lines.*

Figure 2. *Apoptosis in DLD-1 and Caco2 cells.*

and rectum-sided cancers may involve, at least partially, different mechanisms (20,21). In colon cancer distant metastasis is the major problem, whereas in rectal cancer it is local recurrence. It is therefore reasonable to suggest that the aetiological factors and the molecular basis may differ between colon and rectal cancer (22). Environmental factors that could potentially favor the development of right- or left-sided colon tumors include diet, physical activity, smoking, cholecystectomy, chemopreventive agents, reproductive and hormonal status (21). Previously, we have demonstrated that growth factors such as EGF and TGFα are expressed more in the left colon than in the right one (23). Different segments in the large bowel may have different susceptibilities to neoplastic transformation. Kinetic changes are more marked in the left colon than in the right colon, as shown by the labelling index of the upper crypt regions (24). Molecular features are responsible for determining tumor phenotype as well as genetic and biochemical factors which may be linked to the anatomical site of tumor. The differences in HMGCoA reductase activity among left, right colon and rectum detected in this study underline the regional variability in the neoplastic cell proliferation and differentiation of the large bowel. The up-regulation of HMGCoA reductase activity in left-sided tumors could be due to an increased demand for cholesterol by neoplastic cells of these tumors. Several clinical and experimental investigations have observed that distal lesions show a higher incidence of chromosomal aberrations and hyperploidy (25), whereas proximal colonic tumors are often associated with normal karyotypes and characterized by diploid DNA content. Multiple linear regression analysis shows that the

association between HMGCoA reductase activity and tumor side is more strong in tumors where lower levels of LDL receptor are present. In agreement with this finding, the HMGCoA reductase activity was previously found to be enhanced in human colorectal cancer that did not express LDL receptors (14), indicating that LDL receptors absence, which deprives colonic neoplastic cells of exogenous sterols, is overcome by an increase in endogenous cholesterol synthesis. The loss of LDL receptors leading to a high endogenous HMGCoA reductase activity determines high levels of cholesterol and mevalonate-derived metabolites, such as isoprenoid compounds. Isoprenoids have been implicated as potential growth regulators and also serve as substrates for the farnesylation of the gene product of the ras oncogene, activated in 30-40% of colorectal cancer. Farnesylation is central to the intracellular localization and proper function of Ras proteins and other members of its superfamily (26). Given the role these proteins play in pathways regulating cell survival, proliferation, differentiation and cytoskeletal organization, it seems likely that altered expression will manifest as a markedly abnormal function. We have found an increased farnesyltransferase (Ftase) activity in human colorectal cancer, showing differences in Ftase activity in relation to histological grading, tumor location and K-ras mutation (27).

The altered cholesterol metabolism in colonic neoplastic cells as compared to patients with colorectal carcinoma has provided the basis for our studies designed to identify the effects of simvastatin, an HMGCoA reductase activity inhibitor, on cell growth and apoptosis of two human colon cancer cell lines. Simvastatin inhibited cell proliferation at

pharmacological doses in DLD-1 and at higher concentration in the Caco2 cell line. The anti-proliferative effect of simvastatin observed in DLD-1 cells might occur *via* the apoptotic pathway. Interestingly, exposure of DLD-1 cells to simvastatin promoted the apoptotic process. The effects of simvastatin on cellular growth and apoptosis were reversed by the addition of mevalonate. No apoptosis was detected in the Caco2 cells. Thus, the antiproliferative effect of simvastatin takes place in Caco2 cells regardless of apoptosis.

Although growth inhibitory effects of HMGCoA reductase inhibitors on different cells have been documented in several studies (28-30), we showed that simvastatin reduces cell proliferation and induces apoptosis at low concentration. The required concentrations of drug to achieve growth inhibition and apoptosis were within therapeutically achievable levels. The aim is to develop therapeutic strategies using the HMGCoA reductase inhibitors at a lower dose as anti-cancer compounds.

Moreover, future epidemiological studies and clinical trials concerning colorectal cancer would be advised to take into account the possibility of tumor site differences in their study design. The anatomic site of origin of the neoplasia is a convenient discriminator to identify subgroups of tumors with biological and clinical differences.

Acknowledgements

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