Abstract. Background: Panitumumab, a fully-human monoclonal antibody raised against epidermal growth factor receptor (EGFR), has been approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) for the treatment of patients with EGFR-expressing metastatic colorectal carcinoma (mCRC) after failure of standard chemotherapy. Additionally, the guideline of the EMEA includes the use of panitumumab in patients with wild-type KRAS. The goal of the current study was to evaluate the effect of panitumumab on colon cancer cells, proliferation, apoptosis, necrosis, cell cycle arrest and autophagy. The effect of panitumumab on the redox status of the cells was also studied. Materials and Methods: The cell lines Caco-2, DLD-1 and HT-29 which differ in their expression of EGFR and HER-2 were used. Cell proliferation and apoptosis/necrosis were measured by methyl tetrazolium (MTT) assay and annexin V/propidium iodide assay, respectively. Cell cycle arrest was estimated by propidium iodide assay and autophagy was detected using Western blot analysis. Spectrophotometrical quantification of glutathione (GSH) levels and an analysis of KRAS sequence were applied. Results: Panitumumab reduced proliferation only in the DLD-1 cells despite the mutated KRAS in this cell line. However, panitumumab did not affect DLD-1 cell apoptosis, necrosis or cell cycle progression. Interestingly, immunoblotting analysis revealed that panitumumab increased protein levels of beclin-1, a marker of autophagy. In addition, an increase in the GSH level was noted following panitumumab treatment reflecting an imbalance in the redox status of the cells. Conclusion: Panitumumab affects colon cancer cell proliferation independently of KRAS mutations and EGFR protein levels, possibly through the induction of autophagy.

Among the most recent advances in the management of colorectal cancer (CRC) are immunotherapies targeting cellular entities such as the epidermal growth factor receptor (EGFR) (1). Panitumumab (Vectibix®), a recombinant fully-human IgG2 monoclonal antibody raised against EGFR, has been approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) (2, 3) for the treatment of EGFR-expressing metastatic CRC (mCRC) as monotherapy after the failure of fluoropyrimidine-, oxaliplatin- and irinotecan-containing chemotherapy regimens (2-7). More specifically, in Europe, panitumumab treatment in patients with mCRC is confined to wild-type KRAS tumors (3, 4). Although the cellular targets of panitumumab are known (8), the mechanisms through which panitumumab exerts its antitumor activity are not as yet well documented.

EGFR inhibitors interfere with a number of biological functions regulated by the EGFR resulting in cell cycle arrest, induction of apoptosis and autophagy and inhibition of angiogenesis and tumor cell invasion and metastasis (9, 10). Previous in vitro data have demonstrated the antitumor effect of panitumumab in many cancer cell lines which express EGFR (11). The inhibition of cell proliferation by panitumumab may involve the induction of apoptosis (12). EGFR is activated upon ligand-induced receptor homo- and heterodimerization which in turn activates Ras-MAPK, PI3K-PKB/Akt, STAT and PLC-PKC pathways (13). The MAPK pathway transmits signals from the receptor to the nucleus via a series of intermediate proteins including RAS, RAF, MEK and ERK. Mutations in KRAS result in the constitutive activation of this pathway indicating that these patients would not benefit from treatment with anti-EGFR agents (14). In fact, it has recently been shown that KRAS status should be determined for selecting patients with mCRC as potential candidates for treatment with panitumumab (4).

Autophagy, an alternative type of cell death (programmed necrosis), is a self-digesting mechanism responsible for the removal of long-lived proteins and damaged organelles by lysosomes (15). Autophagy has a dual role: it may protect cells from apoptosis during stress by removing damaged organelles, but it may also act as a cell killer. Autophagy is
identified by the detection of beclin-1, a 60 kDa protein, whose expression is induced in the early stages of autophagy (16, 17). Moreover, many cancer cells, such as breast, colon, ovarian and prostate expresses beclin-1. More specifically, immunohistochemical analysis has shown that beclin-1 is overexpressed in colorectal and gastric cancer cells compared to normal cells. However, there was no significant difference in beclin-1 expression with respect to histological subtype, or depth of invasion or metastasis. These particular observations indicate that beclin-1 neo-expression occurs at a relatively early stage of colorectal and gastric cancer development and suggests that beclin-1 might contribute to cancer development but not to cancer progression (15).

The gastrointestinal tract, especially the colon, is constantly exposed to reactive oxygen species (ROS) originating from endogenous and exogenous sources (18). An excess of intracellular ROS results in an oxidative environment, which can modulate gene expression and damages cellular molecules. ROS are mainly involved in DNA damage occasionally leading to mutations in tumor suppressor genes. They also act as initiators and promoters of carcinogenesis (19). Some data have shown that ROS including hydrogen peroxide (H₂O₂) are involved in cell proliferation, survival and migration (20, 21). The effects of ROS are balanced by the action of antioxidant enzymes and non-enzymatic antioxidants (22). Glutathione, a multifunctional intracellular non-enzymatic antioxidant, is considered to be the major thiol-disulphide redox buffer of the cell. The reduced form of glutathione (GSH) is oxidised to glutathione disulphide (GSSG) resulting in the conversion of H₂O₂ to water (22). The ratio of GSH/GSSG represents a reliable measure of oxidative stress (23). The modulation of redox-regulated signal transduction, storage and transport of cysteine and regulation of cell proliferation as well as regulation of immune response, are an integral part of the physiological functions of glutathione (24).

The aim of the present study was to investigate the possible effect of panitumumab on three human colon cancer cell lines regarding cell proliferation, apoptosis, necrosis, cell cycle arrest, autophagy and the redox status of the cells.

Materials and Methods

Cell culture and reagents. Caco-2, DLD-1 and HT-29 colon cancer cell lines were purchased from the American Type Culture Collection (ATCC). All cell lines express EGFR as previously documented by Giannopoulou et al. and Calonghi et al. (25, 26). The DLD-1 and HT-29 cells were cultured in RPMI 1640 medium with 2 mM L-glutamine and supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose, 1.5 g/l sodium bicarbonate and 10% fetal bovine serum (FBS) as recommended by the ATCC. Caco-2 cells were cultured in Eagle’s minimum essential medium (EMEM) with Earle’s balance salt solution (BSS) and 2 mM L-glutamine and supplemented with 1.0 mM sodium pyruvate, 0.1 mM non-essential amino acids, 1.5 g/l sodium bicarbonate and 20% FBS as recommended by the ATCC. Both media were supplemented with 2.5 μg/ml amphotericin B, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamycin. The cells were cultured at 37°C, 5% CO₂ and 100% humidity.

Panitumumab was supplied by Amgen (Amgen Inc., CA, USA) and was applied to the cells after their attachment, at doses of 0, 20, 40, 80, 100 or 200 μg/ml. An IgG antibody from human serum (Sigma, Steinheim, Germany) was added to untreated cells as a control. All the experiments were performed in medium supplemented with 10 or 20% FBS as outlined above.

Cell proliferation assay. To determine whether panitumumab affects the proliferation of cells, the 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) assay was used, as previously described in the literature (25). The proliferation assay was performed 48 h after the addition of panitumumab.

Apoptosis assay. Apoptosis and necrosis were assessed using annexin V/propidium iodide detection assay as previously discussed in the literature (25). Cells were analyzed by flow cytometry (EPICS-XL of Coulter, Miami, FL, USA) according to the manufacturer’s instructions (rh Annexin V/FTTC kit; Bender MedSystem, Vienna, Austria), 24 or 48 h after the addition of panitumumab.

Cell cycle assay. The cell cycle progression was estimated with flow cytometric analysis, using Coulter DNA Prep Reagents kit (Beckman, Fullerton, CA, USA). The cells were seeded at 10⁵ cells/well in 6-well plates and panitumumab was added as described above, for 24 and 48 h. At the end of the incubation periods with panitumumab, attached cells were washed twice with phosphate-buffered saline (PBS), trypsinized for 6 min and centrifuged for 4 min at 166 xg. Cells were stained with propidium iodide and analyzed by flow cytometry (EPICS-XL of Coulter, Miami, FL, USA), in accordance with the manufacturer’s instructions.
Immunoblotting. Cells were seeded at 10⁶ cells/Petri dish, panitumumab was added as described earlier and 24 h later the cells were prepared for electrophoresis and immunoblotting, as reported by Giannopoulou et al. (25). Actin was used as an internal control. A goat polyclonal anti-beclin-1 antibody (D-18) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; dilution 1:1000) and a monoclonal anti-actin antibody (Chemicon, Millipore, Temecula, CA, USA, dilution 1:1000) were used. MCF-7 cells were used as a positive control (lane 7, Figure 2). Detection of the immunoreactive proteins was performed by chemiluminescence using horseradish peroxidase substrate SuperSignal (Pierce, Rockford, IL, USA), according to the manufacturer’s instructions.

Figure 2. Western blot analysis of beclin-1 in colon cancer cell lines after their treatment with panitumumab. A) Lanes 1, 3 and 5: cells treated with human IgG antibody; lanes 2, 4 and 6: cells treated with panitumumab 80 μg/ml; Caco-2 cells: lanes 1 and 2, DLD-1 cells: lanes 3 and 4, HT-29 cells: lanes 5 and 6; lane 7: positive control for beclin-1, MCF-7 cells. Representative example of three independent experiments. B) Quantification of Western blot images. **p<0.01, compared to control (unpaired t-test).

Spectrophotometrical quantification of GSH levels. Cells were seeded at 10⁶ cells/Petri dish and panitumumab was added as described above. Forty-eight hours later the cells were lysed with 20 mM EDTA using a sonicator. GSH levels were measured with a photometrical assay as described by Giannopoulou et al. (27). The results were expressed as nmol/mg of total protein quantified by the Bradford assay.

KRAS mutation analysis. DNA was extracted using a QIAamp DNA blood mini kit (QIAGEN, Valencia, CA, USA). DNA was then amplified by PCR using Kapa DNA polymerase (Kapa Biosystems, Cape Town, South Africa) and codons 12 and 13 (28) were sequenced by VBC-BIOTECH (Vienna, Austria).

Statistical analysis. Differences between groups and controls were tested by unpaired t-tests. Each experiment included at least triplicate measurements for each condition tested. All the results are expressed as mean±SEM of at least three independent experiments. The data were analyzed with Origin version 6.0 software (London, UK).

Table I. Effect of panitumumab on apoptosis and necrosis of colon cancer cell lines. Results are expressed as % of annexin V-positive and propidium iodide-positive cells, respectively. C: Control; cells treated with human IgG antibody; Pan/mab: cells treated with 80 μg/ml panitumumab.

<table>
<thead>
<tr>
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<th>Apoptosis (%)</th>
<th>Necrosis (%)</th>
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<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>Caco-2</td>
<td>25±8.8</td>
<td>17±6.2</td>
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<tr>
<td>Pan/mab</td>
<td>24±5.5</td>
<td>15±5.5</td>
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<tr>
<td>DLD-1</td>
<td>16±6.4</td>
<td>12±5.0</td>
</tr>
<tr>
<td>Pan/mab</td>
<td>12±1.6</td>
<td>10±1.8</td>
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<tr>
<td>HT-29</td>
<td>8±1.2</td>
<td>5±0.8</td>
</tr>
<tr>
<td>Pan/mab</td>
<td>7±0.5</td>
<td>4±0.5</td>
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Figure 3. The levels of –SH groups in colon cancer cells after treatment with panitumumab. Results are expressed as mean±SEM of –SH groups (μM)/mg of total proteins. *p<0.05, compared to control (unpaired t-test).
Results

Effect of panitumumab on cell proliferation. Panitumumab inhibited proliferation in the DLD-1 cells in a dose-dependent manner 48 h after its application (Figure 1). All the experiments were performed using 80 μg/ml of panitumumab since this was the most potent inhibitory dose (Figure 1). In contrast, the proliferation of Caco-2 and HT-29 cells was not significantly affected by the treatment with panitumumab.

Effect of panitumumab on apoptosis and cell cycle arrest. No differences were observed in the percentage of apoptotic and necrotic cells 24 or 48 h after treatment of the cells with panitumumab (Table I). Furthermore, 24 and 48 h after treatment, the tested agent did not induce cell cycle arrest (Table II).

Effect of panitumumab on autophagy. Since there were no changes in apoptosis, necrosis or cell cycle arrest, the effect of panitumumab on protein levels of beclin-1 was assessed. Panitumumab caused an increase in beclin-1 levels 24 h after its application to the DLD-1 cells as shown in Figure 2, indicating induction of autophagy. Although there was an increase of beclin-1 protein levels in the HT-29 cells, this result could not be evaluated because of the high standard error.

Effect of panitumumab on GSH. Treatment of the DLD-1 cells with panitumumab for 48 h resulted in an increase in GSH levels as shown in Figure 3, suggesting a reduction of oxidative stress.

KRAS mutation status. The DLD-1 cells had a transition in the second base of codon 13 (GGC13GAC) of KRAS causing a change in the amino acid sequence (Gly13Asp). The Caco-2 and HT-29 cells carried wild-type KRAS.

Discussion

The EMEA approval of panitumumab in mCRC specifies the guideline for the patient population with wild-type KRAS (3, 4). Panitumumab may not be effective in patients with mCRC and KRAS mutations, whereas it has proven efficacy in patients with wild-type KRAS (4, 29, 30). Clinical studies have shown that KRAS status is an important predictive marker of panitumumab efficacy (4, 31), however patients with wild-type KRAS may exhibit resistance to panitumumab treatment. The mechanisms that underlie the differential effect in patients with wild-type KRAS are still unknown (4).

In the present study, three human colon cancer cell lines that express different levels of EGFR (11, 24, 32), Caco-2 and HT-29 with wild-type KRAS, and DLD-1 with mutated KRAS were treated with panitumumab. Surprisingly, panitumumab reduced proliferation in the DLD-1 cells and did not affect proliferation of the Caco-2 and HT-29 cells. The resistance of Caco-2 and HT-29 cells to panitumumab may be relative to the number of EGFR molecules present in each cell type. In vivo experiments with human tumor xenografts from several cell lines have demonstrated a strong inhibition of tumor growth after treatment with panitumumab in cases where tumors expressed 17,000 or more EGFR molecules/cell. In contrast, the growth of tumors with 11,000 or fewer EGFR molecules/cell was unaffected (11). The number of EGFR molecules/cell for HT-29 cells was 9,000 (11) and Caco-2 cells express lower protein levels of EGFR than HT-29 (32). According to these findings, panitumumab should not affect the proliferation of DLD-1 cells, since in these colorectal cancer cells, EGFR protein levels are lower than those found in HT-29 cells. In line with the present results regarding the effectiveness of an anti-EGFR treatment in DLD-1 cells are previous experiments with established DLD-1 tumors in nude mice, which showed that treatment of animals with IMC-C225, an anti-EGFR monoclonal antibody, reduced tumor growth (33). Thus EGFR levels
appear to be correlated with the response to anti-EGFR treatment. The use of EGFR as a marker for selecting an appropriate chemotherapeutic regimen in colorectal cancer remains controversial (34). Therefore, the question remains as to why panitumumab inhibited DLD-1 cell proliferation independently of EGFR levels and KRAS mutations.

The importance of EGFR in the growth of colon cancer tumors has been previously demonstrated (35, 36), but its predictive role has not been confirmed (37). Preclinical data derived from breast cancer cell lines suggested that increased levels of HER-2 expression are associated with increased effectiveness of anti-EGFR therapy (38). More specific, MCF-7 breast cancer cell line was transfected to produce subclones with different HER-2 levels. The proliferation of MCF-7 subclones with high HER-2 levels was inhibited by AG1478, an EGFR inhibitor, while the proliferation of subclones with low or intermediate levels of HER-2 was not affected. These findings could potentially explain the present results since DLD-1 cells are known to express higher levels of HER-2 compared to Caco-2 and HT-29 cells (32).

Regarding KRAS mutation status, although KRAS is constitutively activated in DLD-1 cells, MEK and ERK1/2 are not phosphorylated (39). The endogenous disruption in RAS signaling might explain the lack of panitumumab efficacy. At the same time, EGFR inhibitors may also inhibit PI3K-PKB/Akt and PLC-PKC pathways (40) which also affect cell proliferation. The present findings were in line with the hypothesis that the constitutive activation of KRAS can yield numerous different outcomes depending on the cell context (41). However, this variability has not been observed in the clinical setting (42).

In DLD-1 cells, panitumumab failed to promote cell apoptosis, necrosis or cell cycle arrest at any time-point tested. However, an induction of beclin-1 expression was noted. Only few preclinical studies have shown that another anticancer agent, tamoxifen, may exert its antitumor effect through the induction of autophagy (16, 43). To the best of our knowledge, this is the first study demonstrating that an EGFR inhibitor may induce cell death by a mechanism other than apoptosis, necrosis or cell cycle arrest.

Furthermore, treatment with panitumumab resulted in an imbalance of the redox status of the DLD-1 cells through an increase in GSH levels. Previous studies have shown that GSH levels in the blood serum of patients with CRC decrease as the disease progresses (15). The main role of GSH is to protect the cell from increased levels of intracellular ROS. Furthermore, GSH may bind to proteins resulting in glutathionylation, a post-translational modification, which stabilizes many proteins (44). The increase in GSH levels may, therefore, indicate reduced stability of many proteins, promoting cell death as has previously been suggested for DLD-1 cells (45).

To conclude, panitumumab reduces colon cancer cell proliferation through autophagy and possibly via protein destabilization regardless of EGFR levels and KRAS mutations. This may be explained by the existence of other pathways through which EGFR may lead to tumor growth, independently of KRAS.

Acknowledgements

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References