Expression of *p65* gene in experimental colon cancer under the influence of 5-fluorouracil given alone and in combination with hormonal modulation*

G. MELEN-MUCHA[1], E. BALCERCZAK[3], S. MUCHA[2], M. PANCZYK[3], S. LIPA[3], M. MIROWSKI[3]

[1]Department of Experimental Endocrinology and Hormone Diagnostics, e-mail: g.m-mucha@wp.pl, [2]Department of Clinical Endocrinology, Institute of Endocrinology, Medical University of Lodz, Poland 91-425 Lodz, Poland; [3]Department of Pharmaceutical Biochemistry, Molecular Biology Laboratory, Faculty of Pharmacy, Medical University of Lodz, Poland

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The effect of tamoxifen (TAM), lanreotide (LAN) and 5-fluorouracil (5-FU), given separately or together, on *p65* gene expression in murine Colon 38 cancer was investigated by RT-PCR method. The findings were compared with cell proliferation determined by bromodeoxyuridine (BrdU) labeling index, apoptosis visualized by TUNEL method and tumor mass.

It was found that in the control group (mice bearing colon cancer without treatment) the expression of *p65* gene was present in 57% of investigated samples. In the groups treated with TAM or LAN *p65* gene expression was detected in 87.5% and 83.3% of analyzed cases, respectively. Both these substances increased apoptotic index in Colon 38 cancer and LAN also decreased the proliferation index. After a combined treatment with TAM and LAN a percentage of *p65* positive cases was similar to that of the control group and equaled approximately 60%. This treatment did not increase proapoptotic effects of these drugs, and even reduced the antiproliferogenic effect of LAN. In the group treated with 5-FU and LAN *p65* gene expression was also close to the control value (about 66%). Similarly in this group the combined treatment with these two drugs did not cause any favorable effect on proliferation and apoptosis. Moreover, in this group even reduced antiproliferogenic effect of LAN was observed. In the group with 5-FU alone the expression of *p65* was present in about 80% of samples. The treatment with 5-FU increased apoptotic index and did not change proliferation. In the group treated with a combination of TAM and 5-FU all analyzed cases showed the presence of *p65* gene expression. Previously, we observed in this group the most pronounced and synergistic effect of these substances on the inhibition of cell proliferation and tumor mass reduction. Based on these findings we conclude that *p65* gene expression in murine Colon 38 cancer tissues can be modulated via chemotherapy (5-FU) and also via hormonal modulation (TAM and LAN).

Key words: p65 gene, 5-FU, hormonal modulation, Colon 38 cancer

Colorectal cancer belongs to the family of social diseases because of its high incidence and mortality (the second leading cause of cancer death) [6]. The annual incidence of colorectal cancer has increased in many countries, and this trend continues. Its etiology is multifactorial and depends on age, diet, genetic, and other factors. It has been found that many growth factors and some hormones e.g. gastrins [13], estrogens [9, 21], somatostatin (SS) [14] influence the growth of colon cancer, although colorectal cancer does not belong to hormone-dependent cancers. Apart from surgery 5-FU remains as the reference chemotherapy treatment for colorectal cancer, but its efficacy is unsatisfactory (5–20% response rates) [23] and limited by resistance, either spontaneous (50%) or secondary (in almost all cases). This data prompted the researchers to search for new alternative therapeutic agents in colon cancer treatment, or substances which may enhance the therapeutic effect of 5-FU.

We found in our previous studies that SS analogs, lanreotide and octreotide, inhibited proliferation and induced apoptosis in murine Colon 38 cancer [14, 15]. We also ob-

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served the synergistic effect of the combined treatment with octreotide and 5-FU (but not with lanreotide) on tumor mass reduction [15]. However, the combined treatment with these SS analogs and 5-FU did not enhance their effect on apoptosis and even abolished SS analogs influence on the reduction of cell proliferation [15]. We also noticed the synergistic effects of tamoxifen and 5-FU on the inhibition of cell proliferation and tumor mass in Colon 38 cancer [16].

Similarly due to the lack of satisfactory treatment options, there are still not enough of reliable tumor-markers for prognosing and monitoring colon cancer patients. A 65 kDa oncofetal protein (P65) seems to be a new biomarker of colon cancers. It has been isolated from a culture medium of human breast cancer cells (MCF-7) [18]. Its increasing levels have been shown in the serum as well as in tumor tissue taken from patients suffering from different cancers [10, 19, 22, 25]. Moreover, it has been also found that P65 assessed together with CEA and CA 19-9 in the serum of patients noticeably improve the detection of colorectal cancer when compared to CEA or CA 19-9 assessed alone [26]. On the basis of partial amino acid [18] as well as nucleotide sequences [11] it has been supposed that gene encoding P65 protein belongs to the steroid/thyroid hormone receptors family. It has been shown [20] that the combined treatment with carboplatin and amifostine of HL60 cells in culture may stimulate *p65* as well as *bax* and *c-myc* genes expression.

The aim of this study was to examine the influence of tamoxifen (a partial estrogen antagonist), lanreotide (SS analog) and 5-FU, given separately or together, on the expression of *p65* gene in Colon 38 cancer cells and to compare *p65* gene expression with proliferative and apoptotic indices.

Material and methods

Experimental model. An experiment was carried out on male mice of B6D2F1 strain, weighing 20 ± 5 g. This strain is the first generation of the crossbred between C57BL/6 and DBA/2 strains. The animals were subcutaneously (sc) inoculated with 33% suspension of Colon 38 cancer cells to induce tumors. Seven days after the tumor implantation, mice were divided into groups (8–10 animals each) and treated as follows:

Group I (control (C)) – received sc 0.2 ml of dimethyl sulphoxide on day 1st, 4th, 7th and 10th of the experiment,

Group II – 5-fluorouracil (5-FU, Fluorouracil, Polpharma S.A., Poland) at a dose of 70 mg/kg b.w. i.p., on the 2nd and 8th day of the experiment,

Group III – lanreotide (LAN, Somatuline L.P., Ipsen Biotech) in a single injection, at a dose of 1 mg/animal on the 1st day of the experiment, intramusculary (i.m.),

Group IV – tamoxifen (TAM, Sigma), at a dose of 20 mg/kg b.w. s.c., on day 1st, 4th, 7th and 10th of the experiment,

Group V – LAN and TAM at the above doses,

Group VI – LAN and 5-FU at the above doses,

Group VII – TAM and 5-FU at the above doses.

On the eleventh day, the animals were killed by spinal cord dislocation after receiving, 90 min earlier intraperitonealy (i.p.) a single injection of bromodeoxyuridune (BrdU, Sigma) at a dose of 50 mg/kg b.w.. All detectable tumors were removed, and then fixed and embedded in paraffin (for assessment of proliferation and apoptosis).

Cell proliferation assay. The BrdU labeling index was used as an index of cell proliferation. Bromodeoxyuridine is a pyrimidine analog and like thymidine is incorporated into DNA when cells enter the S phase of the cell cycle. Therefore, cells in the S phase can be identified by immunocytochemical staining with anti-BrdU monoclonal antibody. The number of BrdU-immunopositive cell nuclei detected by immunostaining using the Cell Proliferation Kit (Amersham) counted per 1000 chosen at random was considered as the BrdU labeling index.

TUNEL assay. The *in situ* labeling of nuclear DNA fragmentation according to TUNEL method was considered as an apoptotic index. The laboratory protocol was adopted from GAVRIELI et al [8] and performed with the reagent of In Situ Cell Death Detection Kit, POD (Boehringer Mannheim). The apoptotic index equals the number of cells containing apoptotic bodies or nuclei assessed per 1000 randomly scored tumor cell nuclei (based on staining and the characteristic features of the apoptotic nuclei).

RNA isolation. After deparafinization of tissue slides, RNA was isolated by RNA Prep Plus Minicolumn Kit (A&A Biotechnology, Poland) using the CHOMCZYNSKI's modified method as described earlier [7]. The quality and quantity of isolated RNA was characterized by spectrophotometer assessing absorption in 260/280 wavelengths.

RT-PCR. The complementary DNA (cDNA) was obtained by RevertAid^{TM} cDNA Synthesis Kit (MBI Fermentas) with M-MuLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase). Reaction mixture containing total RNA (1 μg), oligo(dT)18 primers (0.5 μg/l) and deionized, nuclease free water was prepared. After short centrifugation the mixture was incubated at 70 °C for 5 min, then chilled on ice and the following components were added: 4 μl reaction buffer, 1 μl ribonuclease inhibitor (20 U/μl), 2 μl dNTP mix (10 mM). The mixture was incubated in 37 °C for 5 min and 1 μl of RevertAid M-MuLV RT (200 U/μl) was added and incubated at 42 °C for 60 min. The reaction was stopped by heating the mixture at 70 °C for 10 min.

PCR. The primers were designed for *p65* gene expression on the basis of amino acid sequences of N-terminal fragments of P65 molecule (foreword) and its internal peptide, which after CNBr treatment and electrophoresis migrate as band with 51 kDa (reverse) [2-4]. PCR mixture contained 1.5 mM $MgCl_2$, mix dNTPs, 0.5 U Taq Polymerase, reaction

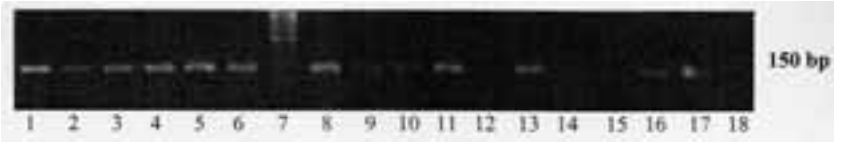

Figure 1. An example of *P65* gene expression analysis by PCR technique in mouse colon cancer treated with: tamoxifen and 5-fluorouracil (TAM + 5-FU) – lane 1 to 3; tamoxifen (TAM) – lane 4 to 6; 5-fluorouracil (5-FU) – lane 8 and 9; lanreotide (LAN) – lane 10 and 11; lanreotide and 5-fluorouracil (LAN + 5-FU) – lane 13 to 15; tamoxifen and lanreotide (TAM + LAN) – lane 16 to 17; molecular size markers – lane 7; *P65* negative case – lane 12; negative control – lane 18.

buffer and 0.5 μM of each primer. DNA was amplified in 35 cycles using the following parameters: denaturation (94 °C, 30 s), annealing (57 °C, 30 s), elongation (72 °C, 30 s). As a control β-actine cDNA was amplified according to the methods described in literature [24]. The PCR products were analyzed by 2% agarose gel electrophoresis (Fig. 1).

Statistical analysis. Statistical analysis was carried out with the use Chi-square and McNemar's fraction tests.

Results

The percentage of *p65* positive cases (the presence of *p65* gene's mRNA) was analyzed by RT-PCR technique and is summarized in Table 1, together with proliferative and apoptotic indices, proliferation/apoptosis ratio and tumor mass of Colon 38 cancer.

It was found that in animals of the control group the expression of *p65* gene was present in 4 out of 7 investigated samples (57%). The proliferation index (a number of BrdU-immunopositive cell nuclei counted per 1000 tumor cell nuclei chosen at random) was approximately 230‰. The apoptotic index (a number of cells containing apoptotic bodies or nuclei assessed per 1000 randomly scored tumor cell nuclei) was calculated to be about 26‰. The proliferation/apoptosis ratio was estimated as about 9. Median tumor mass in the control group reached the value about 300 mg (Tab. 1).

In the group treated with TAM the *p65* gene expression was detected in 7 out of 8 samples (87.5%), whereas in LAN treated group in 5 out of 6 samples (83.3%). Both these substances increased apoptotic index up to 40‰ in the case of TAM and up to 50‰ in the case of LAN. A proliferation index reduction was observed only in the case of LAN treated animals. A decrease of proliferation/apoptosis ratio was observed in both groups and was estimated as 6.2 in the case of TAM (a difference not statistically significant) and 3.4 in LAN treated animals. Surprisingly, tumor mass induction was observed in the group treated with TAM (665 mg) (Tab. 1). A combined treatment with both above mentioned compounds (TAM and LAN) reduced percentage of *p65* positive samples to the control value (about 60%). In this group the proliferation index was similar to that of the control group (about 240‰) but the apoptotic index was only slightly higher than in cases treated with each compound separately, and reached the value of about 58‰. The proliferation/apoptosis ratio for this group was estimated as 4.2. A combined treatment with both compounds (TAM + LAN) resulted in a slight tumor mass reduction (about 200 mg) in comparison to the control group (about 300 mg, Tab. 1).

In the group treated with 5-FU alone, *p65* gene was expressed in 4 out of 5 samples (80%). The treatment with 5-FU increased the apoptotic index (about 60‰), did not change the proliferation (about 230‰) and reduced the proliferation/apoptosis ratio (3.7).

In the group of animals treated both with 5-FU and LAN percentage of *p65* positive samples was also close to the control value (about 66.6%). This combined treatment (5FU + LAN) did not have any favorable effect on proliferation (about 200‰). In this group apoptosis reached the highest value (about 70‰) and the lowest proliferation/apoptosis ratio (3.1‰). Moreover, tumor mass was reduced twice to less than a half of the control group value.

The highest percentage of *p65* gene expression (100%) was observed in the group treated with combination of 5-FU and TAM. In this group we observed the most pronounced inhibition of cell proliferation (about 170‰) and tumor mass reduction (84 mg). The apoptotic index reached the value of about 40‰ and the proliferation/apoptosis ratio was over twice lower than in the control group.

However, we did not find any statistically significant correlation between *p65* gene expression and indices of cell proliferation, apoptosis or tumor mass in each of the experimental groups.

Discussion

The majority of patients with colon cancer is diagnosed in an advanced stage and apart from surgery requires even chemotherapy. 5-Fluorouracil remains the gold standard for these patients, but its efficacy is still unsatisfactory. An enormous effort has been undertaken over the last three decades to transform both basic and clinical findings into earlier and more accurate diagnosis, and to improve therapy of this site of cancer. In many experimental attempts the researchers try to enhance cytostatic effect of 5-FU on colon cancer via combined treatment with different agents, such as: lovastatin [1] or interferon-γ [12]. Hormonal modulation seems to be one of these methods, which could enhance cytotoxic effect of 5-FU on colon cancer. In the series of our earlier experiments we have found the synergistic inhibitory effect of 5-FU on murine Colon 38 cancer when given together with different hormonal modulators, such as: oc-

Table 1. Changes in p65 gene expression, proliferation, apoptosis, proliferation/apoptosis ratio and tumor mass in Colon 38 cancer under the influence of 5-FU and hormonal modulation

Experimental group	*p65* gene expression (% of positive cases)	Proliferation* (‰) (mean ± SEM)	Apoptosis* (‰) (mean ± SEM)	Proliferation/ apoptosis* (mean ± SEM)	Tumor mass* (mg) median (min-max)
Control	57 n=7	232.6 ± 20.5 n=7	26.5 ± 2.9 n=8	8.8 ± 0.9 n=6	308 (40–505) n=9
TAM	87.5 n=8	241.1 ± 36.3 n=8	39 ± 3.4 n=8	6.2 ± 1.8 n=7	665 (308–1527) n=9
LAN	83.3 n=6	169.1 ± 11.3 n=9	50.4 ± 3.4 n=8	3.4 ± 0.5 n=8	446 (40–886) n=9
TAM + LAN	60 n=5	239.1 ± 22.2 n=7	57.6 ± 4.8 n=8	4.2 ± 0.8 n=7	196 (0–1120) n=9
5-FU	80 n=5	233.2 ± 30.0 n=5	62.3 ± 7.1 n=5	3.7 ± 0.2 n=5	228 (0–405) n=7
LAN + 5-FU	66.6 n=5	207 ± 36.4 n=5	67.3 ± 5.4 n=5	3.1 ± 0.7 n=5	142 (0–718) n=7
TAM + 5-FU	100 n=5	168.4 ± 27.3 n=8	41.8 ± 2.6 n=7	4 ± 1.0 n=6	84 (29–514) n=9

5-FU – 5-fluorouracil, TAM – tamoxifen, LAN – lanreotide, n – number of animals, * – data taken from [7 and 8].

treotide – on tumor mass [15], tamoxifen – on proliferation and tumor mass [16], proglumide (non-specific blocker of gastrin/cholecystokinin receptor) – on proliferation and apoptosis in colon cancer [16]. But in the short-term experiment tamoxifen and proglumide did not potentate 5-FU action on Colon 38 cancer [17]. The important role of estrogens and other steroid hormones in colon carcinogenesis has been emphasized in some other papers [9, 21], and the most convincing evidence is a reduced risk of colorectal cancer in women taking postmenopausal hormone replacement therapy [9].

It has been postulated on the basis of a partial amino acid sequence analysis that *p65* gene belongs to the family of genes encoding steroid hormone receptors [11, 18]. It seems interesting that *p65* gene is expressed in murine Colon 38 cancer and that its expression changes under hormonal modulation and chemotherapy. In patients with colon cancer serum levels of P65 protein facilitate an accurate detection of colorectal cancer much better than serum levels of CEA or CA19-9 alone [26]. Moreover, P65 blood levels better discriminate healthy volunteers from colon cancer patients than CEA, because P65 is detected only in 4% of healthy men while 28% of healthy volunteers show the presence of CEA in the blood [26].

Recently *p65* gene expression was investigated in human colon cancer cases using RT-PCR technique. About 47% of the cases were *p65* positive. The fact that about a half of the investigated carcinomas revealed expression of *p65* gene indicates that its expression is not always involved in colorectal neoplastic transformation. None of mucosa specimens derived from healthy patients revealed the expression of *p65* gene [5].

The data of the presented paper indicate that *p65* gene expression in murine Colon 38 cancer tissues is detectable in a similar percentage (about 50%) to that of the human colorectal cancers. In this study we show that *p65* gene expression is modulated via chemotherapy (5-FU), and also via hormonal modulators e.g. tamoxifen and lanreotide. The modulation of *p65* gene expression after anticancer therapy (carboplatin alone and carboplatin in combination with cytoprotective agent amifostine) investigated *in vitro* in human acute promyelocytic leukemia cells HL-60 was similar to those of some other apoptotic genes [20]. Proliferation of HL-60 cells exposed to carboplatin was decreasing with the increasing dose of the drug. This effect was slightly stronger when carboplatin in combination with amifostine was used. Furthermore, the expression of *bcl-2*, *c-myc* and *bax* genes involved in apoptosis as well as *p65*, which function in this process is unknown, was determined by semi-quantitative RT-PCR technique. The HL-60 cells treated with carboplatin in combination with amifostine, showed an increased expression of *p65* as well as *bax* and *c-myc* genes when compared to the cells treated only with carboplatin.

In this study the highest percentage of *p65* positive samples was observed in the group treated with 5-FU and TAM. In this group we also found the most pronounced tumor

mass reduction and the lowest cell proliferation index [14, 15]. Moreover, high percentage of *p65* gene expression was noticed also in the groups treated with TAM and LAN separately. Both these substances caused an increase of apoptotic indices, and in the case of LAN a cell proliferation reduction was noticed, too [14, 15]. However, contrary to the data from MIROWSKI et al [20], we did not find in this study any correlation between *p65* gene expression and proliferative and apoptotic indices, or tumor mass reduction. Perhaps, this may be due to a small number of Colon 38 tissue samples in which we assessed the *p65* gene expression, or to a various role of *p65* gene in different tissues. The presence of *p65* gene in almost all except one samples of Colon 38 tissues in the group treated with tamoxifen and in all samples in the group of animals treated with tamoxifen together with 5-FU seems that the main regulator of *p65* gene expression in this study is tamoxifen. Perhaps, P65 protein is a target for a cytostatic action of tamoxifen in colon cancer tissues. This hypotesis is tempting because of similarities in partial amino acid as well as nucleotide sequences between P65 protein and its gene and steroid receptors protein and genes. Further studies are needed to confirm, if P65 protein may function as a target for antiestrogen therapy. It should also be clarified whether the presence of *p65* gene in colon tumors may be a predictive prognostic factor in diagnosis, and correlate with growth parameters of colon tumors.

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