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ANTI-CANCER EFFECT OF COMBINED ACTION OFANTI-MUC1 AND ROSMARINIC ACID IN AGSGASTRIC CANCER CELLS

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ABSTRACT

MUC1 seems to be promising target in cancer cells due to its abundant and specifically altered expression as well as differential distribution pattern relative to normal tissues. Rosmarinic acid (RA) is a natural, polyphenolic compound with pharmacological activities, including anti-cancer. Herein, we aim to explore the effect of combined action of anti-MUC1 and RA on selected cancer-related factors in AGS gastric cancer cells. Cancer cells were treated with 100, 200 µM rosmarinic acid, 5 µg/ml anti-MUC1 and acid together with antibody. Western blotting, ELISA and RT-PCR were used to assess the expression of MUC1, selected sugar antigens, enzymes participating in protein glycosylation, Gal-3, p53, pro- and anti-apoptotic factors, and caspases3,-8,-9 incancer cells. MUC1 mRNA was significantly suppressed by combined action of anti-MUC1 and RA. Such treatment markedly inhibited expression of cancer- related Tn, T, sialyl Tn, sialyl T, and fucosylated sugar antigens as well as mRNA expression of enzymes participating in their formation: ppGalNAcT2, C1GalT1, ST6GalNAcT2, ST3GalT1 and FUT4. C1GalT1 was suppressed also on protein level. Gal-3, factor likely participating in metastasis, was significantly suppressed on mRNA level by RA administrated with anti-MUC1. Pro-apoptotic Bax protein and Bad mRNA were significantly induced, and anti- apoptotic Bcl-2 mRNA expression was inhibited by such treatment. Combined action of mAb and RA markedly increased caspase-9 mRNA expression. Results of the study indicate that combined action of anti-MUC1 and RA is more effective than monotherapy in relation to examined cancer related factors. Such treatment can be considered as new, promising strategy in gastric cancer therapy.

KEYWORDS: Anti-MUC1, Apoptosis, Gastric cancer, Glycosylation, MUC1, Rosmarinic acid.

INTRODUCTION

MUC1 is membrane-bound, heavily O-glycosylated protein, overexpressed and underglycosylated in many cancers. MUC1 expression is often associated with resistance to anti-cancer drugs and poor patient prognosis. Altered MUC1 glycosylation and differential distribution pattern relative to normal tissues can enable its interaction with various receptors and promote cell differentiation, proliferation, invasion, and metastasis (Beckwith and Cudic, 2020; Birrer et al., 2019). Specifically modified MUC1 makes it an attractive tumor-associated factor for diagnostic, drug delivery, and immunotherapy.

Targeted therapy based on monoclonal antibodies directed to tumor associated molecules seems to be promising approach in cancer therapy. Antibody binding to its cellular ligand should result in internalization of the antibody-antigen complex via macropinocytic pathway and by this, the reduced expression of targeted molecule could be observed.mechanism of mAb-based therapy effectiveness is based on inducing of signal arrest leading to apoptosis by binding with their selected receptors, promoting their modulation or interfering in ligand binding. There are anti-MUC1 antibodies which are promising as a strategy for cancer treatment, several have been entered into preclinical studies. One of them is PankoMab-GEX, antibody that passed a phase I clinical trial recently, but its effectiveness remains to be assessed.

Rosmarinic acid (RA; α-o-caffeoyl-3,4-dihydroxyphenyl lactic acid) is anatural polyphenolic compound isolated from medicinal species of Boraginaceae and Lamiaceae like mint, rosemary or perilla frutescens It has been reported to have anti-oxidative, anti-inflammatory, anti-angiogenic, and anti-neoplastic activities. It has been reported to have anti-oxidative, anti- inflammatory, anti-angiogenic, and anti-neoplastic activities. RA inhibited pro-inflammatory cytokines and inactivated inflammatory pathway in MKN45 gastric cancer cells. In leukemia U937 cells RA sensitized TNF-α- induced apoptosis through the suppression of NF-κB and reactive oxygen species. RA inhibited the migration of MDA-MB-231BO bone-homing breast cancer cells mostly via the receptor activator of NF-κB ligand (RANKL)/RANK osteoprotegerin pathway). In our recent study we determined potential anti-cancer properties of RA in CRL-1739 gastric cancer cells based on its inhibitory action toward MMP-9, TIMP-1, MUC1 extracellular domain, Tn and T antigens.

MATERIAL AND METHODS

1. Cell culture

Human gastric adenocarcinoma AGS (CRL-1739) cells (ATCC, Manassas, VA, USA) were maintained in F-12 medium supplemented with 10% Fetal Bovine Serum (FBS) 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in humidified atmosphere containing 5% CO_2. Then the cells were seeded in six-well plates (5×10^5 cells/well) and cultured for 24 h in F-12 FBS-free medium supplemented with RA (100 and 200 µM), anti-MUC1 (BC2) (5 µg/mL), RA + anti-MUC1 (100 µM + 5 µg/ml and 200 µM + 5 µg/ml). Anti-MUC1 reacts with a 5 amino acid epitope ofthe MUC1 core protein with a low affectivity for glycosylation. Stock solution of RA was 2 mg RA/50 µl DMSO (Sigma, St. Louis, MO, USA) and it was stored at 4 °C. The cells were washed with Phosphate Buffered Saline, pH 7.4 (PBS) and lysed 20 min at 4 °C with RIPA buffer supplemented with protease inhibitors (Protease Inhibitor Coctail; Sigma, St. Louis, MO, USA), diluted 1 : 200 in RIPA buffer. The lysates and collected culture media were centrifuged at 1000×*g* for 5 min at 4°C. The supernatants were frozen at −70 °C and then used for analysis. For real- time PCR determinations the monolayers were washed three times with sterile 10 mM PBS and cell membranes were disrupted using sonicator.

Aliquots of the homogenate were used for RNA isolation. Cells without RA addition were taken as control.

2. Cell viability test

Cell viability assay, in the presence of anti-MUC1 monoclonal antibody, was carried out with MTT Cells, cultured for 24 h with 2.5–40 µg/ml of anti-MUC1 mAb in 6-well plates, were incubated in 1 ml of MTT solution (0.5 mg of MTT/ml of PBS) at 37 °C in 5% CO_2 for 4 h. Then absorbance of converted dyein living cells was measured at 570 nm. Viability of gastric cancer cells treated with anti-MUC1 was determined as a percentage of the cells without antibody (negative controls with 100% cell viability).

3. Western blotting

To detect MUC1 cytoplasmic domain, C1GalT1, ST3GalT2, FUT4, Gal-3, p53, caspase-3,-9, Bax and Bcl-xL protein expressions in gastric cancer cells, equal amounts of protein (20 µg) were separated by SDS-PAGE and transferred to an Immobilon P Then 5% skim milk in Tris Buffered Saline, pH 7.4 (TBS) with 0.05% Tween 20 (Sigma, St. Louis, MO, USA) (TBS-T)

was applied to block the membranes (1 h at room temperature (RT)). The proteins were analyzed using specific monoclonal antibodies, proper secondary horseradish peroxidase (HRP)- conjugated antibodies and an enhanced chemiluminescence with Westar Supernova, ECL substrate for Western blotting. The intensities of the bands were determined by densitometric analysis with the Gene Tools program. To exclude non-specific bindings TBS-T buffer instead of primary antibodies was applied. The β-actin expression served as protein loading control.

4. ELISA tests

To determine the relative level of Tn, T, sialyl Tn, sialyl T, and fucosylated sugar antigens in culture media ELISA-like tests with biotinylated lectins were applied. The following lectins (5 µg/mL) (were used: VVA, PNA, SNA (from *Sambucus nigra* with affinity to SAα2,6-Gal/GalNAc (sialyl Tn antigen)), MAAII (from *Maackia amurensis* with affinity to SAα2,3-Gal (sialyl T antigen)), and UEA (from *Ulex europaeus* with affinity to Fucα1,3-GlcNAc/Fucα1,2-Gal). 50 µl of media (100 µg protein/ml) were coated on microtiter plates at RT overnight. Then, after blocking (with 100 µl of 1% blocking reagent for ELISA; Roche Diagnostics, Mannheim, Germany) and washing steps (three times in 100 µl of washing buffer – PBS, 0.05% Tween) the plates were incubated with 100 µl of specific lectins (2 h at RT). In the next step there was incubation with 100 µl of HRP avidin D (Vector, Burlingame, CA, USA). The colored reaction was developed with 100 µl of ABTS (2,2′-azino-bis(3-ethylbenzthiaxoline-6-sulfonic acid)) (Sigma, St. Louis, MO, USA), liquid substrate for HRP. Absorbance at 405 nm was read after 30–20 min. The samples were analyzed in triplicate. 1% solution of BSA (instead of sample) and washing buffer (instead of lectin solutions) were applied as negative controls.

5. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA Mini Plus Concentrator (A&A Biotechnology, Gdynia, Poland) was used to isolate total RNA. Purity and concentration of RNA was assessed spectrophotometrically cDNA was synthesized from 1 µg of total RNA using Tetro cDNA Synthesis Kit (Bioline, London, UK) following the manufacturer's instructions. Briefly, 20 µl of the reaction mixture with 1 µl oligo $(dT)_{18}$ primer,1 µl of dNTP mixture (10 mM each), 5 µl of 5 x RT Buffer, 1 µl of RiboSafe RNase Inhibitor (10 u/µl), 1 µl of Tetro Reverse Transcriptase (200 u/µl) and DEPC-treated water was kept for 30 min at 45 °C and then incubated at 85 °C for 5 min. RT-PCR assay was carried out in CFX96 Real-time set (Bio-Rad) using SensiFAST™ SYBR Kit

(Bioline, London, UK). The reactions consisted of 2 µl of diluted twice cDNA template, 0.8 µl of each primer (10 µM), 10 µl 2 x SensiFAST SYBR Mix and nuclease-free water (in 20 µl ofa final volume). Primers) were synthesized by Genomed (Warsaw, Poland).

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was evaluated as housekeeping gene. The following reaction parameters were applied: 1 min at 95°C to activate the DNA polymerase, then 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and extension at 72 °C for 20 s. Then the reaction was exposed to a melting protocol from 55 °C to 95 °C with a 0.2 °C increment and 1 s holding at each increment to confirm the specificity of the amplified products. Single product production was proved by melting point assessment and agarose gel electrophoresis. Water instead of mRNA samples was used as negative control. Reactions were run in triplicates and statistical analysis of the CT-values was performed by the ΔΔCT method. The levels of relative gene expression were standardized with those measured in the untreatedcontrol.

RESULTS

Effects of anti-MUC1 mAb and rosmarinic acid on the viability of AGS gastric cancer cells

The results of MTT test indicated 80% of the viability of the gastric cancer cells in the presence of 2.5 µg/ml anti-MUC1, ending with some percentage of the viability in the presence of 40 µg/ml anti-MUC. In the experiments performed in the study 5 µg/ml anti-MUC1 concentration was used what was about 55% of gastric cells viability.

Effect of rosmarinic acid and anti-MUC1 on MUC1 mRNA and MUC1 cytoplasmic domain in gastric cancer cells

In this study, RT-PCR and Western blotting analysis were applied to check the expression of MUC1 mRNA and MUC1 cytoplasmic tail respectively. The gastric cancer AGS cells were incubated for 24 h with 100, 200 µM rosmarinic acid, 5 µg/ml anti-MUC1 mAb alone and rosmarinic acid combined with anti-MUC1. We observed significant increase of MUC1 mRNA expression after incubation of cells with 100 µM RA, no influence of 200 µM RA and significant decrease of MUC1 mRNA after combined treatment of mAb with RA as compared to control. Expression of cytoplasmic tail of MUC1 mucin was significantly inhibited by RA, antibody and their combined action, however without induced impact of mAb when combined with RA.

Effect of rosmarinic acid and anti-MUC1 on Gal-3 expression

Gal-3, a β-galactoside binding protein was significantly inhibited by 200 μM RA, anti-MUC1 and both molecules combined on mRNA level in comparison to untreated control. Addition of mAb enhanced RA action. Surprisingly, there was no effect of RA and mAb on Gal-3 protein in cell lysates, and only slight stimulatory effect of both 200 μM RA and anti-MUC1 was observed.

Effect of rosmarinic acid and anti-MUC1 on p53 expression

p53 transcription factor, is one of the most important tumor suppressors. RA with both concentrations significantly induced p53 mRNA. Anti-MUC1 alone revealed similar effect, however effect induced by RA was much stronger. After combined therapy, low, significant increase of *p53* gene in comparison to control was observed only in the presence of mAb and 200 μM RA. p53 on protein level was stimulated similarly to *p53* gene but significant changes were observed only upon action of both concentrations of RA and after combined treatment of 200 μM RA with anti-MUC1.

Effect of rosmarinic acid and anti-MUC1 on pro-apoptotic Bax, Bad and anti-apoptotic Bcl-xl, Bcl-2 expression

To assess how the examined compounds impact apoptotic factors, the expression of pro-apoptotic Bax, Bad, and anti-apoptotic Bcl-xl, Bcl-2 were examined. Bax mRNA was significantly induced by 200 μM RA as well as by combined action of anti-MUC1 and 200 μM RA. mAb used separately did not affect *Bax* gene. The increase after 100 μM RA combined with mAb was not significant) when compared with untreated control. Bax protein was significantly induced by 200 μM RA, anti-MUC1 used separately and also by combined treatment of both RA concentrations and mAb. There was no influence of 100 μM RA on Bax protein. Bad was assessed only on mRNA level.

Effect of rosmarinic acid and anti-MUC1 on caspase-3, -8, -9 expression Initiatory caspase-8, -9 and executive caspase-3 were examined in the study. *Caspase-8* gene expression significantly increased upon both RA doses and RA with anti-MUC1, however without stimulatory effect of mAb while combining with acid. *Caspase-9* expression was significantly stimulated by RA with two concentrations and by RA combined with mAb. Anti-MUC1 enhanced the effect. Pro-caspase-9 protein expression was significantly inhibited and cleaved caspase-9 was simulated by the examined compounds. The effect of reduction of pro-caspase-9 was the weakest in case of 200 μM RA used together with anti-MUC1. The

strongeststimulatory effect on cleaved caspase-9 was observed for anti-MUC1.However, combined action of mAb and RA revealed stronger inducingeffect in comparison to RA monotherapy. Caspase-3 mRNA was notaffected by 100 μM RA, significantly stimulated by 200 μM RA andsignificantly inhibited by anti-MUC1 and mAb combined with RA.

CONCLUSION

Targeting altered glycosylation as therapeutic strategy seems to be among major fields of surveys in the fight against cancer. MUC1 has been extensively examined as a target for directed cancer therapy and as a marker of disease progression. Thus, the concept of utilizing anti-MUC1 antibodies in cancer treatment seems to be highly reasonable. The loss of normal MUC1 composition expressed by tumors renders this molecule more available to anti-MUC1 antibodies. In this study, we examined the effectiveness of combined action of anti-MUC1 and rosmarinic acid, the flavonoid with anti-cancer ability, in AGS gastric cancer cells, towards several cancer related factors, such as MUC1, enzymes of protein glycosylation, cancer-related antigens, Gal-3, p53, and apoptosis associated proteins. BC-2 anti-MUC1 antibody used in our experiments reacts with a 5 amino acid epitope of the MUC1 core with lower effectiveness for glycosylation than the epitopes detected by other antibodies. We demonstrated inhibitory effect of antibody administrated with RA on MUC1 mRNA and assumed that such combined treatment achieved the goal as according to Birrer at al. (2019) antibody binding to its ligand must resultin internalization of the antibody-binding complex. Surprisingly, 100 μM RA notably increased MUC1 mRNA. We suggest that 100 μM RA activated mechanisms which were not triggered by 200 μM RA. We showed that the applied therapy inhibited MUC1 cytoplasmic domain expression, nonetheless, without enhanced effect of anti-MUC1 administered with RA. In this review the results support the general conception of anti-mAb actionas the expression of targeted molecule was reduced).

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