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The Effects of Plasma-Activated Saline and Medium on Colorectal Cancer in Rat Models and the Human Colon Adenocarcinoma Cell Line COLO 205

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Abstract: Colorectal cancer affects a large number of people. There is an urgent need to develop novel treatments for this disease. In recent years, research on medical applications of atmospheric low temperature plasma (ALTP) has been actively conducted, and cancer treatment is one of the targets. In this study, we focused on colorectal cancer and evaluated plasma as a new treatment method. In vivo and in vitro experiments were conducted using plasma-treated saline (PTS) and plasma-treated medium (PTM) prepared by submerged bubbling treatment using ALTP. In vivo experiments using a rat model of colorectal cancer revealed that PTS administration to the colon slowed tumor progression based on endoscopic and histopathological observations. To investigate the cause of the inhibition of tumor progression, we evaluated the impact of ALTP on colon cancer cells. As a result of culturing colorectal cancer cells with PTM, cell proliferation was inhibited. Also, cell death was induced by cell swelling. qPCR revealed that PTMs induced cell death of cancer cells through signaling of tumor necrosis factor α (TNF-α), an inflammatory cytokine. Therefore, the inhibition of cancer progression by PTS in the rat model occurs by inducing cell death through the dissolution of ALTP components in the liquid.

Keywords: Adenocarcinoma, Tumor Necrosis Factor-alpha, Therapeutics, Reactive Oxygen Species, Atmospheric Low Temperature Plasma

1. Introduction

Colorectal cancer is one of the leading causes of cancer-related death in adults. In the U.S, over 140,000 patients are diagnosed with colorectal cancer every year [1]. Therapeutic endoscopy or surgical removal is possible up to stage T1, however, surgical treatment is no longer possible as cancer progresses, thus the prognosis is poor [2]. There is an urgent need to develop new therapies to target advanced cancer or inhibit cancer progression [3]. Several treatment methods for cancer currently exist, and in recent years, "plasma medicine" which uses plasma directly or indirectly, has been attracting attention. Hemostasis, ablation, and sterilization are the primary applications for plasma [4, 5]. However, in recent years, many studies exploring ALTP for treatments, have broadened plasma applications in medicine [6, 7]. ALTP can generate higher-density plasma than low-pressure plasma, without requiring a vacuum chamber. ALTP exists at room temperature (~293 K) and can be irradiated directly against the body. The main components of ALTP are electrons, ions, radicals, and light [8]. Radicals, in particular, induce physiological actions in cells and tissues [9]. Research by G. Fridman et al. explored the use of dielectric barrier discharge (DBD) for wound healing [10]. The researchers exposed diabetic necrosis lesions, which were

not likely to heal on their own, to DBD, and evaluated their progression. DBD promoted healing of the lesions, which were completely healed after two months. This was thought to be due to the induction of cell growth factors and stimuli towards damaged cells, promoting cell proliferation, leading to a robust therapeutic effect. S. Takeda et al., is exploring the use of atmospheric plasma to treat cancer, specifically examining cells rather than tissues [11]. By culturing these cancer cells in a culture medium irradiated with atmospheric plasma, cancer cell apoptosis will be caused by molecular mechanisms specific to the cells [12, 13].

Fundamentally, cancer can be divided into solid tumors and blood cancers. Solid tumors are further classified into carcinomas that occur in the epithelial cell lineage (lung cancer, breast cancer, stomach cancer, colon cancer, uterine cancer, ovarian cancer, and head and neck cancer), and sarcomas that occur in non-epithelial cell lineages such as bone and muscle (osteosarcoma, fibrosarcoma, liposarcoma, and angiosarcoma). In addition to occurrence in a variety of sites and organs, cancers also have different biological properties, which are targeted by varied treatment methods. When *in vivo* experiments are successfully reproduced *in vitro*, it is possible to determine the mechanisms of cancer cell development, proliferation, and apoptosis. Differences will also be clarified by conducting both the *in vivo* experiments with small animals, and the in vitro experiments with cultured cells at the same time.

In this paper, we conducted basic experiments using "plasma medicine" with a goal to develop regenerative medicine methods in the future. We used ALTP to directly irradiate living tissue and cultured cells and evaluated the use of PTS and PTM as a therapeutic tool for colorectal cancer.

2. Experimental Methods

2.1. PTS and PTM

As shown in Figure 1, ALTP has a structure in which ring electrodes for high voltage application and grounding are attached to the glass capillary from the outside. The PTS solution is made by irradiating saline with plasma. It contains plasma radicals dissolved in the saline. It is possible to administer PTS to locations such as the colon, which cannot be directly irradiated with plasma. We administered PTM to the colons of colorectal cancer model rats and observed its effects on the inhibition of colorectal cancer progression. When we cultured colon adenocarcinoma cells in a PTM, there was a significant decrease in colorectal cancer cells. The PTS and (PTM were prepared by bubbling a plasma flow in 6 mL of each solution. The following conditions were used to generate plasma: applied voltage: V_{ap}=8 kV, frequency: f=3 kHz, helium (He) gas flow rate: F=1 L/min, APP irradiation time: t=90 s, and irradiation distance (tip of glass capillary from medium): L=5 mm [14].

2.2. In Vivo Experiments Using Model Rat

The Kyoto Apc delta (KAD) rats (Japan SLC, Inc., Japan) used in the in vivo experiments have a point mutation in the APC gene [15, 16]. The adenomatous polyposis coli (APC) gene is the causative gene for familial adenomatous polyposis, and it is mutated in 70-80% of all colon and rectal cancer [17]. Mutations in APC produce cleaved proteins, which stabilize β-catenin and induce β-catenin/Tcf-mediated transcriptional activation to induce intestinal tumors [18, 19]. KAD rats show significant colorectal cancer susceptibility in colorectal cancer induction test systems using carcinogens [20]. The method for producing a model rat is as follows; Carcinogens: azoxymethane (AOM) (Sigma Aldrich, USA) was administered subcutaneously at 20 mg/kg to KAD rats (male, 5 weeks old). In addition, 1 week after subcutaneous injection of AOM, rats were administered a 2% -dextran sulfate sodium (DSS) solution (Sigma-Aldrich, USA), which is a tumor promoter, for 1 week. In particular, DSS is known to cause ulcerative colitis. Since enteritis increases the production of nitric oxide along the mucosa of the large intestine, DSS can promote carcinogenesis due to oxidative stress. The number of rats used in the experiment was 6 in total, non-treated saline (control, n=2) and PTS-treated (n=4). Preliminary endoscopic observations showed that tumors formed in the large intestine of rats treated with AOM for 18 weeks. For *in vivo* experiments, histopathological specimens of tissue fragments were stained with hematoxylin & eosin (H&E).

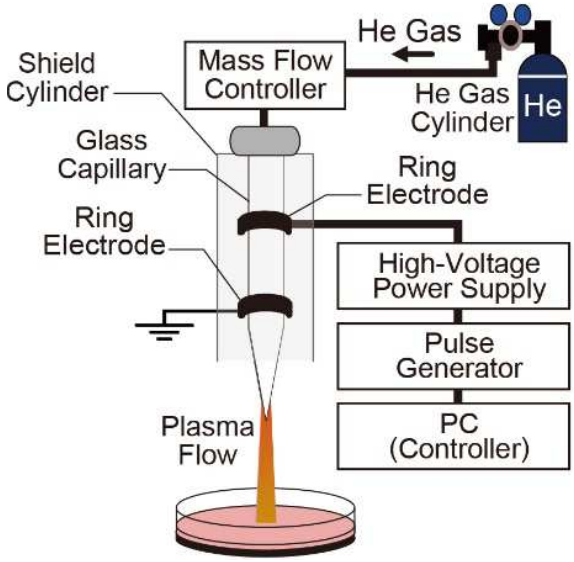

Figure 1. *Schematic of ALTP devices used to generate PTM and PTS.*

2.3. In Vitro Experiments Using Cell

In vitro experiments using COLO 205 cells (KAC Co., Ltd, Japan) were conducted to confirm the effect of ALTP on colorectal cancer. COLO 205 cells are human-derived colorectal cancer cells that have been used as a model for colorectal cancer in many studies [21]. 1 mL roswell park memorial institute (RPMI) 1640 medium containing COLO 205 cells (cell number: 1.0×10^5 cells / mL) and 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc., USA) and was seeded on plates. Well plates with PTM containing

COLO 205 cells were incubated in a CO_2 incubator (temperature: 37°C, CO_2 concentration: 5%). Hemocytometer cell counts were performed after 12, 24, 28, and 72 h. The following conditions were used to generate plasma: V_{ap}=8 kV, f=3 kHz, F=1 L/min, t=90 s, and L=5 mm.

2.4. Cell Gene Expression Analysis by Real-Time RT-PCR

To compare the effect of ALTP on the expression of inflammatory cytokines, gene expression analysis by real-time polymerase chain reaction (RT-PCR) was performed. An RT-PCR system (StepOne™, Applied Biosystems, USA) was used for gene expression analysis. Here, we focused on tumor necrosis factor α (TNF-α) as a marker for inflammatory cytokines. TNF-α is involved in the expression of cell adhesion molecules and the induction of apoptosis, and overexpression leads to the development of rheumatoid arthritis. [22-24] Cells were harvested 24, 48, and 72 h after ALTP irradiation to extract total RNA. 800 µL of ISOGEN (Nippon Gene Co., Ltd.) was added to the samples followed by homogenization. 160 µL of RNA-free water was then added and the samples were incubated at room temperature for 15 min, then centrifuged at 12,000g for 15 min (4°C). The supernatant was mixed with 300 µL of isopropanol and allowed to stand at room temperature for 10 min, followed by centrifugation at 12,000g for 10 min (4°C). After removing the supernatant, 500 µL of 70% ethanol was added, and the samples centrifuged at 8,000g for 3 min. After removing the ethanol, 10 µL of RNA-free water was added to the residual precipitate, and the resuspended sample was used as extracted RNA in the experiment. *TNF-α* (forward: TCCTTCAGACA CCCTCAACC, reverse: AGGCCCAGTTTGAATTCTT) was used as a marker for inflammatory cytokines, and *β-actin* (forward: AGAGCTACGAGCTGCCTGAC, reverse: AGCA CTGTGTTGGCGTACAG) was used as a housekeeping gene.

3. Experimental Results

3.1. Endoscopic and Pathological Anatomy

Figure 2 shows an endoscopic image of normal rat and the KAD rat colon and histopathological specimens (hematoxylin & eosin (H & E) staining). When comparing Figure 2(b) and 2(c), it was determined that tumor progression (circled area) in PTS-treated KAD rats was slower compared to the control. KAD rats treated with saline for colon cleansing were diagnosed with adenocarcinoma as abnormal glandular tissue was observed (arrow in Figure 2(d)). On the other hand, Figure 2(e) was diagnosed as adenoma as the glandular tissue (circled area) had fewer visual abnormalities than the control. Here, adenocarcinoma is a cancer of the secretory gland and a malignant tumor. Although adenomas are benign tumors of the secretory glands, they are likely to become cancerous.

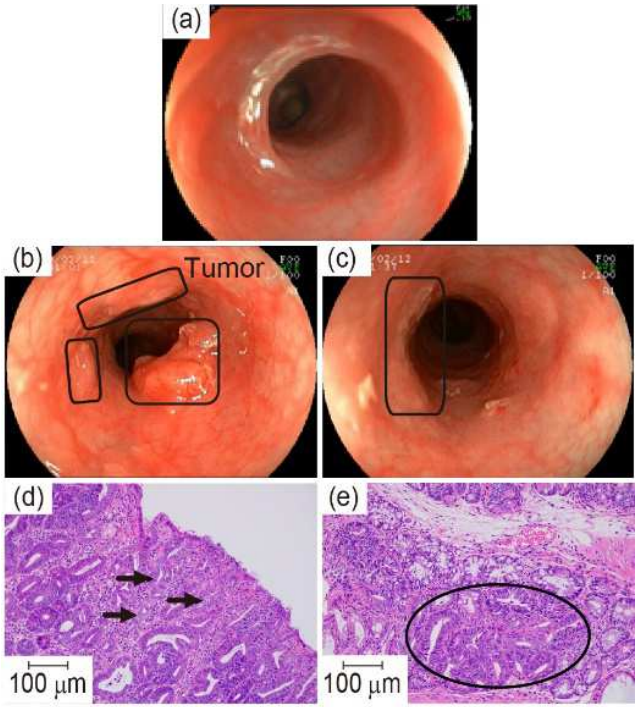

Figure 2. *Endoscopic and pathological anatomy. (a) normal rat. Inside of a rat intestine at 17 weeks after AOM administration [(a) Control and (b) PTS treatment]. Pathological anatomical at 18 weeks after AOM administration [(c) control-adenomatous and (d) PTS treated adenocarcinoma].*

3.2. Cell Growth Curves of COLO 205 Cells

Figure 3 shows the cell growth curves of COLO 205 cells with and without PTS treatment. In the case of control, the cell numbers decreased within 24 h, but tended to increase after 24 h. On the other hand, with PTS treatment, there was almost no increase after the initial decrease within 12 h. Therefore, PTS treatment was clearly found to have an inhibitory effect on COLO 205 cell proliferation.

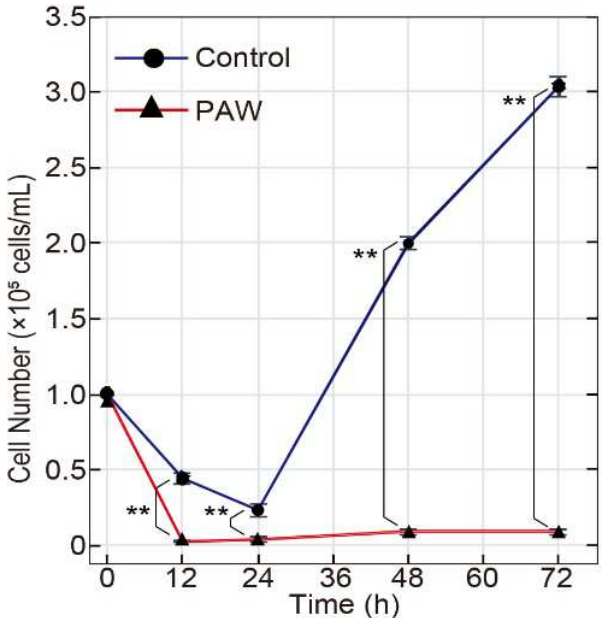

Figure 3. *Growth curves of COLO 205 cells for control and PTS treatment. (X ± SD of seven rats, *: $p < 0.05$, **: $p < 0.005$).*

3.3. TNF-α Expression

Figure 4 shows changes in *TNF-α* expression levels after 12, 24, 48, and 72 h. Here, the housekeeping (endogenous control) gene is a gene that is commonly expressed in a certain amount in tissues. Although it is possible to compare gene expression levels with the real-time PCR method, it is necessary to correct the target gene with a housekeeping gene. Furthermore, although housekeeping genes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine-guanine phosphoribosyl transferase (HGPRT), which are enzymes and proteins, are present in cells, β-actin was selected in this experiment. The reason is that not only B-act was used in some papers, but it is often expressed more stably than GAPDH.

There was no significant difference between the control group and PTS until 12 h. After 24 h there was a significant difference. Therefore, it was determined that PTS induces cell death of cancer cells via the TNF-α signaling pathway [25].

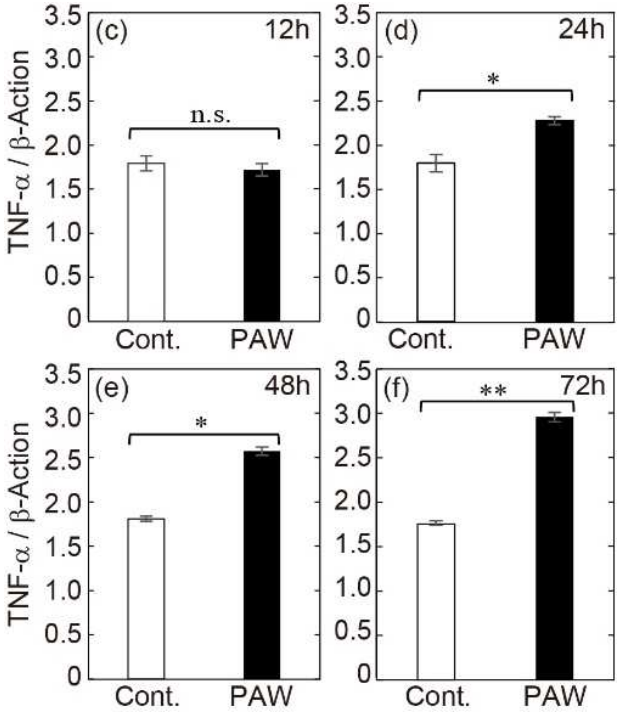

Figure 4. *TNF-α gene expression levels at 12, 24, 28, and 72 h ($\bar{X} \pm SD$ of seven rats, *: $p < 0.05$, **: $p < 0.005$, n.s.: not significant).*

3.4. Reactive Species Measurement Formed by Plasma

The simplified water analysis tool: PACKTEST (KYORITSU CHEMICAL-CHECK Lab., Corp., JAPAN) was used to measure the ion concentration in the solution derived from the reactive species formed by plasma irradiation. Here, we focused on H_2O_2, NO_2^- and NO_3^-, which are mainly formed by plasma irradiation. Figure 5 shows the H_2O_2, NO_2^- and NO_3^- concentrations in the medium at different humidity (control (55), 65, and 95%). The following irradiation conditions were used: V_{ap}=8 kV, f=3 kHz, F=1 L/min, t=60 s, and L=10 mm.

The H_2O_2 concentration tended to increase as the humidity increased. As cell proliferation was promoted at high humidity, it can be stated that the H_2O_2 concentration significantly affected the cell proliferation. It has also been reported that low concentrations of H_2O_2 are generally deeply involved in angiogenesis and epithelial tissue formation [26, 27]. However, although H_2O_2 significantly affects cell/tissue proliferation, differentiation, and migration, the detailed mechanism has not been elucidated [28, 29]. NO_2^- and NO_3^- are less dependent on the changes in humidity. A spectrum corresponding to the second positive system of nitrogen (N_2 SPS, C3Πu → B3Πg) was detected at 300–500 nm, and a spectrum corresponding to N_2 first negative and the N_2 second positive systems was detected at 600–800 nm [30]. N_2 SPS (C3Πu → B3Πg) and OH radicals were generated in the turbulent region of contact between the medium surface and plasma stream, forming reactive species such as H_2O_2, NO_2^- and NO_3^-. According to the experiments in which our and other groups measured OH radicals using laser-induced fluorescence (LIF), it has been confirmed that plasma irradiation on the water surface or in a high humidity environment promotes the generation of OH radicals [31, 32].

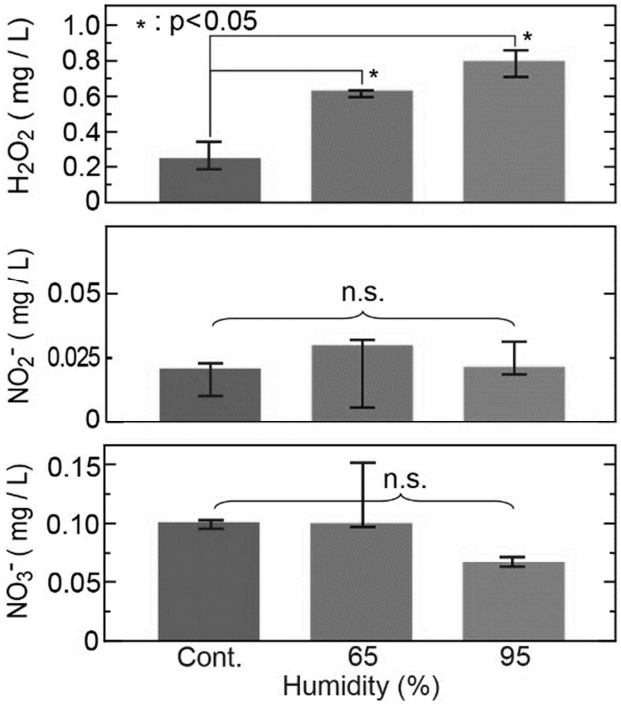

Figure 5. *Humidity dependence of H_2O_2, NO_2^-, and NO_3^-. *: $p < 0.05$.*

Our current theory is that reactive oxygen species (ROS) and reactive nitrogen species dissolved in PTW permeate the cell membrane and damage the mitochondria. ROS are metabolized by enzymes such as superoxide dismutase, catalase, and peroxidase in healthy cells, but cancer cells do not have a strong ability to do so [33]. Thus, they are unable to metabolize all of the ROS, which in turn induces cell death [34, 35]. In addition to ROS, reactive nitrogen species (RNS) are also present in the plasma products.

4. Conclusion

"*In vivo*" experiments with PTS administration to KAD colorectal cancer model rats showed a tendency to inhibit the progression of colorectal cancer. On the other hand, according to the "*in vitro*" experiment with COLO205 cells using PTM, there was a significant difference in the PTM group compared with the control group. In particular, changes in TNF-α expression levels using RT-PCR also suggest that cell death is induced via the TNF-α signaling pathway. The reactive oxygen species (ROS) (mainly H_2O_2) and active nitrogen species (RNS) (mainly NO_2^- and NO_3^-) contained in PTS and PTM penetrate the cell membrane and damage mitochondria. Furthermore, since cancer cells have a lower metabolic function than normal cells, they cannot metabolize all ROS and induce cell death.

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