Effects of atmospheric low temperature plasma on the rat model of colorectal cancer

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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. There is an urgent need to develop new therapies to target advanced cancer or inhibit cancer progression. Several treatment methods for cancer currently exist, and in recent years, "plasma medicine" which uses plasma directly or indirectly, has been attracting attention. In this paper, we evaluate the use of Plasma Treated Saline (PTS) generation to study the therapeutic application of plasma to colorectal cancer.

Keywords: Atmospheric pressure low temperature plasma, colorectal cancer, cell death.

(Received: 5 November 2021, Revised: 18 January 2022)

1. Introduction

Cell division in a normal organism is controlled by necessity. A mass of cells that escapes control and grows autonomously is called a tumor. There are two types of tumors, benign and malignant, and the latter is synonymous with cancer. Among tumors, malignant tumors are defined as those with characteristics such as invasion, metastasis, cachexia, and avoidance of apoptosis [1]. Among cancers, which are the leading cause of death in Japan, lung cancer, colorectal cancer, and gastric cancer are among the leading causes of death by site. Among them, the number of patients with colorectal cancer has been increasing every year, and it has been the most common cancer since 2012. Since the number of patients with colorectal cancer is expected to increase in the future, this study will focus on colorectal cancer, which has many both deaths and cases.

Currently, plasma is applied to surface processing, sterilization technology, and also to living organisms. Atmospheric Low-Temperature Plasma (ALTP) is used for direct treatment of living organisms. ALTP is a plasma generated by electrical discharge under atmospheric pressure. Compared to low pressure plasma, atmospheric pressure plasma can produce high density plasma and does not require a vacuum device. Plasma production While high-temperature plasmas range from several thousands to several tens of thousands K, low-temperature plasmas exist at about 293 K, which is about room temperature, and can be irradiated to living bodies [2]. Current medical applications of plasma include the treatment of allergic rhinitis and dental care. In addition, recent studies suggest that ALTP may be useful for cancer cells [3]. Therefore, we believe that atmospheric pressure and low temperature plasma may be effective for colorectal cancer, and we will investigate the effect of atmospheric pressure and low temperature plasma on colorectal cancer. In this study, we prepared Plasma Treated Saline (PTS) by irradiating ALTP in saline solution, and examined the effects of intracellular and tail vein administration.

The main types of cell death that occur in vivo are necrosis and apoptosis. In necrosis, the cell is damaged and ruptures, scattering its contents and triggering an inflammatory response in the surrounding area, which can be harmful. Apoptosis, on the other hand, is a planned cell death, in which the contents are taken up by phagocytes without dispersal so as not to cause harm to the surrounding cells. Therefore, we believe that apoptosis is preferable for inducing cell death.

Apoptosis is carried out by a group of enzymes called the caspase family. The exogenous pathway, which is one of the mechanisms that induce apoptosis, is called the Mitogen Activated Protein Kinase (MAPK) cascade [4]. MAPK is phosphorylated and activated by MAPKK, and MAPKK is phosphorylated and activated by MAPKKKs (MAP3K) (Fig. 1). one of the MAP3Ks, ASK1, binds to the reduced protein thioredoxin in the steady state and remains in an inactive form.

Activation of ASK1 activates the MAPK MEK4 and MEK7 and MEKK3 and MEKK6, which in turn activate the MAPK JNK and p38, respectively. Both activated JNK and p38 cleave and activate caspase-3. Both JNK and p38 are activated by cleaving caspase-3, which ultimately leads to apoptosis by cleaving proteins necessary for cell survival. Therefore, we hypothesized that the oxidative stress of reactive oxygen species in PTS could induce activation of ASK1 and induce apoptosis. Extracellular ROS-induced apoptosis by ALTP is important, but the possibility of endogenous ROS also needs to be investigated. When ROS stimulate cells, the immune response system acts to form a protein complex
called inflammasome, which stimulates the production of IL-1β, and IL-1β in turn stimulates the production of ROS, resulting in positive feedback. In other words, we thought that ROS produced by ALTP could exogenously activate ASK1, which in turn could produce endogenous ROS, and thus further effects could be expected, so we also investigated IL-1β.

In addition, the Wingless-type (Wnt)/β-catenin pathway has been associated with the promotion of tumor development, growth, metastasis, and invasion in cancer. Therefore, we investigated the effect of PTS by examining the activity of Wnt inhibitory factor 1 (Wif1), an inhibitor of the Wnt/β-catenin pathway.

### Table 1 Primer sequences used for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>AGAGCTACGAGCTGCCCTGAC</td>
</tr>
<tr>
<td>β-actin</td>
<td>AGCAGCTGTGTGGCCTACAG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>GAACTGGATCCGGATCGGAC</td>
</tr>
<tr>
<td>IL-1β</td>
<td>TCCGTCCGTCATCAATCCA</td>
</tr>
<tr>
<td>Wif1</td>
<td>AGCCATCCCGTCATCAATCCA</td>
</tr>
<tr>
<td>Wif1</td>
<td>TCTGCCATATGCGCTTTATCCA</td>
</tr>
</tbody>
</table>

![Fig. 1 Mechanism of ROS-induced apoptosis by ASK1 pathway.](image)

![Fig. 2 Schematic of ALTP devices used to generate PTS.](image)

![Fig. 3. Experimental procedure.](image)

### 2. Methods

#### 2.1 Method for making PTS

PLS was prepared by using the plasma generator shown in Fig. 2. The saline solution was irradiated to 3 [mL] for intestinal cleansing and 6 [mL] for intravenous injection. ALTP has a structure in which ring electrodes for high voltage application and grounding are attached to the glass capillary from the outside. The following conditions were used to generate plasma: applied voltage: 13 kV, frequency: 3 kHz, helium (He) gas flow rate: 1 L/min, and ALTP irradiation time: 300 s.

#### 2.2 PTS intestinal administration and tail vein administration to KAD rats

The Kyoto Apc delta (KAD) rats (Japan SLC, Inc., Japan) used in the in vivo experiments have a point mutation in the APC gene [5]. 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. Mutations in APC produce cleaved proteins, which stabilize β-catenin and induce β-catenin/Tcf-mediated transcriptional activation to induce intestinal tumors. KAD rats show significant colorectal cancer susceptibility in colorectal cancer induction test systems using carcinogens.

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 [6]. 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 = 3), administration of PTS to the large intestine (n = 3) and PTS tail vein injection (n = 4). 6 mL of PTS was administration of PTS to the large intestine of rats, and 0.5 mL of PTS was injected into the tail vein twice a week in both groups. The Preliminary endoscopic observations part of the endoscope system FTS4400 (FUJIFILM Co., Ltd.) was inserted through the anus of the rats once a week to observe the intestinal conditions.

Twenty weeks after the administration of AOM, the colon was removed from the rats and tissue specimens were prepared by hematoxylin eosin (H&E) staining. Tumor status was evaluated by histopathological examination of the prepared tissue specimens.

All experiments were performed under anesthesia using a mixture of Vetorphale (Meiji Seika Pharma Co., Ltd.), Domitor (Nippon Zenyaku Kogyo Co., Ltd.), and Dormicum Injection (Marushi Pharmaceutical Co., Ltd.).

2.3 Detection of tumor suppressor-related genes by RT-qPCR

Another pathway that stimulates ASK1 is the activation of FasL upstream of ASK1 by Nitric Oxide (NO) [7]. The increase in intracellular NO is divided into exogenous and endogenous, and since ALTP has also been shown to generate NO, it is likely that FasL is activated by exogenous NO. In addition, IL-1β promotes the generation of endogenous NO and the tumor-killing effect of macrophages, so we analyzed the expression of IL-1β [8]. We also analyzed the expression of WIF1, an inhibitor of the Wingless-type (Wnt)/β-catenin pathway.

1000 µL of ISOGEN (Nippon Gene Co., Ltd.) was added to the samples followed by homogenization. 200 µL of chloroform (FUJIFILM Wako Pure Chemical Co., Ltd.)RNA-free water was then added and the samples were incubated at room temperature for 15 minutes, then centrifuged at 12,000 ×g for 15 min (4 °C). The supernatant was mixed with 300 µL of isopropanol and allowed to stand at room temperature for 10 minutes, followed by centrifugation at 12,000×g for 10 min (4 °C). After removing the supernatant, 500 µL of 70% ethanol was added, and the samples centrifuged at 8,000×g 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. Then, cDNA synthesis by reverse transcription reaction was performed. FastGene™ ScriptaseII 5X ReadyMix Odt (LS65) (NIPPON Genetics Co., Ltd.) 2 [µL], total RNA solution for 1 [µg] of RNA, and RNA-free water were added and mixed to make 10 µL in total. The reverse transcription reaction (25 °C for 10 min, 42 °C for 60 min, and 85 °C for 5 min) was performed in a thermal cycler to obtain cDNA.

Real-time PCR was performed using TBGreen Premix Ex TaqTM II ROX plus (Takara Bio Co., Ltd.) and StepOne TM Real-Time PCR system (Thermo Fisher SCIENTIFIC Co., Ltd.). A PCR reaction solution of 10 µL was prepared by mixing 5 µL of TBgreen, 0.4 µL of

Fig. 4  Endoscopic image of the colon at 20 weeks after AOM administration
(a) Control (Rat C).
(b) Administration of PTS to the large intestine (Rat D)
(c) Tail vein injection (Rat J).
forward primer, 0.4 µL of reverse primer, 0.2 µL of ROX difference dye II, 3 µL of RNA-free water, and 1 µL of quadruple-diluted cDNA solution. The reverse transcription reaction was performed in StepOne at 42 °C for 5 minutes and 95 °C for 10 seconds, and then the PCR reaction was performed by repeating 45 cycles of 95 °C for 5 seconds and 60 °C for 30 seconds. The primers are shown in Table 2 [9]. In this experiment, β-actin was used as an internal standard [10]. All the samples compared were tumor sites. IL-1β was compared in the control group, intestinal administration group, and tail vein injection group, and Wif1 was compared in the control group and micro vein injection group for each individual.

3. Results

3.1 PTS intestinal administration and tail vein injection in KAD rats

The endoscopic images shown in Fig. 4 showed that the tumor enlargement tended to be suppressed in the intestinal cleansing group and the tail vein injection group compared to the control rats.

The histopathological images shown in Fig. 5, the atypia of the intestinal gland was strong in the Control group, the administration of PTS to the large intestine group, and the tail vein injection group, and the rats were diagnosed as adenocarcinoma.

On the other hand, the degree of invasion in the tail vein injection group tended to be lower than that in the other groups (Fig. 6-8, Table 2, G and H).

3.2 Detection of tumor suppressor-related genes by RT-qPCR

Fig. 9 shows that the expression of IL-1β was higher in the administration of PTS to the large intestine group and tail vein injection group than in the control group. In addition, we compared the expression of Wif1 between the tail vein injection group and the control group, and found that the expression of Wif1 was higher in the tail vein injection group (Fig. 10). The expression of Wif1 was higher in the tail vein injection group than in the control group, especially in 1 and J, suggesting that the Wnt/β-catenin pathway was strongly inhibited.

4. Discussion

Although all rats were diagnosed as having adenocarcinoma, tail vein injection tended to inhibit metastatic invasion (Fig. 4, 5, Table 2). In addition, rat A in the Control group died in the middle of the treatment without waiting for the cancer to grow, which indicates that the rats were under considerable stress compared to the other two groups. In terms of the number of colorectal cancers, although it is necessary to consider the effects of individual differences in rats, the maximum number of tumors in each group was lower in the other two groups than in the Control group, suggesting the effect of ALTP. The reason for the lack of suppression of metastasis in the in-
The PTS that flowed into the intestine during cleansing was quickly expelled from the body, and only a small amount of PTS was absorbed from the intestinal wall.

Initially, we aimed to promote apoptosis of cancer cells from the activation of ASK1 by ROS of ALTP, but the results of this experiment did not show that the generated cancer cells shrank or disappeared. We cannot deny the possibility that apoptosis occurred because the number of tumors in the vein injection group was suppressed, but even so, the effect was not sufficient to allow cancer treatment.
The cause of the decrease in the number of tumors and invasive capacity in the PTS microvascular group is related to \( IL-1\beta \) and \( Wif1 \). \( IL-1\beta \) is one of the factors that promote tumorigenesis and promotes inflammation by activating NF-\( \kappa \)B, a transcription factor. On the other hand, there is a report that \( IL-1\beta \) induces macrophages and has tumoricidal effects. Therefore, the suppression of the number of tumors by PTS and the increase in \( IL-1\beta \) expression in the present experiment suggest that macrophages promote tumor killing and apoptosis. In addition, \( Wif1 \), an inhibitor of the Wnt/\( \beta \)-catenin pathway, was activated in the tail vein group, indicating that PTS suppressed tumor growth.

5. Conclusion

The results of this study showed that the degree of tumor invasion was similar in rats treated with PTS in the intestine compared with control rats that were not treated with plasma. However, tumor invasion tended to be slower in the tail vein injection group. In addition, increased expression of \( IL-1\beta \) and \( Wif1 \) was observed in the PTS-treated group. It was suggested that the ROS produced by plasma generation had tumoricidal and tumor suppressive effects.

The presence of E-cadherin is also important in the reduction of cancer invasiveness, although the pathway could not be determined in this experiment [10]. E-cadherin is also important in inhibiting tumor progression because its expression has been shown to be increased by experiments to increase endogenous ROS.

As a future research direction, we will not only apply ALTP as a cancer therapy, but also apply it to adjuvant therapies that inhibit cancer progression and metastasis by using it in combination with other existing therapies. Therefore, it is necessary to investigate more specifically the biological safety of ALTP, its mechanism of action, and the extent to which it has a tumor-killing effect on macrophages and induction of apoptosis.

Acknowledgment

This study was supported by a Grant-in-Aid for Scientific Research (No.19KO3814) from the Ministry of Education, JAPAN.

Animal Rights

All institutional and national guidelines for the care and use of laboratory animals were followed and approved by the appropriate institutional committees. No human studies were carried out by the authors for this article.

References