의학박사 학위논문

저온플라즈마의 흑색종, 대장암 동종 마우스 모델에서의 항암효과

Anti-cancer effect of cold atmospheric plasma in melanoma and colon cancer syngeneic mouse models

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정 준 민

Anti-cancer effect of cold atmospheric plasma in melanoma and colon cancer syngeneic mouse models

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이 논문을 의학박사 학위 논문으로 제출함

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국문요약

배경 및 목적: 현재 저온플라즈마 (cold atmospheric plasma) 의 의료관련응용분야에는 피부 오염 제거, 치아 표백, 상처 치유 및 암 치료 등이 포함된다. 세포의 DNA 를 손상시키고 세포 사멸을 유도하는 활성 산소종을 생산하는 저온플라즈마의 능력은 저온플라즈마가 다양한 유형의 종양치료에 이용될 수 있음을 의미한다. 본 연구는 동종 마우스 모델에서 저온플라즈마의 항암효과를 평가하였다.

방법: 먼저 실험을 통해 저온플라즈마가 정상 인간 멜라닌 세포, 흑색종 세포 및 대장암 세포의 생존에 미치는 영향을 평가하였다. 다음으로 $5x10^5$ B16F10 흑색종 세포를 20 마리의 마우스에 주사하거나 $5x10^5$ MC38 대장암 세포를 18 마리의 마우스에 주사함으로써 동종 마우스 모델 (murine syngeneic cancer model) 을 만들었다. 종양이 성장한 후, 격일로 5 회의 저온플라즈마를 처리하였다. 종양의 부피는 각 시점에서 측정되었다. 10 일째에 종양의 무게를 측정하고 종양성장억제 (Tumor growth inhibition, TGI) 를 계산하고 모든 종양을 절제하여 조직학적으로 평가하였다.

결과: 저온플라즈마 처리는 흑색종과 대장암 세포주의 생존력을 현저하게 감소시켰으며, 정상 인간 멜라닌 세포의 생존력에는 영향이 미미하였다. 동종 마우스 모델에서 저온플라즈마 처리는 두 암 모델 모두에서 종양 부피와 종양 무게를 줄였으며, 종양이 감소된 정도는 저온플라즈마의 치료 기간과 횟수에 의존적이었다. B16F10 흑색종 모델에서, 2 분, 5 분 및 15 분 동안 저온플라즈마로 처리 된 마우스의 10 일째 종양성장억제 (TGI) 는 각각 3%, 43.4% 및 47.0%였다. MC38 대장암 모델에서, 2 분 및 5 분 동안 저온플라즈마로 처리 된 마우스의 10 일째 종양성장억제 (TGI) 는 각각 35.8% 및 58.3%였다. 조직학적검사는 두 종양 모델에서 저온플라즈마의 종양 억제 효과를 보여주었다.

결론: 저온플라즈마는 실험실적으로 마우스 흑색종 및 대장암 세포주의 성장을 억제했을 뿐만 아니라 흑색종 및 대장암 동종 마우스 모델에서 항암효과를 보여주었다.

핵심 단어: Cold atmospheric plasma, Anti-cancer effect, Melanoma, Colon cancer, Murine syngeneic cancer model

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INTRODUCTION

The medical applications of cold atmospheric plasma (CAP) have been expanding and now include skin decontamination, tooth bleaching, wound healing, and cancer therapy.(1) CAP has a significant impact on cellular biology, mainly by inducing the production of reactive oxygen species (ROS),(2) which act as second messengers in redox biology and can induce cellular differentiation and structural changes.(3) ROS produced by CAP can damage cellular DNA, leading to apoptosis.(4) Numerous cancer cells have been treated with CAP *in vitro* to assess the potential role of CAP in cancer treatment, with studies showing that CAP effectively inhibits cancer cell growth without resistance.(5) Exposure of cancer cells to CAP *in vitro* was found to reduce cell migration and induce cellular apoptosis.(6, 7) Cancer cells may be more vulnerable to CAP exposure than normal cells, indicating that CAP treatment can selectively reduce the numbers of cancer cells while having less destructive effects on adjacent normal cells.(8-10) This selective effect may be dependent on baseline intracellular ROS, the expression level of aquaporins, and/or the cholesterol composition of the cell membrane.(11, 12) CAP may also activate the immune response to tumor cells, inducing immune-mediated death of tumor cells and enhancing the function of macrophages.(13, 14) Furthermore, CAP was reported to have a selective anti-tumor effect against oral squamous cell carcinoma by triggering nitric oxide (NO)-induced dysfunction of epidermal growth factor receptor (EGFR).(15) This potential selectivity of CAP toward cancer cells suggests that CAP may be a promising new treatment modality in oncology. For example, CAP can be applied to unresectable tumors located in critical anatomical areas, with minimal potential risks of unintended local destruction of normal structure.

Few *in vivo* studies and clinical trials have evaluated the efficacy and safety of CAP in cancer treatment. In addition, to optimize its use in cancer therapeutics, the effectiveness of different types of CAP must be compared in each type of cancer. Additional studies are required to objectively evaluate the role of CAP in cancer therapeutics and to design reliable treatment protocols.

Melanoma is a fatal cutaneous malignancy that has been shown to be resistant to various medical therapies.(16) Because CAP can be easily applied to melanomas, most of which are located on the skin, CAP may be an appealing alternative when complete tumor removal that includes adequate margins is not achievable. In addition, the refractoriness of advanced

melanoma to treatment suggests a need for new combination therapies. Thus, CAP can be readily combined with other systemic treatments, such as EGFR inhibitor. Melanomas express several receptor tyrosine kinases, including EGFR family members. Although the role and clinical significance of their expression in melanoma is not fully understood, EGFR may play a role in the progression and metastasis of a subset of melanomas.(17) Therefore, targeting EGFR may be a potential therapeutic option for patients with these tumors.(18)

Recent developments in plasma sources make it possible for CAP to propagate through a flexible endoscope,(19) allowing CAP to be used to treat tumor types such as colon cancer.

This study was performed to evaluate the ability of direct CAP treatment to inhibit the growth of melanoma and colon cancer cells *in vitro* and to reduce targeted tumor volumes *in vivo* using syngeneic mouse models. In addition, the possible therapeutic role of a combination of CAP treatment with EGFR inhibitor was assessed in melanomas.

MATERIALS AND METHODS

Experimental system

The CAP device employed in this study was the MediPL® plasma torch system (2.5 W, argon flow, 2.0 l/min) developed and built by MediPL, Inc., Seoul, Korea.

Cell cultures

Normal human epidermal melanocytes (NHMs) were cultured in Medium 254, supplemented with Human Melanocyte Growth Supplement (Thermo Fisher Scientific, Inc.) at 37 °C and 5% CO_2. NHMs were used at passages between 3 and 7. B16F10 murine melanoma cells were obtained from the Korean Cell Line Bank (Seoul, Korea). Human melanoma cells (A375, A2058) and colon cancer cells (MC38) were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin in 5% CO_2 at 37°C.

Cell viability assay

The viability of NHMs, B16F10, A375, A2058, and MC38 cells was measured 24 h after CAP treatment for the indicated lengths of time using crystal violet assays according to the standard protocols recommended by the manufacturer (Sigma-Aldrich, St. Louis, MO, USA). Cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature and rinsed four times with distilled water. The crystal violet retained by adherent cells was extracted with 95% ethanol, and the absorbance was determined at 590 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). To facilitate data analysis, relative cell viability was calculated based on the measured absorbance at 590 nm by dividing absorbance between the experimental and control groups. To evaluate the anti-cancer effect of EGFR inhibitor combined with CAP treatment, cells were pretreated with the EGFR inhibitor AG1478 (Sigma-Aldrich) for 1 h followed by CAP treatment for 120 sec.

After 24 h, the cells were again treated with CAP for 120 sec, with cell viability assessed 24 h after the last CAP treatment.

Western blot analysis

Western blot analysis was performed using antibodies against Bcl-2, cleaved caspase 3, heme oxygenase-1 (HO-1), Nrf2, p53 (Cell Signaling, Danvers, MA, USA), β-actin (Sigma-Aldrich), phospho-EGFR, EGFR, phospho-AKT, AKT, phospho-ERK, and ERK (Cell Signaling). Epidermal growth factor (EGF) was purchased from Sigma-Aldrich. A monoclonal anti-β-actin antibody was used as an internal loading control. Levels of protein expression were quantified by densitometry after normalization to the optical density of β-actin using ImageJ software (National Institute of Health, Bethesda, MA, USA).

In vivo experiments

All animal experiments were performed in compliance with the Principles of Laboratory Animal Care, formulated by the Institutional Animal Care and Use Committee (IACUC) of the Asan Institute for Life Sciences, Asan Medical Center, Seoul, Korea and were consistent with the guidelines of the Institute of Laboratory Animal Resources (ILAR).

Thirty-eight C57BL/6 mice were purchased from ORIENT BIO, Inc. (Seongnam, Korea). At age 6 weeks, their fur was removed using small animal clippers, and 5×10^5 B16F10 or MC38 cells were injected into each mouse intra-dermally using a 2.5 μl Hamilton syringe (Hamilton, Reno, NV, USA). The mice were followed up routinely, and tumor size was measured throughout the study period using calipers. Tumor volume was calculated as (length × width^2)/2. For CAP treatment, the mice were anesthetized using isoflurane; CAP was applied 1 ± 2 mm above the skin for the designated time while continuously moving the probe to cover the entire tumor, with 0.5 cm margins. Tumor growth inhibition (TGI) was defined as the difference between the mean tumor volumes of a treated and a control group, and was calculated as TGI (%) = (mean tumor volume of control group - mean tumor volume of treated group)/mean tumor volume of control group × 100. On day 10, the tumors were excised to measure their weight, and skin samples were harvested for histological

evaluations. The skin samples were fixed in 10% paraformaldehyde (in 0.1 M PBS, pH 7.4), mounted in paraffin and sectioned into 5 μm thick paraffin sections, which were stained with hematoxylin and eosin (H&E).

Statistical analysis

The statistical significance of the differences between groups was evaluated by Student's t-test, with $P < 0.05$ considered statistically significant. All statistical analyses were performed using R version 3.5.3 (R Foundation for Statistical Computing) software. In this study $P < 0.05$, $P < 0.01$, $P < 0.001$ represent *, **, and ***, respectively.

RESULTS

Selective anti-cancer effect of cold atmospheric plasma

The anti-cancer effects of CAP on cells of the melanoma cell lines B16F10, A375, and A2058, and the colon cancer cell line MC38, were investigated by applying CAP for up to 150 sec and measuring cell viability 24 h later. To assess whether CAP had selective effects on cancer cells, NHMs were treated with CAP for up to 150 sec. CAP treatment significantly reduced the viability of each cancer cell line in a treatment duration-dependent manner (Fig. 1A, B, C, D) but had negligible effect on the viability of NHMs (Fig. 2). These results suggested that CAP has more potent activity against cancer cell lines than against normal cells, although the degree of response varied between cancer cell lines.

Treatment with cold atmospheric plasma is associated with cancer cell apoptosis

The cleaved, activated form of caspase 3 is an indicator of the activation of pro-apoptotic signaling pathways,(20) whereas heme oxygenase-1 (HO-1) expression is induced by oxidative stress and reported to mediate apoptosis.(21) Treatment of B16F10 and MC38 cancer cells with CAP upregulated the levels of expression of cleaved caspase 3 and HO-1 (Fig. 3), suggesting that CAP treatment induces apoptosis in cancer cell lines through a mechanism involving oxidative stress.

Cold atmospheric plasma and epidermal growth factor tyrosine kinase inhibitor have an additive anti-melanoma effect

To explore whether EGFR inhibitor, alone or combined with CAP, has anti-melanoma effects, B16F10 and A375 cells were treated with EGFR inhibitor alone or EGFR inhibitor plus CAP. The combination of CAP treatment and EGFR inhibitor had an additive anti-cancer effect on both melanoma cell lines (Fig. 4A, B), suggesting that a combination of systemic EGFR inhibitor therapy and local CAP treatment is a potential option for treating patients with melanoma. EGFR inhibitor was found to downregulate the EGFR pathway in

melanoma cells, as it effectively reduced EGF-induced phosphorylation of EGFR, AKT, and ERK in A375 cells (Fig. 5).

The anti-cancer effect of cold atmospheric plasma in vivo

The anti-cancer effect of CAP treatment was evaluated using syngeneic mouse models. Twenty mice were inoculated with B16F10 melanoma cells and 18 with MC38 colon cancer cells. Following the growth of B16F10 melanomas, five mice each were treated five times, once every other day, with CAP for 2 min, 5 min, or 15 min (i.e., on days 0, 2, 4, 6, and 8), whereas five untreated mice served as controls. Mean tumor volumes on day 0 did not differ significantly, being 38.82, 37.62, 40.05, and 36.35 mm^3 in the control group and in mice treated with CAP for 2 min, 5 min, and 15 min, respectively. Tumor volumes were measured on days 0, 3, 5, 7, and 10. In addition, TGI was calculated and tumor weights were measured on day 10. CAP treatments for 5 min and 15 min reduced tumor volumes and weights on day 10 (Fig. 6A, B, C). TGIs in mice treated with CAP for 2 min, 5 min, and 15 min were 3.0%, 43.4%, and 47.0%, respectively. Histological examination showed necrotic areas of CAP-treated tumors under relatively unaffected epidermal and dermal layers (Fig. 7A, B, C, D).

In the MC38 colon cancer model, 18 mice were inoculated with MC38 colon cancer cells. Following tumor growth, six mice each were treated five times, once every other day, with CAP for 2 min or 5 min (i.e., on days 0, 2, 4, 6, and 8), whereas six untreated mice served as controls. Because tumor volume and weight reduction did not differ markedly between B16F10 melanoma-inoculated mice treated with CAP for 5 min and 15 min, the latter time point was omitted from the MC38 colon cancer model. Mean tumor volumes did not differ significantly on day 0, being 29.53, 28.57, and 28.39 mm^3 in the control group and in mice treated with CAP for 2 min and 5 min, respectively. As in the B16F10 melanoma model, tumor volumes were measured on days 0, 3, 5, 7, and 10, and TGI was calculated and tumor weights were measured on day 10. CAP treatments for 2 min and 5 min reduced tumor volumes and weights on day 10 (Fig. 8A, B, C). TGIs in mice treated with CAP for 2 min and 5 min were 35.8% and 58.3%, respectively, relative to control mice. Histopathological examination also revealed that CAP treatments had tumoricidal effects but, unlike in the B16F10 melanoma model, the preservation of the epidermal or dermal layer was not

observed because these tumors were located very superficially (Fig. 9A, B, C).

DISCUSSION

CAP has shown potential as a promising anti-cancer modality, as it has been shown to inhibit the growth of numerous cancer cell lines *in vitro*, including breast cancer,(22) colon cancer,(23) glioblastoma,(24) hepatocellular carcinoma,(22) lung cancer,(6) melanoma,(25) neuroblastoma,(26) and pancreatic cancer(27) cell lines. Few studies, however, have assessed the anti-cancer efficacy of CAP treatment *in vivo*.(5)

There are two basic methods of applying CAP as an anti-cancer modality. One is directly applying CAP to target cells *in vitro* or to tumors *in vivo*.(28) Another approach is indirect, using CAP-stimulated solutions. These solutions can inhibit the growth of cancer cells during the cell culture process (29) or the growth of tumors when injected directly into tumor tissue.(30) The relative superiority of these methods in suppressing the growth of individual cancer cell lines has not yet been determined, although most previous studies have only assessed the efficacy of the direct method.(5)

Clinical application of CAP requires an understanding of its anti-cancer potency both *in vivo* and *in vitro*. The present study therefore assessed the anti-cancer effects of CAP applied directly to tumors in syngeneic mice inoculated with melanoma and colon cancer cell lines. Because the immune system is thought to be involved in the anti-cancer effects of CAP,(13, 14) we utilized syngeneic models, in which a functional immune system is present, rather than xenograft models.(31)

The viability of NHMs was barely affected by CAP treatments for up to 150 sec. By contrast, the viability of both melanoma and colon cancer cell lines was significantly reduced by CAP treatment, demonstrating that CAP has a selective anti-cancer effect. The reasons that CAP is more potent against cancer cells than against normal cells, and that cancer cells differ in their degree of sensitivity to CAP, remain largely unknown. These differences, however, may be due to differences in ROS levels after CAP treatment.(32, 33) Basal intracellular ROS levels are thought to be higher in cancer cells than in normal cells because the former have a stronger metabolism.(34) Therefore, an additional influx of ROS in response to CAP treatment can result in ROS levels that cannot be tolerated by cancer cells.(35) The levels of expression of aquaporins may also contribute to different levels of intracellular ROS.(11) More aquaporins were found to be expressed on the cytoplasmic membranes of cancer cells

than of normal cells.(36) Higher expression of those channels may result in faster diffusion of CAP-originated H_2O_2, activating the downstream pathway leading to cellular apoptosis.(11) Similarly, the intracellular antioxidant system can contribute to the differences between cell lines in sensitivity to CAP.(37)

Apoptosis is the active and physiological form of cell death, involving the destruction of cellular components, whereas necrosis is described as passive and accidental cell death caused by sudden environmental changes.(38) Our results suggest that the anti-cancer effects of CAP are mediated by cellular apoptosis, as cleaved caspase 3, a key factor for apoptosis, was increased by CAP exposure in a treatment duration-dependent manner.(39) Members of the Bcl-2 family control the intrinsic pathway of apoptosis.(39) Because CAP did not alter the level of expression of Bcl-2, the CAP-induced anti-cancer effect may involve the extrinsic pathway of apoptosis.

EGFR protein expression is a marker of metastasis in melanomas.(40) Advanced or metastatic melanoma is a debilitating cutaneous cancer with aggressive behavior and a poor prognosis.(41) Although preliminary, our findings suggest that an EGFR inhibitor, whether alone or in combination with CAP treatment, may play a role in future anti-melanoma therapy.

In vivo experiments also showed that CAP treatment had tumoricidal effects. The degree of TGI, however, differed in the two cancer models tested. In contrast to *in vitro* results showing that B16F10 cells were more sensitive to CAP treatment than MC38 cells, TGI in response to the same duration of CAP treatment was higher in MC38 colon cancer than in B16F10 melanoma cells. CAP treatments for 2 min each effectively decreased the growth of MC38 colon cancer cells but had little effect on the growth of B16F10 melanoma cells. However, tumor growth rates were much higher in tumors induced by B16F10 melanoma than MC38 colon cancer cells. The mean volumes of B16F10 melanoma in the control group on days 0 and 10 were 38.82 and 1700.62 mm^3, respectively, whereas the mean volumes of MC38 colon cancers in the control group on days 0 and 10 were 29.53 mm^3 and 384.91 mm^3, respectively. Consequently, the mean amount of CAP exposure per tumor volume was much higher in the MC38 colon cancer model. These results demonstrate the gap between *in vitro* and *in vivo* studies, reinforcing the requirement for more *in vivo* experiments before

treatment of patients with CAP. Sensitivity to CAP at the cellular level does not necessarily indicate good responses to CAP *in vivo* or in clinical trials. Other factors, such as tumor volume and growth rate, should be considered. Increasing the duration of exposure to CAP from 5 min to 15 min had little effect on the B16F10 melanoma model, suggesting that the ability of CAP to inhibit tumor growth and the propensity toward tumor growth had reached equilibrium. Our findings suggest that a combination of direct and indirect exposure to CAP delivery should be considered when treating large volume tumors, as the depth of effective tissue penetration of direct CAP can be limited.(42, 43) In addition, a selective anti-cancer effect of direct CAP treatment was demonstrated histologically in the B16F10 melanoma model, as exposure to CAP for 15 min had little effect on adjacent normal tissue, indicating that CAP treatment was relatively safe and that physical factors, such as thermal effect, radiation, and electromagnetic fields, played minor roles in interactions between CAP and tissues. The effect of physical factors on the *in vivo* application of CAP is largely unknown, although increased temperature of CAP-treated tumor tissue was found to have a negligible effect,(44) consistent with our results. In the MC38 colon cancer model, however, tumors were located too superficially to assess the selectivity of CAP treatment.

The limitations of this study include the lack of full elucidation of the mechanisms underlying the anti-cancer activity of direct CAP treatment and EGFR inhibitor. Another limitation is that the anti-cancer effect of the combination of CAP treatment and EGFR inhibitor was not demonstrated *in vivo*.

In conclusion, this study demonstrated that direct CAP treatment had selective anti-cancer activity, both *in vitro* and *in vivo*. Direct application of CAP *in vivo* effectively inhibited B16F10 tumor growth by 50% and inhibited MC38 tumor growth by more than 50%, suggesting that CAP effectively inhibited the growth of these kinds of cancers. Direct CAP treatment has a potential to be used as adjuvant or neo-adjuvant therapy to reduce tumor burden after or before primary resection, as well as for palliative purposes. In addition, EGFR inhibitor combined with CAP is a candidate modality for a subset of melanomas.

Figure 1. Anti-cancer effect of cold atmospheric plasma on (A) B16F10 murine melanoma cells, (B) A375 human melanoma cells, (C) A2058 human melanoma cells, and (D) MC38 murine colon cancer cells. The viability of each cancer cell line was measured 24 h after treatment with cold atmospheric plasma for the indicated times. Exposure to cold atmospheric plasma effectively decreased cancer cell viability in a treatment duration-dependent manner. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control, untreated cells.

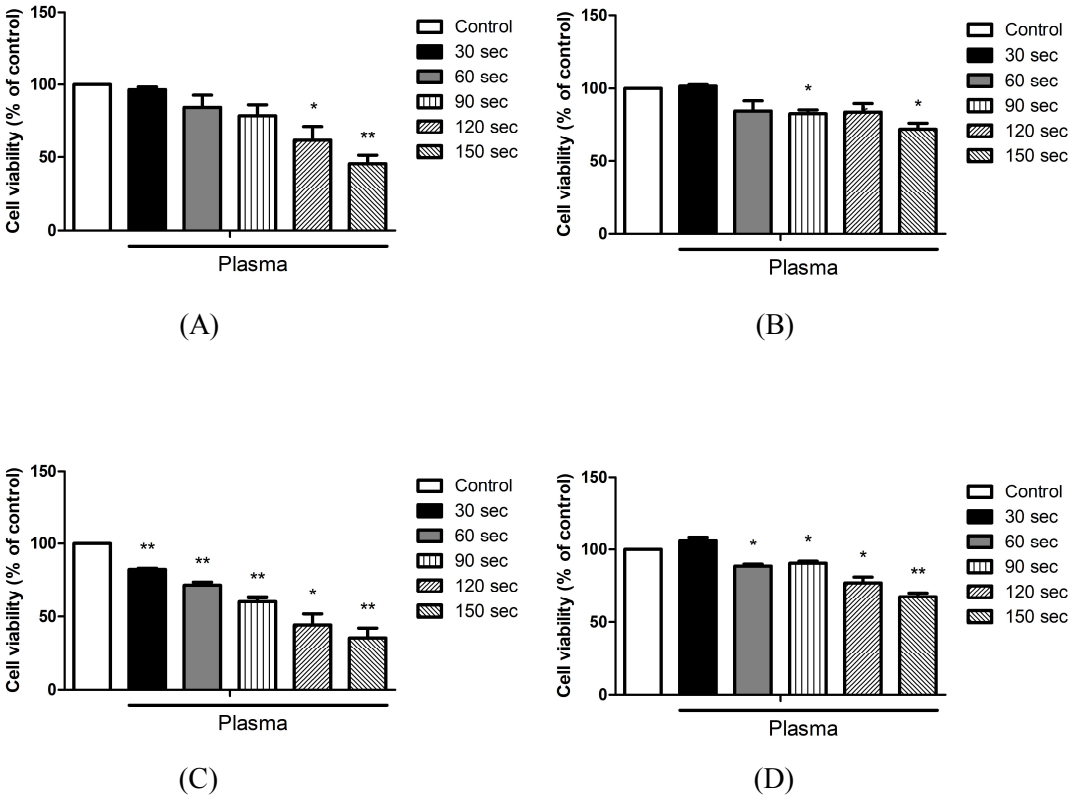
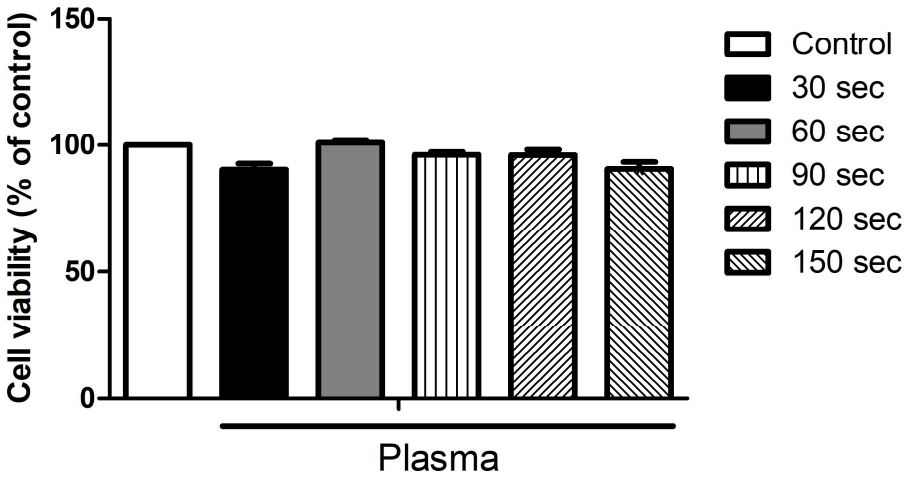


Figure 2. Minimal effect of cold atmospheric plasma on normal human melanocytes, indicating selective anti-cancer activity of cold atmospheric plasma. Viability of normal human melanocytes was measured 24 h after cold atmospheric plasma treatment for indicated times (lower panel, original magnification: ×100).



Control Plasma 30 sec Plasma 60 sec

Plasma 90 sec Plasma 120 sec Plasma 150 sec

Figure 3. Western blot analysis of the levels of expression of Bcl-2, cleaved caspase 3, heme oxygenase-1 (HO-1), Nrf2, p53, and β-actin 24 h after treatment of B16F10 and MC38 cells with cold atmospheric plasma for the indicated times. Levels of protein expression were quantified by densitometry after normalization to the optical density of β-actin. Cold atmospheric plasma treatment upregulated the expression levels of cleaved caspase 3 and HO-1 in both B16F10 and MC38 cancer cells.

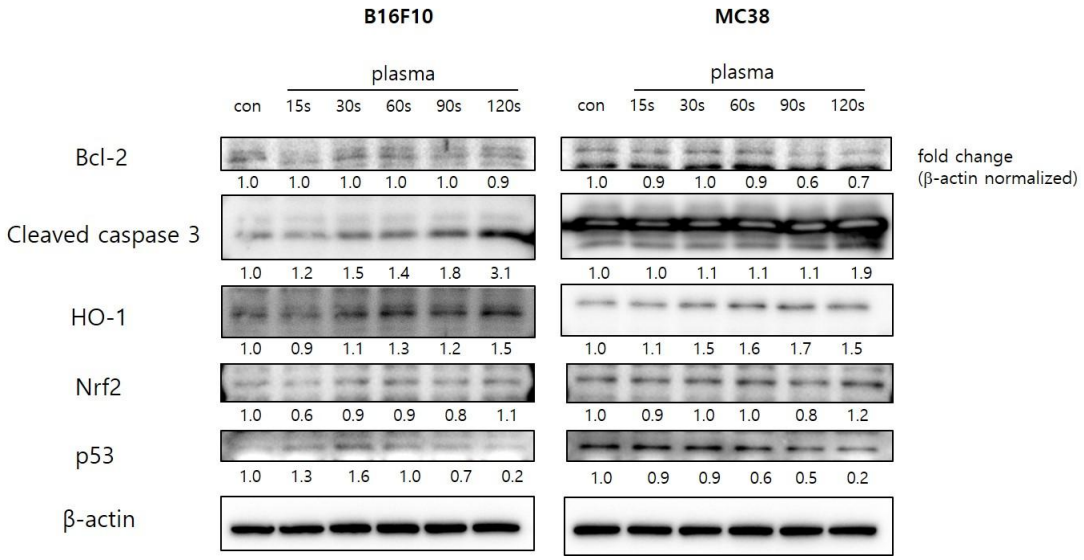
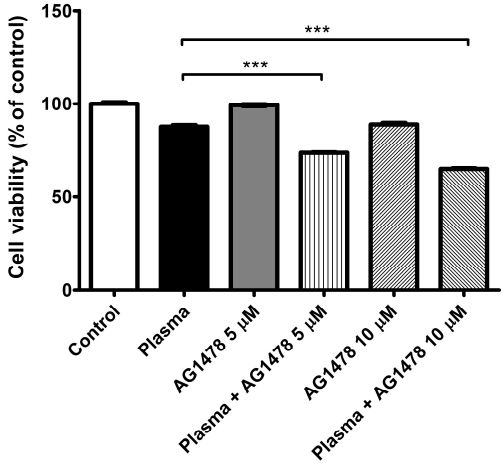
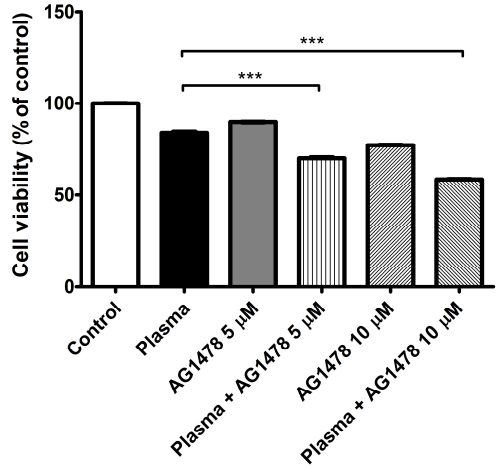


Figure 4. Anti-melanoma effect of the selective inhibitor of epidermal growth factor receptor inhibitor AG1478, alone or combined with cold atmospheric plasma. (A) B16F10 and (B) A375 cells were pretreated with AG1478 at indicated concentrations 1 h before cold atmospheric plasma treatment. Cells were treated twice with cold atmospheric plasma for 120 sec each at a 24 h interval, and cell viability was assessed 24 h after the second treatment. The combination of cold atmospheric plasma and epidermal growth factor receptor inhibitor significantly reduced the viability of both melanoma cell lines compared with cold atmospheric plasma treatment alone. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



(A)



(B)

Figure 5. Cellular effects of epidermal growth factor receptor (EGFR) inhibitor through the downregulation of the EGFR pathway in A375 human melanoma cells. A375 cells were pretreated with AG1478 (2 μm), a selective inhibitor of EGFR, 1 h before administration of epidermal growth factor (EGF) at concentrations from 0 to 50 ng/ml. Western blot analysis at 24 h after EGF treatment revealed that AG1478 effectively reduced EGF-induced phosphorylation of EGFR (P-EGFR), AKT (P-AKT), and ERK (P-ERK), but not total EGFR (T-EGFR), AKT (T-AKT), and ERK (T-ERK). β-actin (Actin) was used as an internal loading control.

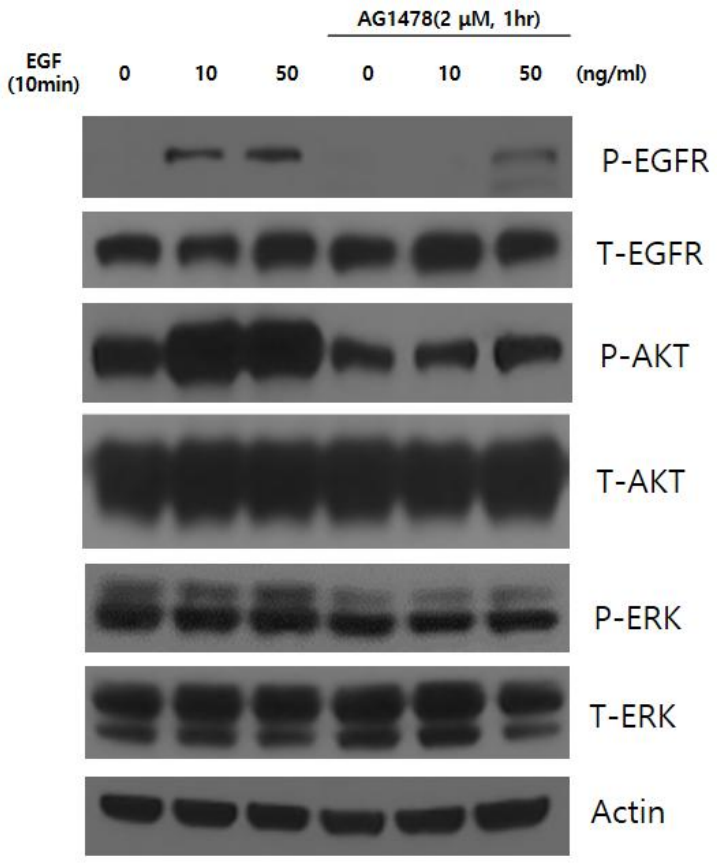
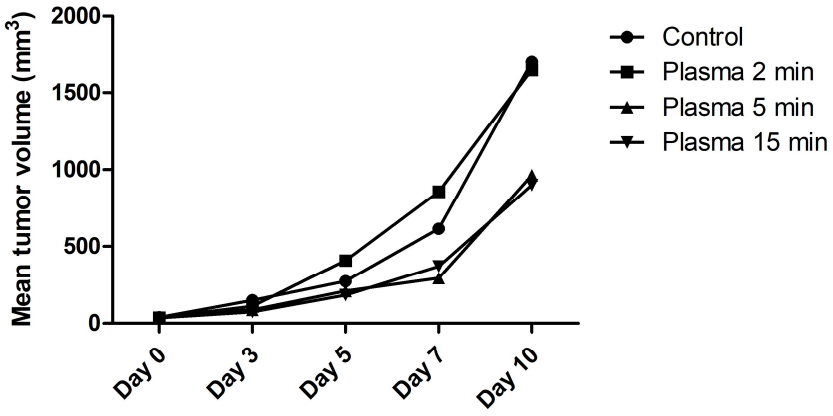
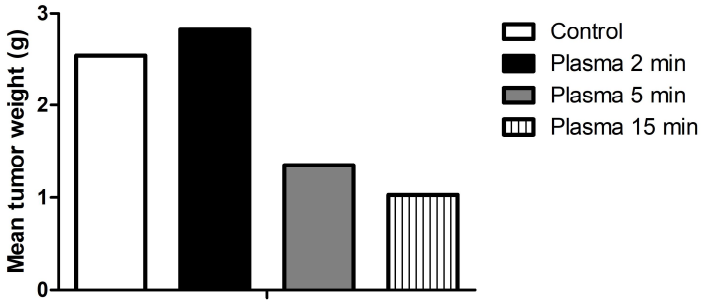


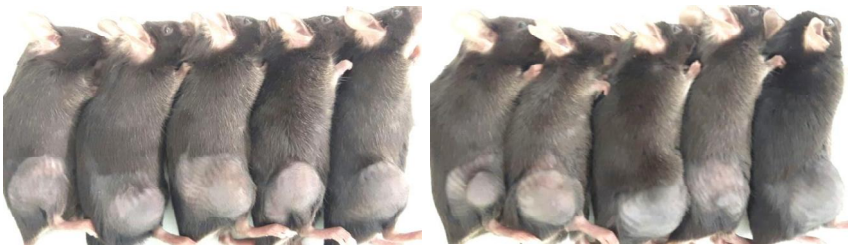
Figure 6. Anti-cancer effect of cold atmospheric plasma (CAP) in a syngeneic mouse model with B16F10 melanoma cells. After the growth of tumors, five mice each were treated five times, once every other day, with CAP for 2 min, 5 min, or 15 min (i.e., on days 0, 2, 4, 6, and 8), whereas five untreated mice served as controls. (A) Tumor volumes measured on days 0, 3, 5, 7, and 10. (B) Tumor weights measured on day 10. (C) Photographs on day 10 of control mice (left) and mice treated with CAP for 5 min (right).



(A)

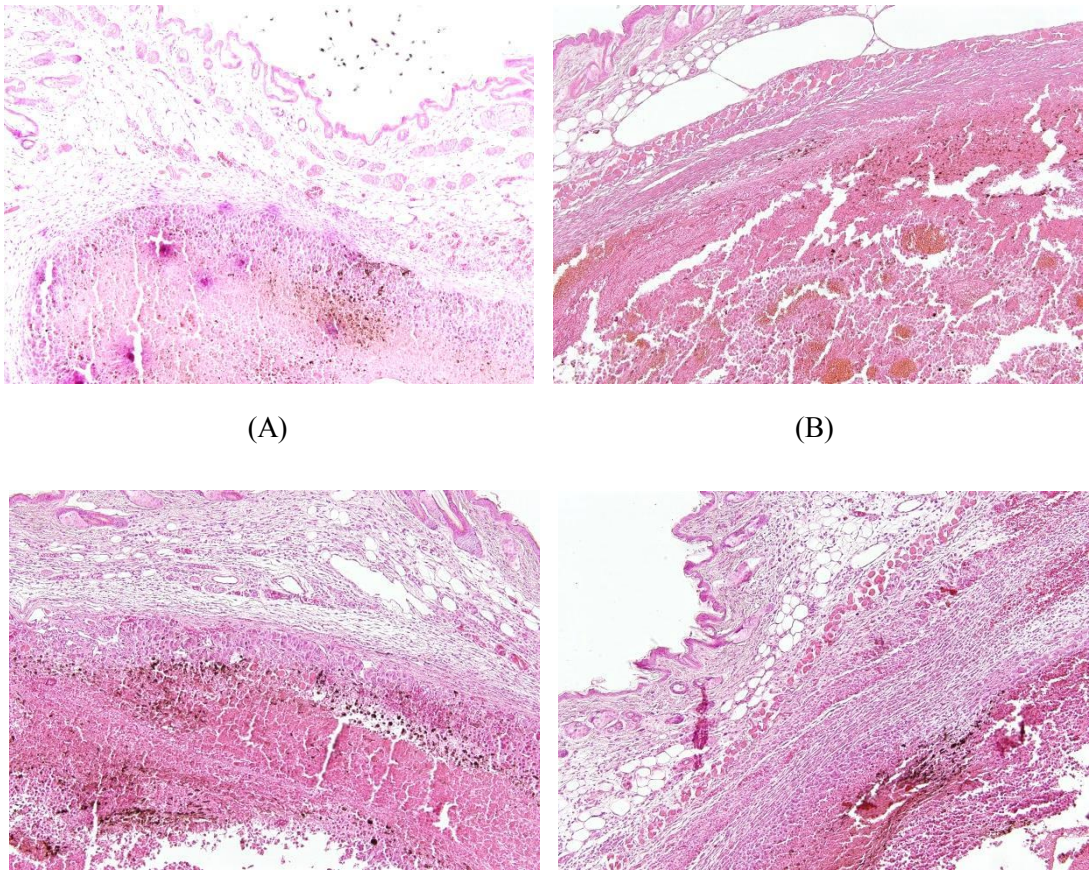


(B)



(C)

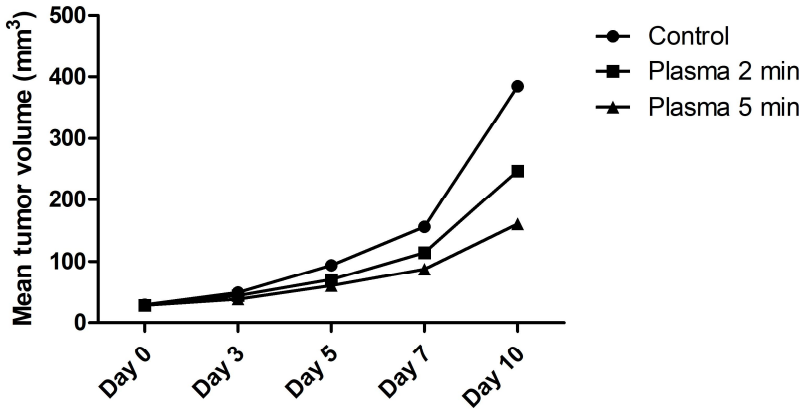
Figure 7. Representative histological images of B16F10 melanomas on day 10 from a control mouse (A) and from mice treated with cold atmospheric plasma for 2 min (B), 5 min (C), and 15 min (D). Original magnification: ×100.



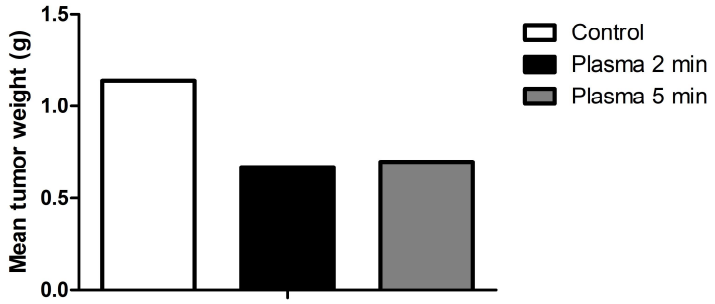
(A) (B)

(C) (D)

Figure 8. Anti-cancer effect of cold atmospheric plasma (CAP) in a syngeneic mouse model with MC38 colon cancer cells. After the growth of tumors, six mice each were treated five times, once every other day, with CAP for 2 min or 5 min (i.e., on days 0, 2, 4, 6, and 8), whereas six untreated mice served as controls. (A) Tumor volumes measured on days 0, 3, 5, 7, and 10. (B) Tumor weights measured on day 10. (C) Photographs on day 10 of control mice (left) and mice treated with CAP for 5 min (right).



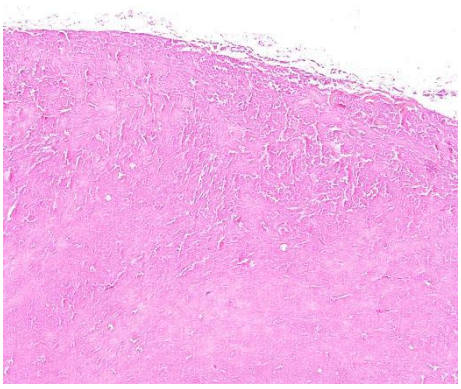
(A)



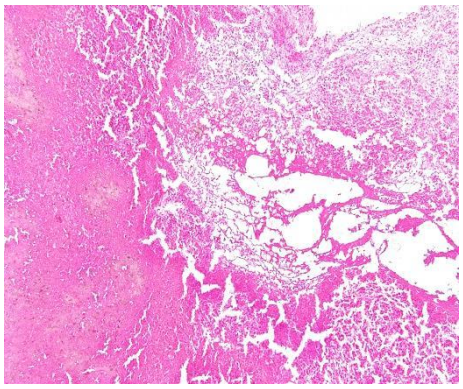
(B)

(C)

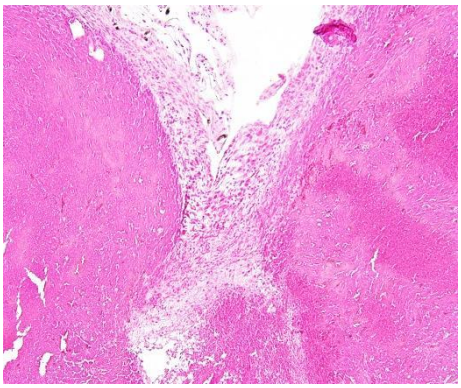
Figure 9. Representative histological images of MC38 colon tumors on day 10 from a control mouse (A) and from mice treated with cold atmospheric plasma for 2 min (B) and 5 min (C). Original magnification: ×100.



(A)



(B)



(C)

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ABSTRACT

Background and Objectives: Current medical applications of cold atmospheric plasma (CAP) include skin decontamination, tooth bleaching, wound healing, and cancer therapy. The ability of CAP to produce reactive oxygen species, which damages cellular DNA and induces apoptosis, suggests that CAP may have oncological applications in various tumor types. The present study assessed the anti-cancer effects of CAP in syngeneic mouse models.

Methods: The effects of CAP treatment on the viability of normal human melanocytes, melanoma cells, and colon cancer cells were determined *in vitro*. Syngeneic tumors were induced in mice by injecting 5×10^5 B16F10 melanoma cells into 20 individual mice, or 5×10^5 MC38 colon cancer cells into 18 individual mice. After the tumors had grown, the mice were treated with five doses of CAP every other day. Tumor volume was measured at each time point. On day 10, tumor weight was measured, tumor growth inhibition (TGI) was calculated, and all tumors were excised and evaluated histologically.

Results: CAP treatment significantly decreased the *in vitro* viability of both melanoma and colon cancer cell lines, while having a negligible effect on the viability of normal human melanocytes. *In vivo*, CAP treatment reduced tumor volume and tumor weight in both cancer models, with the extent of tumor reduction being dependent on the duration and number of CAP treatments. In the B16F10 melanoma model, TGIs on day 10 of mice treated with CAP for 2 min, 5 min, and 15 min were 3%, 43.4%, and 47.0%, respectively. In the MC38 colon cancer model, TGIs on day 10 of mice treated with CAP for 2 min and 5 min were 35.8% and 58.3%, respectively. Histologic examination showed the tumoricidal effects of CAP in both tumor models.

Conclusion: CAP inhibits the growth of mouse melanoma and colon cancer cell lines *in vitro*, as well as showing tumoricidal effects against mouse models of melanoma and colon cancer *in vivo*.

Keywords: Cold atmospheric plasma, Anti-cancer effect, Melanoma, Colon cancer, Murine syngeneic cancer model