Plasma treatment induces apoptosis in U87 cells *in vitro* and *in vivo*

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**Background**

- Non thermal plasma (a ionized gas) is emerging as a novel tool for *in vivo* treatment of living tissues for biological and medical purposes. Plasma is considered as the fourth state of matter and it is obtained by a supply of energy. The excited gas contains free charges (electrons, ions), free radicals, excited molecules, energized photons (UV) and generates transient electric fields.

- Numerous studies have demonstrated the efficacy of sterilization by plasma application and, more recently, several studies have shown an antitumor effect of plasma in *in vitro* on various cell lines by apoptosis induction.1

- The purpose of this study is to develop an endoscopic plasma treatment (plasma Gun®) for *in situ* treatment for colorectal cancer. As a first step, we need to evaluate non-thermal plasma effects on the potential new antitumor treatment. To this end, we used a friendly using and compact plasma dielectric barrier discharge (DBD) to assess the antitumor effect of plasma in vitro and in vivo using an heterotopic xenograft model. We used U87 malignant glioma xenograft, this cell line being characterized by a high mitotic and chemoresistance and being an adequate model to explore a potential antitumor effect. The aim of this work was also to better understand the mechanisms involved in the antitumor effect of plasma treatment observed on U87 glioma cells in vitro and in vivo.

**Methods**

- **U87-Luc cells were seeded in 24-well plates at 5,103 cells/ml.** Treatment of cells with plasma performed at 2 mm above 500 µl of media.

- **Plasma induced apoptosis** was detected with Caspase-Glo® 3/7 reagent (Promega Corporation). Caspase-3/7 Substrates is a family of kinase substrate protected by the caspase-3 and 7 peptide sequence. After activation of caspase-3 or -7, the DEVD-peptide is cleaved to release luciferase.

- **Cell viability** was determined by MTT and bioluminescence imaging (BLI) with IVE Luminex system.

- **ROS such as H₂O₂, ONOO⁻ and ONO⁻ were detected** by means of an oxidant-sensitive fluorescent probe, DHE is highly selective for O²⁻ ([2′,3′ disobutyl-2-hydroxybenzothiazole; E/λ = 515/525 nm]).

- **Luminescence** of U87 gliomas were xenografted heterotopically onto nude mice. Treatments began when tumors reached 100 mm³. Plasma was applied for 6 minutes during 5 consecutive days in open air with a 1 µS/cm plasma density per second. The treatment was performed 10 min after the last injection. **Bioluminescence imaging** was performed before, during and after plasma treatment completion.

- To follow tumor growth, tumor volume was determined every day with a caliper. Mice were anesthetized and killed by cervical dislocation when tumor reached 1000 mm³, thus defining the "survival time".

**Results**

- **Figure 1:** Effect of non thermal plasma treatment on U87 cells in vitro

  - **Figure 2:** Plasma induced cell death is linked to ROS formation

  - **Figure 3:** Effects of plasma treatment in vivo

  - **Figure 4:** Plasma treatment presents a significant antitumor effect *in vivo*

- **In vitro**
  - U87-Luc cells were seeded in 24-well plates at 5,103 cells/ml. Treatment of cells with plasma performed at 2 mm above 500 µl of media.
  - Plasma induced apoptosis was detected with Caspase-Glo® 3/7 reagent (Promega Corporation). Caspase-3/7 Substrates is a family of kinase substrate protected by the caspase-3 and 7 peptide sequence. After activation of caspase-3 or -7, the DEVD-peptide is cleaved to release luciferase.
  - Cell viability was determined by MTT and bioluminescence imaging (BLI) with IVE Luminex system.
  - ROS such as H₂O₂, ONOO⁻ and ONO⁻ were detected by means of an oxidant-sensitive fluorescent probe, DHE is highly selective for O²⁻ ([2′,3′ disobutyl-2-hydroxybenzothiazole; E/λ = 515/525 nm]).

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  - To follow tumor growth, tumor volume was determined every day with a caliper. Mice were anesthetized and killed by cervical dislocation when tumor reached 1000 mm³, thus defining the "survival time".

- **In vivo**
  - **Figure 3:** Effects of plasma treatment in vivo

- **Discussion**

  - In the present work, we investigated the potential antitumor properties of non thermal plasma treatment in human malignant glioma xenografted onto nude mice. Our findings indicate that plasma presents a significant antitumor activity *in vitro* and *in vivo*. Our *in vitro* results are in accordance with previously published data and confirmed the plasma effect on cell death (2).

  - In this study, a plasma dose-response was observed and 20% of U87 cells was killed. Using this model, we have shown that a major antitumor effect was observed in an increase cell death determined by BLI after treatment. As demonstrated with direct and indirect treatment, different components of plasma have no macroscopic conclusion with antitumor effect (i.e. electric field, UV...). Cell death induction was essentially linked to the cell culture medium. Active species produced by plasma may modify the cell membrane, which in turn interacts with cells but plasma treatment in PBS and in media induced the same antitumor effect, instead that cell medium was not modified. This antitumor effect was completely lost when a ROS scavenger (N-Acetyl-L-cysteine) is used. These results showed the major importance of ROS in plasma induced apoptosis. As demonstrated here, non-thermal plasma produced a large ROS concentration in extracellular medium during treatment and concentration of ROS in media proportionally increased with treatment time.

  - Several studies underlined the good tolerance of plasma application on living tissue and we have previously shown the good tolerance of our treatment on mouse skin. In this study, our plasma treatment induced a significant reduction of tumor volume (-40%) at the end of the treatment period, as compared to non-treated mice. This effect is confirmed by BLI, which showed a stabilization of tumor activity during treatment course (D2-D5). This tumor volume reduction could be linked to changes in the cell cycle distribution after plasma treatment. Indeed, after treatment a decrease of G0/G1 phase and an increase of cells in S phase. Accumulation of tumor cells in the S phase caused by plasma may be explained by single DNA strand breaks or double strand breaks (dsb) formation after plasma treatment. Histological studies are ongoing to evidence apoptosis and DNA-dsb after plasma treatment. In vivo.

  - Several reports have demonstrated the high resistance of U87 bearing mice to the cytotoxic effect of radiation therapy. For example, a treatment schedule of 2 Gy per day during ten weeks has been reported to produce an increase in lifespan about 50% in U87 bearing mice. In our study, plasma showed an increase of 60% of mice lifespan that reflects a high cytotoxic effect of plasma treatment. All plasma parameters (i.e. species, temperature, electric field...) could be toxic to cells, inducing membrane damages and changes in intracellular signaling pathways, finally leading to cell death. As demonstrated in vivo, ROS have a major importance but other plasma components could not be neglected during *in vivo* plasma application.

- **Conclusion**

  - Our study demonstrates a marked antitumor effect of plasma treatment in U87 plasma in situ and in vivo with a significant decrease of tumor volume at the end of treatment and an increase of mouse lifespan of 60%, thus confirming our previous data. These results, obtained in a radiotransfected model, are very promising and highlight the potential of plasma treatment as an antitumor treatment with little or no interferences with side effects. The antitumor effect in vitro is linked to ROS formation and the interaction of ROS with tumor cells. Studies to elucidate mechanisms of ROS induced apoptosis after plasma are ongoing. This antitumor effect induced a tumor volume stabilization with an accumulation of cells in S phase.

  - These encouraging results lead us to develop a plasma for endoscopic application, especially for colorectal cancer treatment.