Chapter 6
Plasma-Activated Solution in Cancer Treatment

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Contents

Abstract Cold plasma-activated solution (PAS), particularly the cold plasma-activated medium (PAM), is a type of chemotherapy used in the cold plasma-based cancer treatment. Compared with the direct cold plasma treatment, PAM can be stored for a long time and can then be used without dependence on cold plasma sources or devices. Many in vitro and in vivo experiments have demonstrated anti-tumor effects of PAM against a variety of cancer cells. PAM contains a variety of reactive oxygen species (ROS), reactive nitrogen species (RNS), other cold plasma-activated species, and the generated compounds. These species either interact with

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M. Keidar (ed.), *Plasma Cancer Therapy*, Springer Series on Atomic, Optical, and Plasma Physics 115, https://doi.org/10.1007/978-3-030-49966-2_6

the cellular membrane or the intracellular function to activate signal pathways and the expression of many genes. Several important signaling pathways are affected by PAM. The effectiveness of PAM has been demonstrated in animal studies using peritoneal metastasis model mice.

6.1 Cold Plasma-Activated Medium in Current Plasma Cancer Treatment

Cold plasma has been studied as a next-generation cancer therapy [1–3]. Although cancer cells in vitro or tumorous tissues in vivo can be treated directly with cold plasma, the indirect cold plasma treatments have also been developed over the past years [4–6]. The indirect cold plasma treatments include the cold plasma-assisted immunotherapy [7] and the cold plasma-activated solutions (PAS) [8]. PAS includes medium, phosphate-buffered saline (PBS), or Ringer's solutions, which are used to inhibit the growth of tumorous tissue by subcutaneous or intraperitoneal injection in vivo. To date, the CAP-activated medium (PAM) is the most extensively investigated PAS. PAM can be made by treating the medium using the CAP jet or DBD just above its surface (Fig. 6.1) [8–14]. Moreover, PAM can also be made by the direct discharge in the medium [15]. A number of studies have reported that PAM is suitable for the use in treating various cancers, not only causing the apoptosis but also suppressing the migration and invasion abilities of cancer cells [3, 11, 16–27]. The anti-tumor capacity of PAM over the colon adenocarcinoma multicellular tumor spheroids (MCTS) has also been demonstrated in vitro [28]. Noticeable DBS occurred at the peripheral part of the PAM-treated MCTS [28].

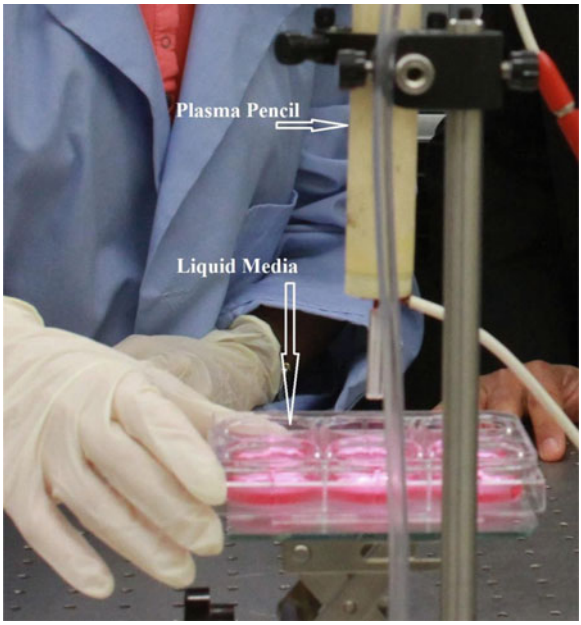


Fig. 6.1 Biological liquid medium being exposed to the plume of a plasma jet to make PAM

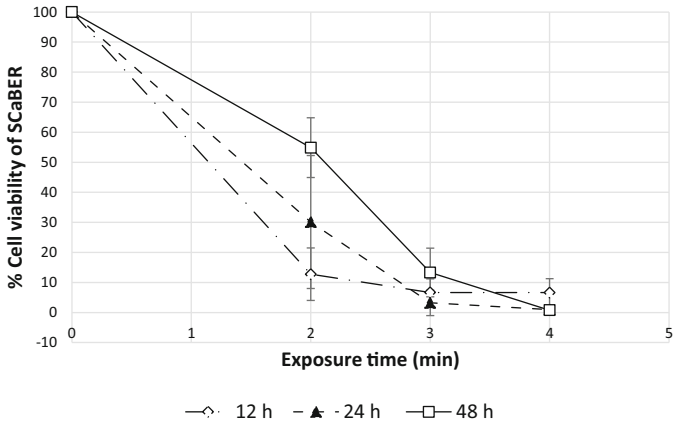


Fig. 6.2 The anti-tumor effect of PAM on SCaBER cells. PAM was produced by exposing 1 mL of MEM growth media (containing 10% calve bovine serum, 1% penicillin-streptomycin-glutamine) to the plasma plume of the plasma pencil, a pulsed plasma jet. The produced PAM was transferred to wells with SCaBER cells, and the suspension was incubated at 37 °C and 5% CO_2. Exposure times were 2, 3.5, and 5 min. Cell viability was assessed at three PAM application times, 12, 24, and 48 h. Experiments were conducted in triplicate and cell viability was normalized to the control and expressed in percent. (Reproduced from Mohades et al. (2016) [29])

As an illustrative example of the effects of PAM on cancer cells, we present in the following section an investigation on the killing of bladder squamous cell carcinoma cells (SCaBER) by PAM (Fig. 6.2). As can be seen in the figure, the PAM created by longer exposure times to cold plasma shows higher kill rates. Cell viability evaluated at all the PAM application times have similar killing trends with the highest killing efficiency (more than 90%) achieved by PAM created by a 5 min of plasma exposure.

Hiromasa Tanaka et al. first demonstrated the selective anti-tumor capacity of PAM over glioblastoma (U251SP) cells [8]. PAM can selectively kill glioblastoma cells rather than normal astrocytes (ACBRI-371) [8]. After that, many studies have validated a similar trend (Fig. 6.3). PAM selectively kills colon cancer cells, lung cancer cells, cervical cancer cells, melanoma cells, breast cancer cells, bladder cancer cells, liver cancer cells, and osteosarcoma cells [12, 13, 15, 29–34]. In addition, the selective anti-tumor effect of PAM has also been observed in a co-culture system with the normal cell line L02 and liver cancer cell line HepG2 [34]. A similar selective anti-tumor effect is also found in a co-culture system with ovarian cancer cell line ES2 and human peritoneal mesothelial cells (HPMCs) [27].

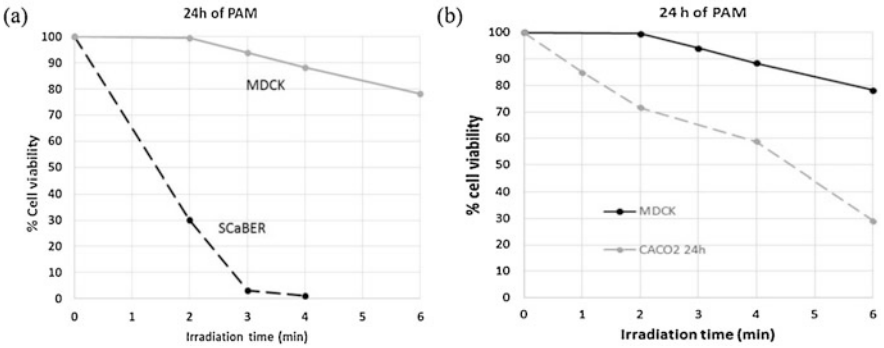


Fig. 6.3 The selective anti-tumor effect of PAM 24 h after the treatment. (**a**) SCaBER (human colorectal adenocarcinoma) cells and non-cancerous MDCK (Madin–Darby Canine Kidney) cells. (**b**) Caco-2 (human colorectal adenocarcinoma) cells and non-cancerous MDCK (Madin–Darby Canine Kidney) cells. Irradiation time refers to the time the media was exposed to plasma to make PAM. It was found that there is an optimum dose of PAM to suppress the viability of cancer cells significantly while inducing minimum damage to non-malignant cells**.** (Reproduced from Laroussi et al. (2019) [30])

6.2 Reactive Species in PAM

The exposure of liquid media to plasma results in diffusion and solvation of ROS and RNS into the treated liquid. The concentration of reactive species produced in the liquid medium is a function of the duration of plasma exposure, gas type and flow rate, and the chemical composition of the liquid [19]. However, many of the reactive species generated by cold plasma in the gas phase (nitric oxide (NO), ozone (O_3), hydroxyl radicals (OH.), singlet oxygen ($^1O_2$), and superoxide anion (O_2^-), etc.) may be short-lived and do not diffuse into the bulk of the liquid [35, 36]. Fortunately, some of the solvated species produce more stable molecules such as hydrogen peroxide, H_2O_2, and peroxynitrite, $ONOO^-$. H_2O_2 is stable and can diffuse into liquid media relatively efficiently (Fig. 6.4).

The anti-tumor effect of PAM is mainly due to the chemical factors, which includes the change of pH, the cold plasma-generated species, and the components generated during the interaction between cold plasma and other components formed in solution. Due to the buffering components in PAM, the CAP treatment only results in a negligible change in pH [37, 38]. ROS and RNS play key roles in the cellular function of PAM. For example, NO in PAM may contribute to its cytotoxicity. The dissolution of NO in PAM significantly increases the cytotoxicity of PAS over HeLa cells [39]. NO_2^- alone will not cause observable cytotoxicity to cancer cells [14].

Among diverse cold plasma-generated species, H_2O_2 plays an important role in PAM (Fig. 6.5) [6, 11, 19, 28, 29, 31, 40–42]. H_2O_2 alone can cause noticeable inhibition and cell death on cancer cells, though it cannot cause the same cytotoxicity as PAM [6]. Even synergistically using H_2O_2/NO_2^- or $H_2O_2/NO_2^-/NO_3^-$

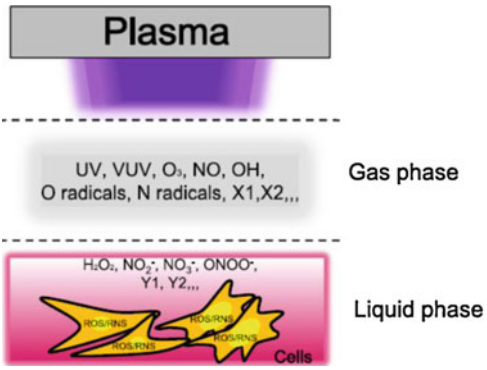


Fig. 6.4 Cold plasma-generated ROS and RNS in the gas phase, liquid phase, and inside cells. The cold plasma interacts with oxygen, nitrogen, and humidity in the air to produce oxygen, nitrogen, and hydroxyl radicals. Various long-lived reactive species are further formed due to the secondary reaction in the liquid phase, such as hydrogen peroxide, nitrites, and nitrates. These species, in turn, will either directly or indirectly trigger the generation of the intracellular ROS or RNS. (Reproduced from Tanaka et al. (2014) [20])

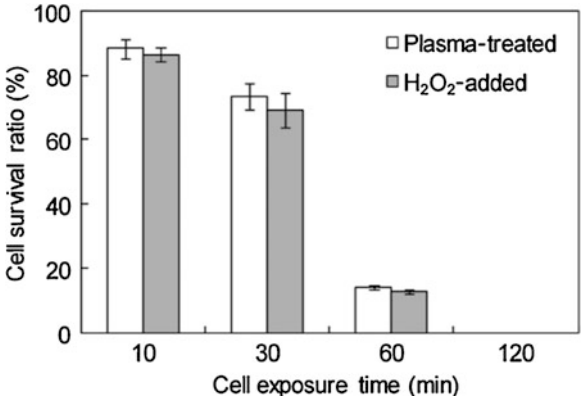


Fig. 6.5 The earliest evidence that the H_2O_2 plays important role in the anti-tumor effect of PAM. Cell survival ratios against the exposure time to cold plasma-treated and H_2O_2-added culture media. The survival ratios are normalized by the absorbance intensity of a control which was exposed to the regular culture medium. (Reproduced from Sato et al. (2011) [11])

in the medium also cannot achieve similar selective cytotoxicity as PAM does [12, 43]. Although H_2O_2 is cytotoxic, the cytotoxicity depends on its concentration. The concentration of H_2O_2 in PAM is typically below the threshold required for synergy between H_2O_2 and NO_2^- that produces a cytotoxic effect (Fig. 6.6).

In addition to reactive species, the cold plasma-activated organic compounds exhibit important anti-tumor effects. As an example, we introduced the study on the cold plasma-activated ringer's lactate solution (PAL) here. PAL exhibits anti-tumor effects on glioblastoma cells [44]. Ringer's lactate solution contains NaCl, KCl, CaCl2, and L-sodium lactate, which functions as an anti-tumor factor upon cold plasma treatment (Fig. 6.7).

Similarly, the cold plasma-treated fetal bovine serum (FBS) has also shown cytotoxicity over cervical cancer HeLa cells and hamster cells CHO-K1 [13]. The

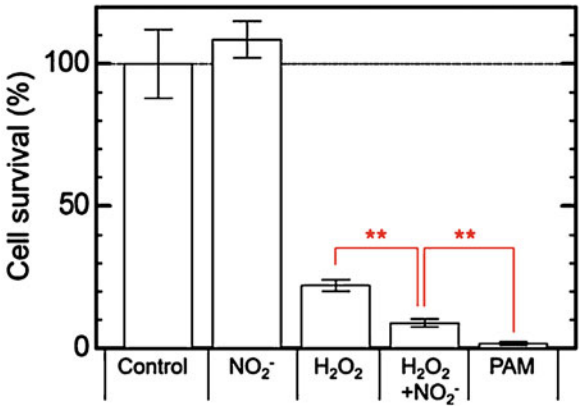


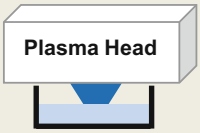
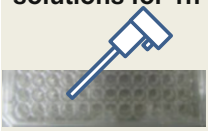
Fig. 6.6 The synergistic anti-tumor effects of H_2O_2 and NO_2^- on glioblastoma cells. For the cold plasma-treated medium (180 s), the PAM contained 63 μM H_2O_2 and 1890 μM NO_2^-. At a concentration of 63 μM, H_2O_2 alone exhibited only weak anti-tumor effect, whereas NO_2^- alone (1890 μM) did not exhibit anti-tumor effects. However, the combination of 63 μM H_2O_2 and 1890 μM NO_2^- exhibited an anti-tumor effect almost equivalent to that caused by PAM (Reproduced from Kurake et al. (2016) [43])

potential chemical modification on the contents of FBS is thought to be the main reason for such cytotoxicity [13]. However, due to the extremely long cold plasma treatment time length, such as 10 min in that study, the thermal deactivation of the proteins in FBS may also happen.

In addition to chemical factors, the carrier of the reactive species, such as medium or PBS, is another factor to affect the anti-tumor efficacy of PAM. Specific cancer cells may be vulnerable to one PAS such as PAM than another PAS such as the cold plasma-activated PBS (Fig. 6.8). The glioblastoma cells U87MG and PA-TU-8988T cells were specifically vulnerable to the cold plasma-activated PBS and PSM, respectively [14]. The specific rise of intracellular ROS in U87MG cells and PA-TU-8988T cells may cause the different vulnerabilities of cancer cells to PAM and the cold plasma-activated PBS [14].

The specific cellular response in the nutrient-starved environment and the nutrient-rich environment may cause these cancer cells to show quite different responses to the extracellular reactive species such as H_2O_2 [14]. The cytotoxicity of reactive species on cancer cells disappears when the PAM was made of simple buffered solutions such as PBS [44, 45]. The dilution of PBS in DMEM drastically weakens the anti-tumor effect of PAM on pancreatic adenocarcinoma PA-TU-8988T cells and breast cancer MDA-MB-231 cells [14]. Similarly, compared with PAM, PAL induced the generation of a lower amount of intracellular ROS (Fig. 6.9). Compared with medium, the ringer's lactate solution contains much less nutrient for cell growth. These results suggest that both PAM and PAL exert intracellular ROS-based anti-tumor effects against glioblastoma cells, albeit by different molecular mechanisms.

(a)

	Plasma-treated 2X solutions	Complementary 2X solutions	Treat cells with mixed 1X solutions for 1h
			
①	N/A	NaCl, KCl, $CaCl_2$, L-sodium lactate	Untreated Lactec
②	NaCl	KCl, $CaCl_2$, L-sodium lactate	NaCl-GOF
③	KCl	NaCl, $CaCl_2$, L-sodium lactate	KCl-GOF
④	$CaCl_2$	NaCl, KCl, L-sodium lactate	CaCl2-GOF
⑤	L-sodium lactate	NaCl, KCl, $CaCl_2$	L-sodium lactate-GOF
⑥	KCl, $CaCl_2$, L-sodium lactate	NaCl	NaCl-LOF
⑦	NaCl, $CaCl_2$, L-sodium lactate	KCl	KCl-LOF
⑧	NaCl, KCl, L-sodium lactate	$CaCl_2$	CaCl2-LOF
⑨	NaCl, KCl, $CaCl_2$	L-sodium lactate	L-sodium lactate-LOF
⑩	NaCl, KCl, $CaCl_2$, L-sodium lactate	Milli-Q	Plasma-treated Lactec
⑪	Milli-Q	NaCl, KCl, $CaCl_2$, L-sodium lactate	Treated water

(b)

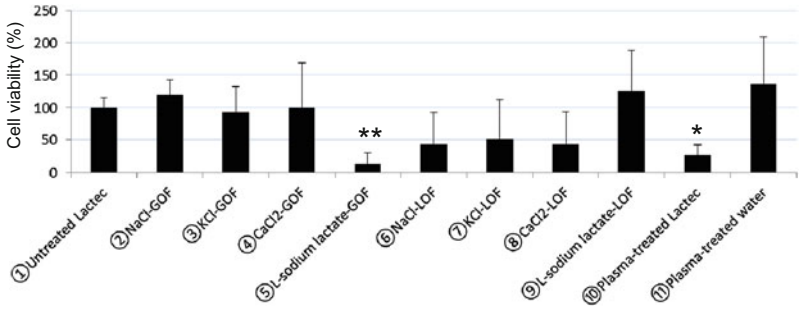


Fig. 6.7 Identification of PAL components exhibiting anti-tumor effects. (**a**) Schematic of experiments for identifying the anti-tumor components in Ringer's lactate solution by cold plasma irradiation. Each doubly concentrated NaCl, KCl, $CaCl_2$, and L-sodium lactate solution were treated with cold plasma for 2 min and then mixed with the complementary doubly concentrated solutions. These solutions are referred to as NaCl-LOF, KCl-LOF, CaCl2-LOF, and L-sodium lactate-LOF (6–9). Doubly concentrated Ringer's lactate solution was treated with plasma and mixed with the same volume of Milli-Q water (10), and vice versa (11). (**b**) 10,000 U251SP cells were seeded in 200 μL medium in a 96-well plate. On the following day, the medium of the cells in the 96-well plate was replaced with 200 μL of the solutions described in (**a**). (Reproduced from Tanaka et al. (2016) [44])

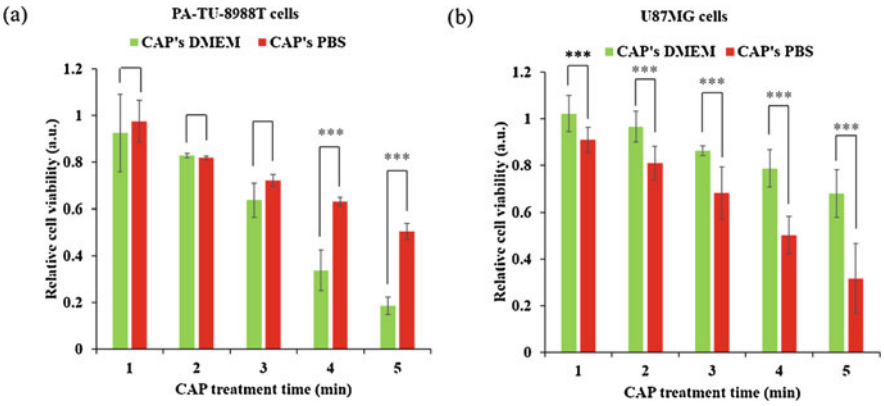


Fig. 6.8 The different anti-tumor effect of the cold plasma-activated DMEM (CAP's DMEM) and the cold plasma-activated PBS (CAP's PBS) on two cancer cell lines. (**a**) PA-TU-8988T cells. (**b**) U87MG cells. Results are presented as the mean ± s.d. of two independently repeated experiments performed in sextuplicate. Student's *t*-test was performed and the significance is indicated as $***p < 0.005$, $**p < 0.01$, and $*p < 0.05$. (Reproduced from Yan et al. (2017) [14])

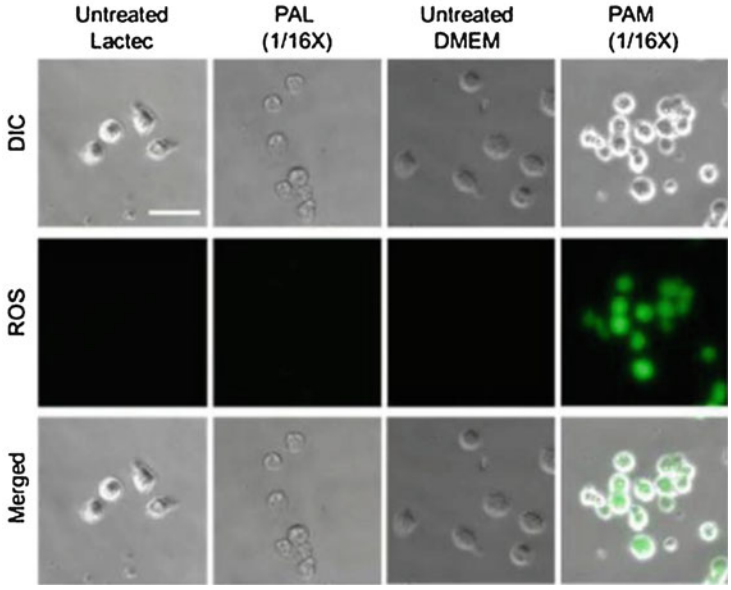


Fig. 6.9 Identification of components in PAL exhibiting anti-tumor effects. Ten thousand U251SP cells were seeded in an 8-well chamber slide. On the following day, the medium of the cells was replaced with 200 μL of CM-H2DCFDA (10 μM) in PBS. Lactate (8 mL) in a 60 mm dish was treated with plasma (L = 3 mm, 2.0 slm), and the PAL was diluted 16 times with lactate. After 1 h, the 200 μL CMH2DCFDA (10 μM) in PBS was replaced with the PAL and untreated lactate. After 2 h, the PALs or untreated lactate were replaced with 200 μL culture medium. After 2 h, the cells were observed using the fluorescent microscope. (Reproduced from Tanaka et al. (2016) [44])

6.3 Intracellular Molecular Mechanism of Cancer Cell Death Induced by PAM Treatment

The long-lived reactive species, such as H_2O_2 and NO_2^-, are thought to play an important role as sources for singlet oxygen formation on the cell membrane [46, 47]. The reaction between H_2O_2 and NO_2^- will generate peroxynitrite ($ONOO^-$), followed by the primary singlet oxygen. Primary singlet oxygen locally inactivates membrane-associated catalase, which leads to the generation of secondary singlet oxygen, thus triggering anto-amplification of singlet oxygen generation. Secondary singlet oxygen is generated by H_2O_2 and $ONOO^-$ supplied by NADPH oxidase-1 (NOX1) and nitric oxide synthase (NOS). NOX_1 generates superoxide anions (O_2^-) extracellularly, whereas NOS generates NO, which is capable of passing through the cell membrane.

The biological effects of H_2O_2 include the peroxidation of lipids, induction of DNA damage, and playing a role in mitogenic stimulation and cell cycle regulation. Exposure of cells to $ONOO^-$ can trigger caspase activation followed by apoptosis, or can cause lipid peroxidation, protein nitration or oxidation, leading to necrosis.

Intracellular levels of ROS are generally elevated in tumor cells, and the treatment with PAM can induce further intracellular ROS generation in these cells. It appears that cancer cells, which are under high oxidative stress, cannot withstand further increase in ROS and RNS such as $OH^{\cdot}$, O, H_2O_2, and $ONOO^-$. A substantial increase in the concentrations of these species can induce cell death, including apoptosis and/or necrosis [30]. The threshold for the apoptosis is lower in the tumor cells than the corresponding non-malignant cell counterparts [48]. This "boost" hypothesis explains the oxidative stress-mediated selective killing of cancer cells.

Intracellular RONS generally damage proteins and DNA and act as signaling molecules. Many oxidative stress responses signal pathways have been studied in the cold plasma-treated and the PAM-treated cells. Interactions between cell membrane components and the reactive species might be important, as the cell membrane serves as an interface between the extracellular environment and the intracellular biochemical processes.

Aquaporins (AQPs) might play an important role in determining the anti-tumor effects of PAM against cancer cells [49]. Indeed, knockdown of the gene encoding AQP8, which functions as an efficient H_2O_2 channel in glioblastoma cells, significantly reduced the anti-tumor effects of PAM. Hydroxyl radical, which is generated from H_2O_2 via the Fenton reaction, is the most harmful ROS, and it was reported that iron mediates PAM-induced cell injury [31].

The development of most cancers likely involves multiple mutations. Mutations in genes associated with cell survival and proliferation signaling networks are especially important in terms of cancer development and therapeutic targeting. Two particularly important signaling pathways associated with survival and proliferation signaling networks are the phosphoinositide 3-kinase (PI3K)/AKT and rat sarcoma (RAS)/mitogen-activated protein kinase (MAPK) signaling pathways. As many as 50% of glioblastoma cells harbor somatic mutations in the PI3K/AKT pathway [50, 51]. An essential regulator of the PI3K/AKT pathway is the phosphatase

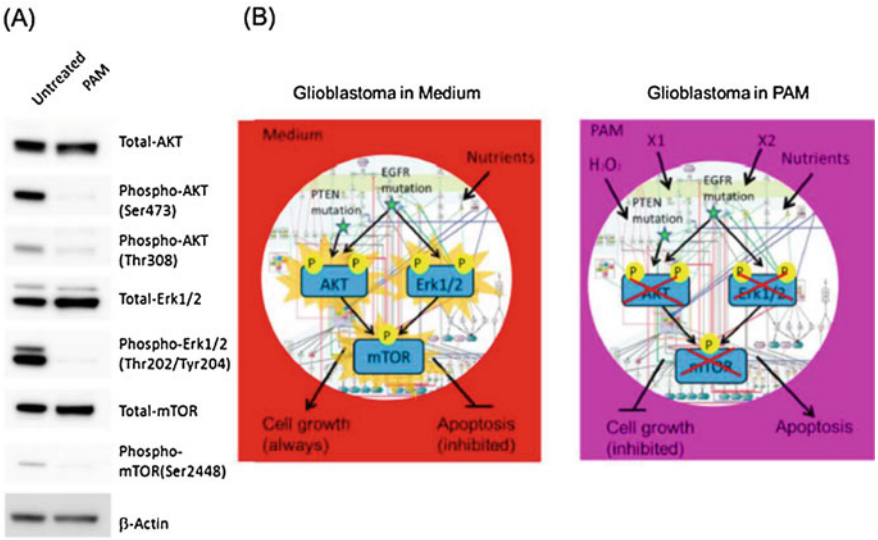


Fig. 6.10 PAM downregulates both the PI3K/AKT and RAS/MAPK signaling pathways in U251SP glioblastoma cells. Western blotting analysis (**a**) and models illustrating the intracellular molecular mechanisms of apoptosis induced by PAM (**b**). (Reproduced from Tanaka et al. (2014) [20])

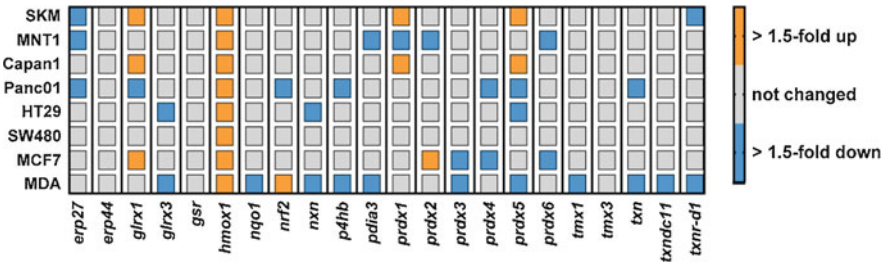


Fig. 6.11 HMOX1 is a biomarker for the treatment of cancer cells exposed to the plasma-treated medium. (Reproduced from Bekeschus et al. (2018) [54])

and tensin homolog (PTEN) tumor suppressor gene [52]. In U251SP glioblastoma cells harboring mutations in survival and proliferation signaling network genes such as PTEN, both the PI3K/AKT and RAS/MAPK signaling pathways are constitutively activated. Treatment with PAM downregulated both the PI3K/AKT and the RAS/MAPK signaling pathways in U251SP cells (Fig. 6.10) [8, 20, 53]. These results suggest that PAM induces the apoptosis of glioblastoma cells by downregulating survival and proliferation signaling networks.

Bekeschus and co-workers conducted a comprehensive screening of eight human cancer cell lines and 27 genes associated with redox regulation. To that end they identified HMOX1 as a biomarker to expose interaction of plasma-treated medium with cell lines (Fig. 6.11) [54]. Interestingly, HMOX1 expression intensity showed

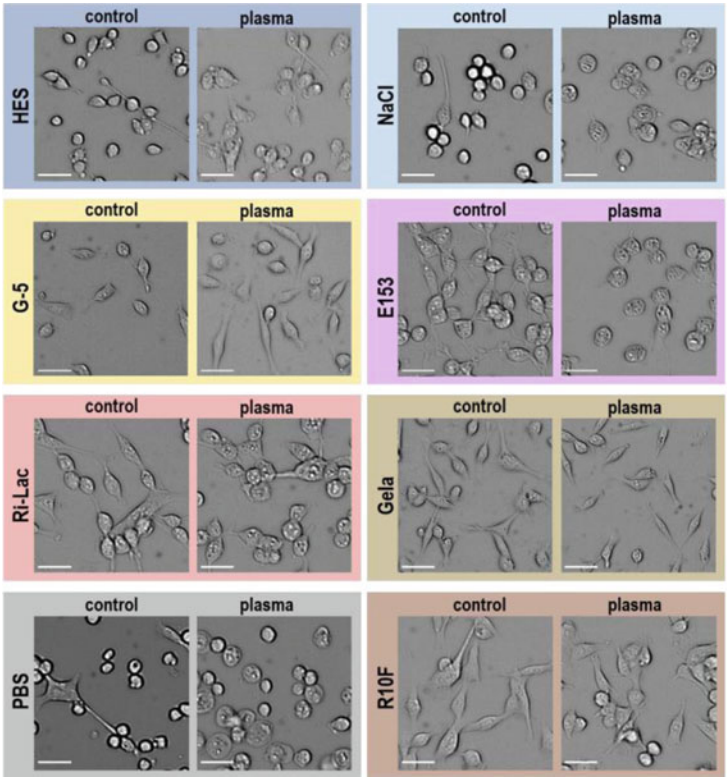


Fig. 6.12 Morphological changes in colon cancer cells exposed to six clinically approved plasma-treated liquids (as well as PBS and RPMI cell culture medium) compared to the untreated counterparts. (Reproduced from Freund et al. (2019) [55])

a significant positive correlation with the tumor cells not responding to plasma-treated medium in cytotoxic fashion. This means that tumors low in expression or upregulation of HMOX1 might be more prone to plasma-treated medium-induced toxicity.

In another mechanistic study from that group, a comprehensive analysis of the "ideal" plasma-treated liquid was performed. This is because the hurdles of receiving the accreditation of complex liquid such as cell culture medium for the clinical practice are high, while at the same time, a number of clinically accredited liquids exist. The study performed storage, redox chemical, cell morphological, and cytotoxic analysis of six clinically accepted liquids, and compared them to PBS and RPMI cell culture medium (Fig. 6.12). The results revealed that sodium chloride and ringer's lactate are the most promising candidates to pursue this promising novel treatment avenue further [55].

Since cancer patients often receive drugs, Bekeschus and colleagues next asked the question whether there might be an additive or synergistic activity with oxidizing liquids and chemotherapy. After screening a drug library of 80 kinase-inhibitors,

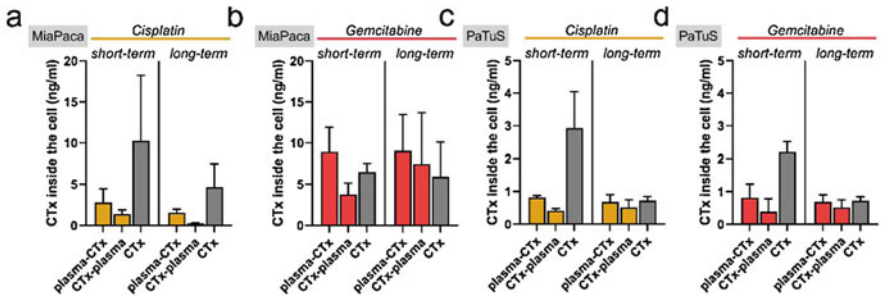


Fig. 6.13 Mass spectrometry analysis of intracellular chemotherapy drug concentrations (Gemcitabine, Cisplatin) upon exposure to plasma-treated Ringer's Lactate solution during short-term and long-term culture in MiaPaca (a,b) and PatuS (c,d) human pancreatic cancer cells *in vitro*. (Reproduced from Liedtke et al. (2020) [57])

they found a number of targets showing promising synergistic activity in several human cancer cell lines assayed under 2D and 3D tumor spheroid conditions [56]. To extend this idea closer to the clinic, a subsequent investigation was concerned with using plasma-treated ringer's lactate in combination with gemcitabine or cisplatin, two drugs applied in the treatment schemes of pancreatic cancer patients in the clinical setting [57]. The main findings were that additive toxicity was present in the combination treatment, leading to a decline in metabolic activity and cell growth, and an increase of apoptosis and cell cycle arrest. Importantly, the additive toxicity was found in both 2D cancer cell lines and 3D tumors grown in the in ovo model. Mechanistically, this study is important because it analyzed intracellular drug concentrations by mass spectrometry. Interestingly, drug concentrations were in the tendency higher in cells receiving drugs alone, while combination treatment with plasma-treated ringer's lactate decreased the concentration (Fig. 6.13). This might be an indicator of accelerated cell death with the combination treatment.

6.4 Some Guidelines to Make PAM

Extending the cold plasma treatment time is the easiest strategy to enhance the anti-tumor capacity of PAM [6, 8, 12, 13, 44]. Three general principles to improve the anti-tumor effect of PAM have been demonstrated by regulating three factors: the gap between the cold plasma source and the surface of the solution, the contacting area between cold plasma and surface of the solution, as well as the volume of solution (Fig. 6.14). Recently, these principles have been tested in other cold plasma-activated solutions such as PBS by other groups [58]. A larger containing area between the cold plasma and the surface of the solution will obtain higher reactive species concentration in PAM [6]. For instance, the PAM made in a well on a 6-well plate will be much more toxic to cancer cells than the PAM made in a well on a 96-well plate. In addition, a shorter gap between the plasma source and medium will

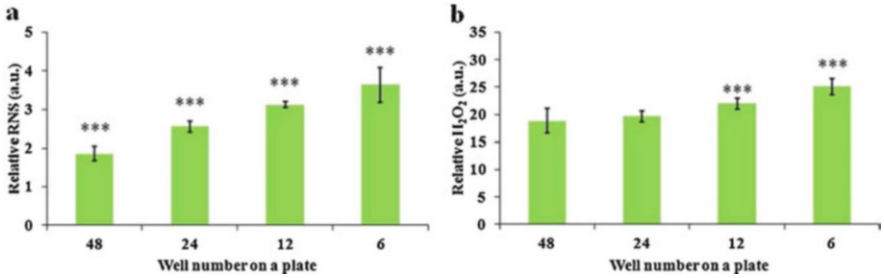


Fig. 6.14 The well size-dependent ROS/RNS accumulation in the PAM. (**a**) Relative RNS concentration in 1 mL of PAM. (**b**) Relative H2O2concentration in 1 mL of PAM. Results are presented as the mean ± s.d. of three repeated experiments performed in triplicate. Student's *t*-test was performed, and the significance compared with the first bar is indicated as $*p < 0.05$, $**p < 0.01$, $***p < 0.005$. (Reproduced from Yan et al. (2015) [6])

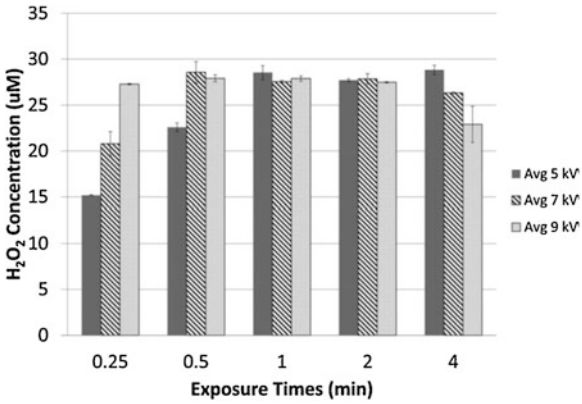


Fig. 6.15 The H_2O_2 concentration in micromoles versus cold plasma exposure time for three different applied voltages. (Reproduced from Mohades et al. (2015) [19])

also make a more toxic PAM with a higher reactive species concentration [6, 58]. The stronger anti-tumor capacity of PAM can also be achieved by using the medium with a smaller volume during the CAP treatment [6, 58].

In addition to these guidelines, the basic operational parameters of cold plasma can also determine the anti-tumor of PAM. For example, the applied voltage to cause the discharge of cold plasma can affect the generation of H_2O_2 in the PAM, which directly affects the anti-tumor effect (Fig. 6.15) [19].

Diverse, complicated reactions exist at the interface between cold plasma and aqueous solutions. Several chemicals are highly reactive with the reactive species in PAM. For example, FBS a standard component of a typical complete cell culture medium, is highly reactive to ROS in PAM. FBS eliminates the anti-tumor effect of PAS on glioblastoma cells [10]. In addition, pyruvate is also highly reactive with the H_2O_2 in PAM [41]. Cysteine is a common component in nearly all standard mediums. Among all 20 amino acids, however, cysteine is most reactive with the H_2O_2 in PAM [10]. In short, an ideal PAM should avoid containing FBS, pyruvate, and cysteine.

6.5 The Storage of PAM

In the earliest study about the anti-tumor effect of PAM, the degradation of PAM during the storage has been observed. The anti-tumor species and corresponding anti-tumor capacity of PAM will be gradually degraded during the storage over a wide temperature range for less than just 1 day, from the room temperature, few degrees above the freezing point of water, and as low as −20 °C ~ −30 °C (Fig. 6.16) [6, 10, 28, 29, 41]. Such rapid degradation can be inhibited by storage at a low-temperature environment (−80 °C in a freezer or −150 °C in the liquid nitrogen) [28, 41, 44]. The ideal storage temperature range of most media on the market is between 2 °C and 8 °C. An ideal PAM should be stably stored under such a temperature range.

The degradation mechanism of PAM has been investigated recently. By comparing the H_2O_2 concentration in the cold plasma-activated PBS and the cold plasma-activated DMEM after the storage at 8 °C, 22 °C, and at −25 °C, it is found that the degradation of PAM was mainly due to the reaction between the cold plasma-generated species and some components in the medium [42]. Cysteine and methionine were the main medium components causing the degradation of PAM (Fig. 6.17). The cold plasma-activated DMEM without cysteine and methionine was much more stable than the cold plasma-activated DMEM during the storage at 8 °C, 22 °C, and at −25 °C [42]. Furthermore, a tyrosine derivative, 3-nitro-L-tyrosine, can improve the stability of PAM at 8 °C even when the medium contains cysteine and methionine (Fig. 6.18). These studies demonstrate that a noticeable degradation

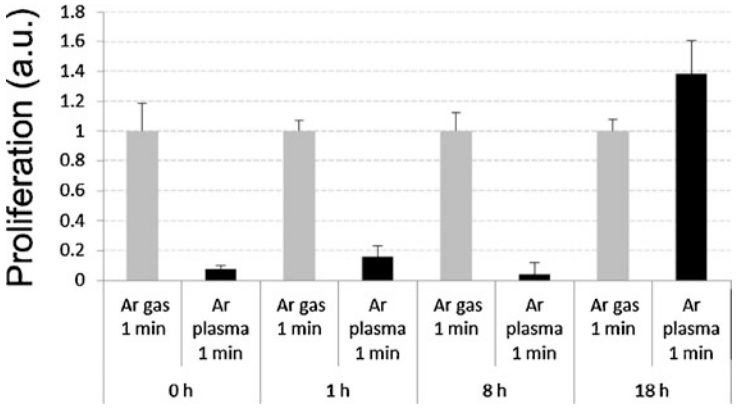


Fig. 6.16 The duration of the effectiveness of PAM. U251SP glioblastoma cells were plated at a density of 1×10^3 cells in 200 μL medium in a 96-well plate. On the following day, 3 mL fresh medium (DMEM with 10% FBS, 1% P/S) in a 6-well plate was treated with cold plasma or Ar gas for 1 min (L = 13 mm, 2.0 slm), and 1, 8, or 18 h after plasma treatment of the medium, 200 μL of this (PAM) was used to replace the medium on the cells in the 96-well plate. On the following day, cell viability was assayed using the MTS assay. (Reproduced from Tanaka et al. (2011) [8])

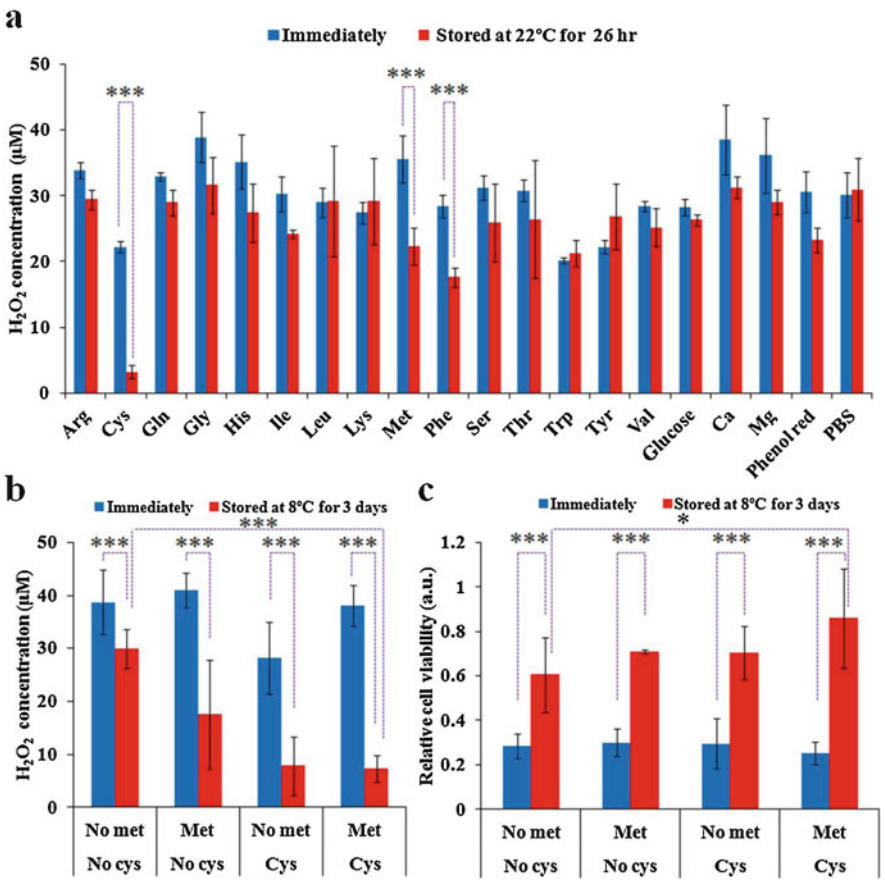


Fig. 6.17 Cysteine and methionine mainly cause the degradation of PAM at 22 °C and 8 °C. (**a**) The change of H_2O_2 concentration in the cold plasma-activated PBS containing a specific component during the storage at 22 °C for 26 h. (**b**) The change of H_2O_2 concentration in the cold plasma-activated cys/met-free DMEM, cys-free DMEM, met-free DMEM, and standard DMEM during the storage at 8 °C for 3 days. (**c**) The change of anti-tumor effect of the cold plasma-activated cys/met-free DMEM, cys-free DMEM, met-free DMEM, and standard DMEM during the storage at 8 °C for 3 days. (Reproduced from Yan et al. (2016) [42])

of PAM at around 8 °C will not occur when the corresponding medium does not contain these reactive components such as cysteine and methionine [42].

6.6 Animal Studies of PAM

The ultimate aim of the study on PAM is the clinical application. The future clinical application of PAM will require its mechanism of action to be fully elucidated and

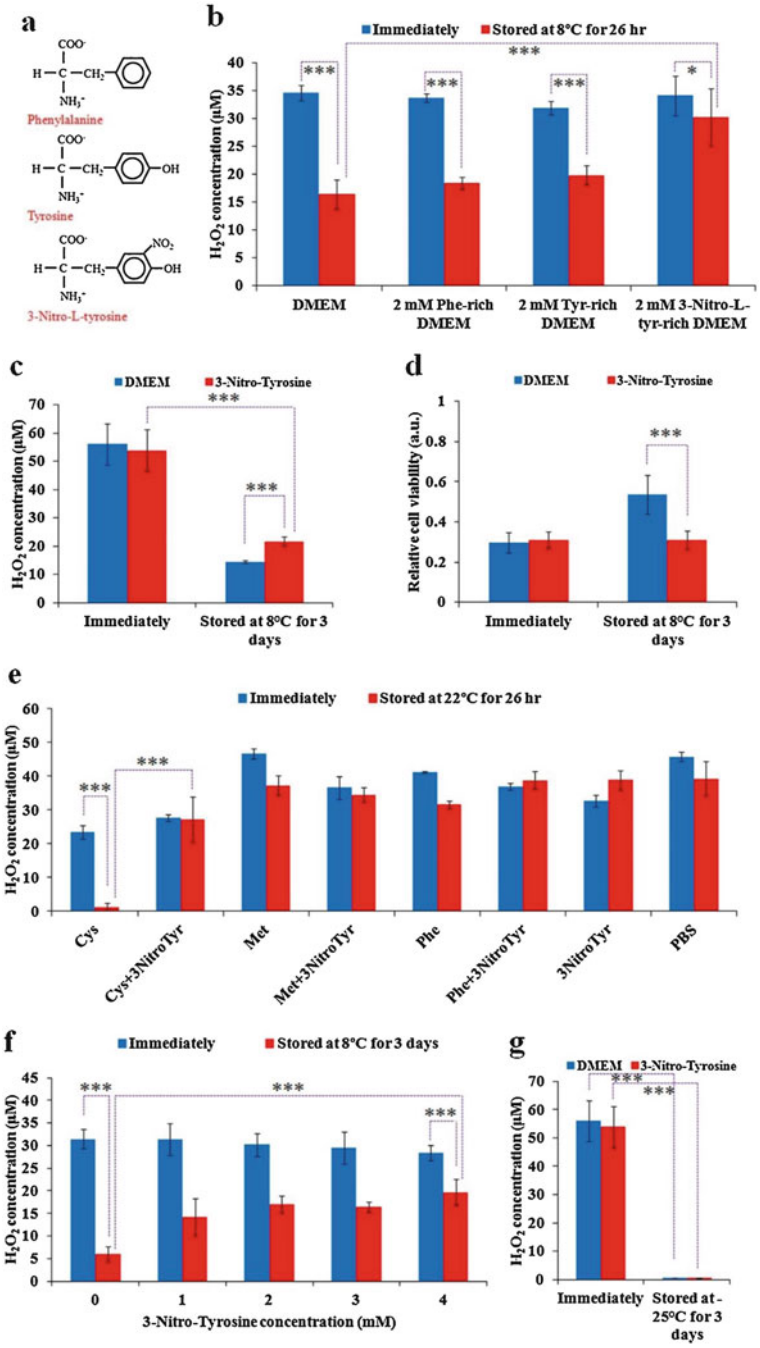


Fig. 6.18 3-Nitro-L-Tyrosine inhibits the degradation of PAM. (**a**) Chemical formulas of phenylalanine, tyrosine, and 3-Nitro-L-Tyrosine. (**b**) The change of H_2O_2 concentration in the cold

its effectiveness and safety to be sufficiently demonstrated in animal studies. The studies on the anti-tumor effect of PAM in vivo are a key step to build such a foundation. The anti-tumor effects of PAM have been demonstrated in various in vivo experiments. Fumi Utsumi et al. first demonstrated that injecting PAM into the subcutaneous tumors grown from the xenografted chemical-resistant ovarian cancer cells could drastically inhibit the tumor growth (Fig. 6.19) [16]. Similar anti-tumor effects of PAM were reported in a xenograft mouse model of pancreatic cancer [59].

Hiromasa Tanaka et al. demonstrated the strong anti-tumor effects of the CAP-activated Ringer's lactate solution via injecting it into the subcutaneously xenografted human cervical cancer cell line (SiHa)-originated tumors in mice. Such a solution effectively reduced tumor volumes and showed no apparent adverse effects on the mice [44]. Recently, the anti-tumor effect of PAM has been tested using intraperitoneal injections in mouse models of intraperitoneal metastasis of gastric cancer [58] and ovarian cancer (Fig. 6.20) [23]. A similar study was shown in Fig. 6.24. Shigeomi Takeda et al. demonstrated that PAM effectively decreased the formation of peritoneal metastatic nodules by 60% in the mouse model without causing observable adverse events [58]. Importantly, PAM treatment did not affect body weight in these studies, suggesting that treatment using PAM is both efficacious and safe. Another study involving a mouse model of intraperitoneal pancreatic cancer demonstrated that PAL also exhibits anti-tumor effects [61].

The biological safety of PAM in clinical trials is paramount. In one recent study, Liedtke, Bekeschus, and colleagues tested the therapeutic efficacy and safety of plasma-treated RPMI cell culture medium in a syngeneic (same genetic background of tumor cells and mice), orthotopic (tumor located at the anatomic location where it would occur in patients) model of peritoneal pancreatic carcinomatosis [62]. Retarded tumor growth was observed by means of magnetic resonance imaging and weighing of the total tumor mass (Fig. 6.21). The tumor lesions showed extensive apoptosis and a significant decline in the number of proliferating cells, with a mean

Fig. 6.18 (continued) plasma-activated DMEM, phe-containing DMEM, tyr-containing DMEM, and 3-Nitro-L-tyr-containing DMEM during the storage at 8 °C for 26 h. (**c**) The change of H_2O_2 concentration in the cold plasma-activated DMEM and 3-Nitro-L-tyr-containing DMEM during the storage at 8 °C for 3 days. (**d**) The change of anti-tumor effect of the cold plasma-activated DMEM and 3-Nitro-L-tyr-containing DMEM during the storage at 8 °C for 3 days. (**e**) The change of H_2O_2 concentration in the cold plasma-activated single amino acids-containing PBS and double amino acids-containing PBS during the storage at 22 °C for 26 h. Corresponding concentration of cys, met, phe, and 3-Nitro-L-tyr in PBS were 0.2 mM, 0.2 mM, 0.4 mM, and 2 mM, respectively. (**f**) The change of H_2O_2 concentration in the cold plasma-activated 3-Nitro-L-tyr-containing DMEM with different concentrations during the storage at 8 °C for 3 days. (**g**) The change of H_2O_2 concentration in the cold plasma-activated standard DMEM and 3-Nitro-L-tyr-containing DMEM during the storage at −25 °C for 3 days. For all experiments, the volume of solution in each well was 1 mL. The treatment time was 1 min for (**b**, **e**, **f**) and was 2 min for (**c**, **d**, **g**), respectively. Results are presented as the mean ± s.d. of three independently repeated experiments performed in triplicate (**b**, **c**, **e**–**g**) or in sextuplicate (**d**). For the cell viability, the data have been normalized to the corresponding control group. Student's *t*-test was performed and the significance is indicated as $*p < 0.05$, $**p < 0.01$, $***p < 0.005$. (Reproduced from Yan et al. (2016) [42])

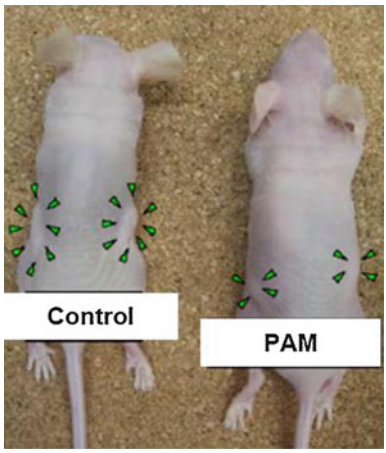


Fig. 6.19 Anti-tumor effect of PAM in a xenograft mouse model of ovarian cancer. (Reproduced from Utsumi et al. (2013) [16])

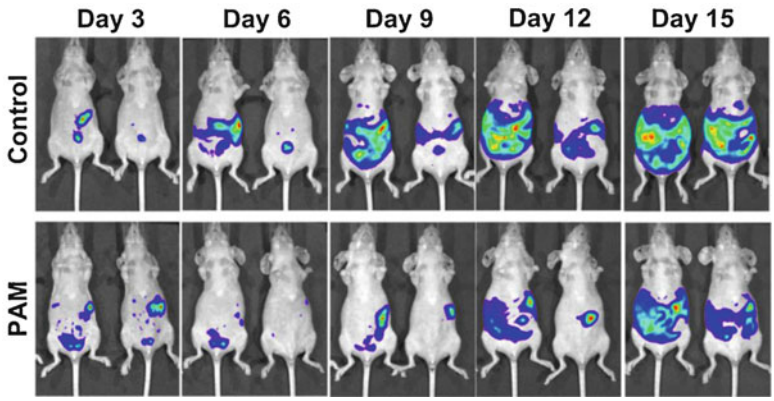


Fig. 6.20 Anti-tumor effects demonstrated in a mouse model of intraperitoneal metastasis of ovarian cancer. (Reproduced from Utsumi et al. (2017) [23])

penetration depth of 250 μm. In the same study in vitro, a selective toxic effect of the plasma-treated medium was confirmed in pancreatic cancer cells against murine primary non-malignant fibroblasts in terms of metabolic activity and abrogation of cellular growth. Simultaneously, the study confirmed the safety of the approach. None of the animals receiving the plasma-treated medium showed off-target side effects. Extensive analysis of all major murine blood leukocyte populations (e.g., neutrophils, monocytes, cytotoxic T-cells, B-cells, NK-cells) did not indicate a negative influence of plasma-treated cell culture medium compared to animals not receiving such treatment. Moreover, over a dozen of hematological parameters were assessed (e.g., red blood cell count, hematocrit, and mean platelet value) and, similar to the analysis of a range of blood cytokines (e.g., IL-6, IL-10, IL-12, and IFNγ), no significant changes were observed in the group of mice receiving repeated injections of plasma-treated medium. In a follow-up study of the Bekeschus lab

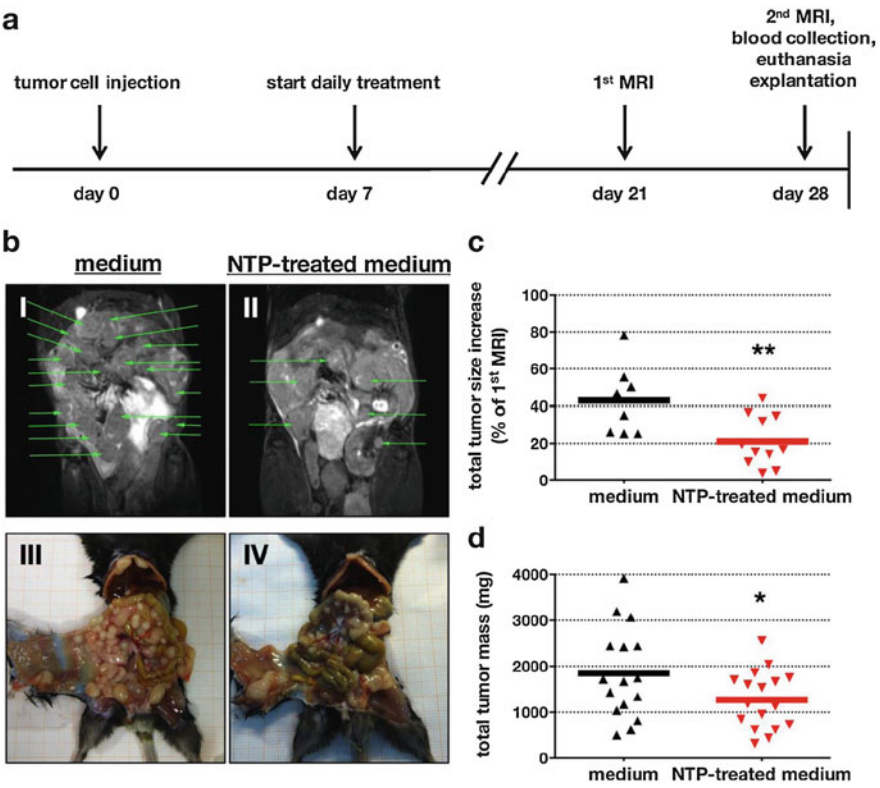


Fig. 6.21 PAM decreases the number, growth, and size of pancreatic lesions in vivo. The experimental timeline (**a**) is given. The number of tumor nodes in the peritoneum was followed by MRI and was elevated in the control animals (I) compared to the NTP-treatment group (II) at the end of treatment (d28). Also, corresponding macroscopic findings for control (III) and NTP-treated (IV) animals are shown (**b**). MRI-based calculation of tumor volume revealed a significantly decreased total tumor growth in the treatment group (**c**). On day 28, animals were sacrificed and tumor nodes were excised and weighed, showing a significantly decreased total tumor mass (**d**). Representative images of 13 animals are given; green arrows indicate tumor nodes (one green arrow per lesion) (**b**). Data are presented as mean of 8–17 animals (**c**, **d**). (Reproduced from Liedtke et al. (2017) [62])

in Greifswald using tumor lesions from this experiment, the Immunobiology of the therapy with the plasma-treated medium was explored [63]. It was discovered that the treatment group tumor lesions contained significantly more macrophages compared to the animals not receiving medium alone (without plasma treatment). Macrophage infiltration in pancreatic cancer, however, is known to correlate with a poor outcome in patients. This is because of the macrophages acquiring a so-called tumor-associated macrophage (TAM) M2-phenotype that supports tumor growth [64]. By contrast, our study revealed a significant decrease of the M2-macrophage marker CD206 in the tumor lesions exposed to plasma-treated medium,

indicating their tumor-toxic nature that might explain the growth retardation seen with the treatment. Multicolor flow cytometry of digested tumor nodules moreover revealed a significant increase of T-cells in mice receiving plasma-treated medium. These cells are known to have the ability to promote anti-tumor effects. This corresponds to the findings of elevated calreticulin exposure, a protein known to enhance pro-immunogenic effects [65], in pancreatic tumor nodules exposed to the plasma-treated medium.

While these results were promising, the applicability of the plasma-treated medium in a clinical setting may be low. This would mainly be because of the organic compounds cell culture medium contains, making accreditation of such liquid problematic. To this end, Bekeschus et al. tested plasma-treated PBS for its activity against pancreatic cancer [66]. The selective apoptosis-inducing effects towards pancreatic cancer cells vs. non-malignant fibroblasts, as found with plasma-treated medium, were replicated when using plasma-treated PBS. This was not only the case for cells cultured in 2D but also in a three-dimensional in ovo tumor model, showing growth retardation and apoptosis (Fig. 6.22).

This study was further extended, testing against colon cancer and elucidating the cellular and molecular mechanisms of action. To investigate the storage conditions, the solution was stored at −20 °C and found to maintain its activity up to 3 weeks post plasma treatment [67]. Plasma-treated PBS oxidized the tumor cells, decreased growth and metabolic activity, led to morphological changes not associated with epithelial-to-mesenchymal (EMT) transition, mediated apoptosis and cell cycle arrest, reduced the cells' motility, and induced hallmarks of the immunogenic cancer cell death as shown by an increased expression of calreticulin, heat-shock protein 70, and HMGB1. The results were replicated in three cancer cell lines and selective when compared against non-malignant HaCaT keratinocytes. Interestingly, incubation with concentration-matched H_2O_2 was able to replicate many of the effects observed. This, however, was not true for cytotoxic effects in 3D tumor spheroids, where plasma-treated PBS outperformed H_2O_2 by far. Importantly, plasma-treated saline also effectively reduced the tumor burden in vivo in a syngeneic, orthotopic model of peritoneal colon carcinomatosis (Fig. 6.23).

In another recent study, the safety of PAW in an immunocompromised animal model has been investigated [60]. Xu et al. examined the safety of immune-deficient nude mice by oral lavage treatment of PAW. The growth status, main organs, and biochemical blood indexes were investigated [60]. The acute toxicity test results showed that the maximum dose of plasma treatment for 15 min had no lethal effect and other acute toxicity. There were no significant changes in body weight and survival status of mice after 2 min and 4 min of plasma-activated water (PAW) treatment for 2 weeks. After treatment, the major organs, including heart, liver, spleen, lung, and kidney, were not significantly changed in organ coefficient and tissue structure (Fig. 6.24). Biochemical blood markers showed that blood neutrophils and mononuclear cells were slightly increased, and the others remained unchanged. Liver function, renal function, electrolytes, glucose metabolism, and lipid metabolism were not affected by different doses of PAW treatment. The above

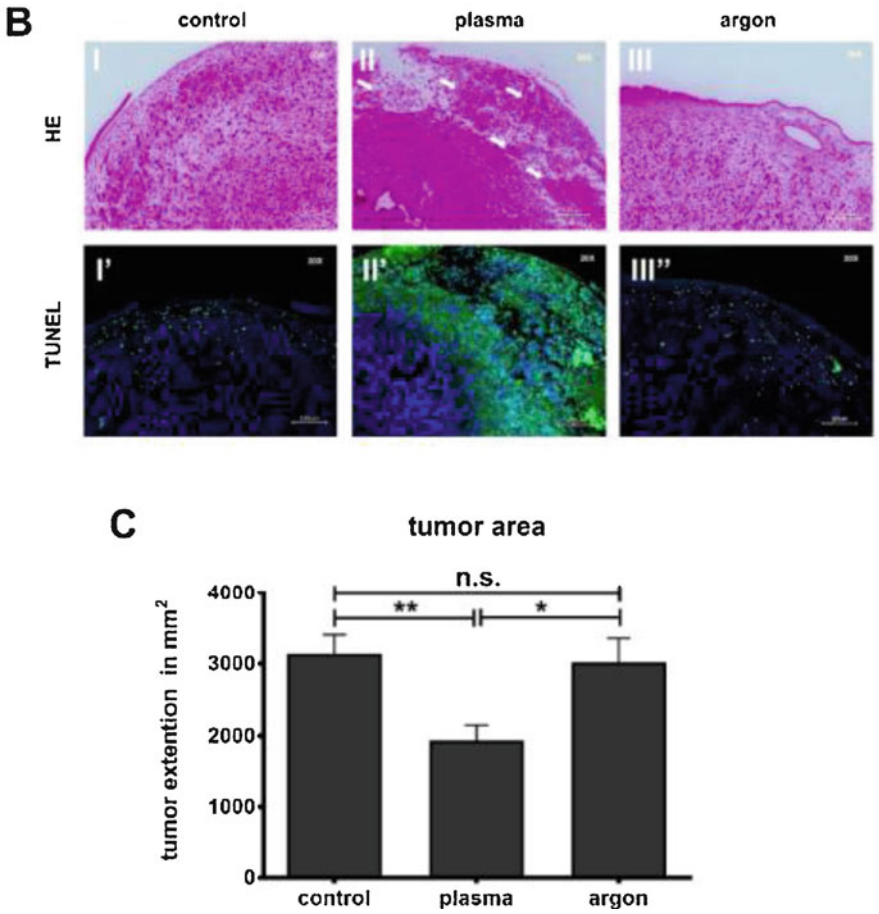


Fig. 6.22 Reduced pancreatic cancer tumor growth with plasma-treated PBS in an in ovo model. (Reproduced from Bekeschu et al. (2018) [66])

results indicate that PAW treatment can be used to treat immuno-deficient nude mice without significant safety problems [60].

6.7 Future Directions of PAM Studies

Cancer therapy using PAM could be combined with personalized cancer treatments in the future. Certain treatments exhibit better effects for some patients than for others, depending on the genetic characteristics of both the patient and the tumors. The development of new types of PAM could enable effective treatments that involve different molecular mechanisms. For example, PAM and PAL exhibit anti-

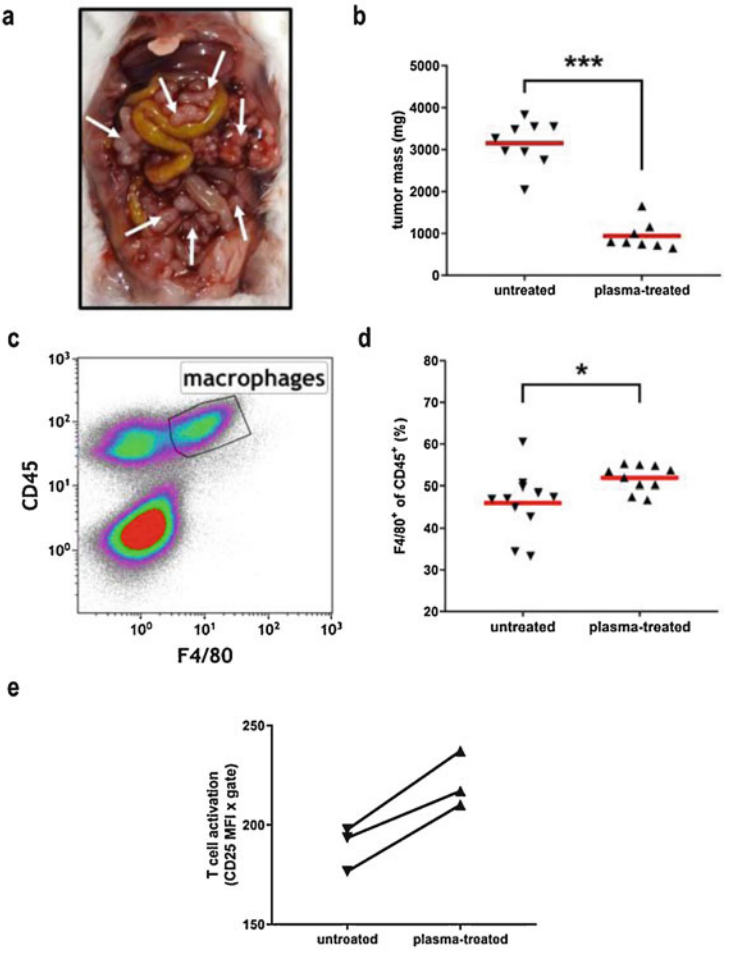


Fig. 6.23 Plasma-treated saline solution effectively controlled the growth of peritoneal metastasis in a syngeneic, orthotopic tumor model of colon carcinomatosis in mice. The treatment also had an immunological dimensions as seen with an increase in immunogenic cancer cell death, intratumoral macrophages, and enhanced activity of intratumoral T-cells. (Reproduced from Freund et al. (2013) [67])

tumor effects on glioblastoma cells via different mechanisms. Obtaining a more-complete understanding of the mechanism of PAM-induced death of cancer cells could ultimately help make it possible to treat cancer patients using personalized approaches.

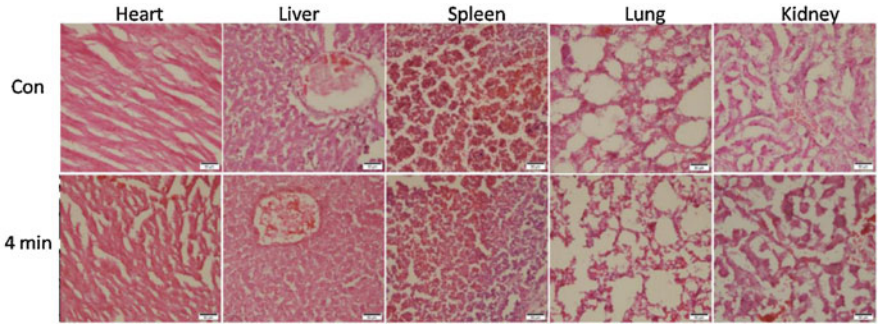


Fig. 6.24 Effect of cold plasma-activated water (PAW) on the vital organs. (Reproduced from Xu et al. (2018) [60])

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