The effect of hyperbaric oxygen treatment, alone or in combination with 5-FU, on a MDA-MB-231 human mammary tumor model

By

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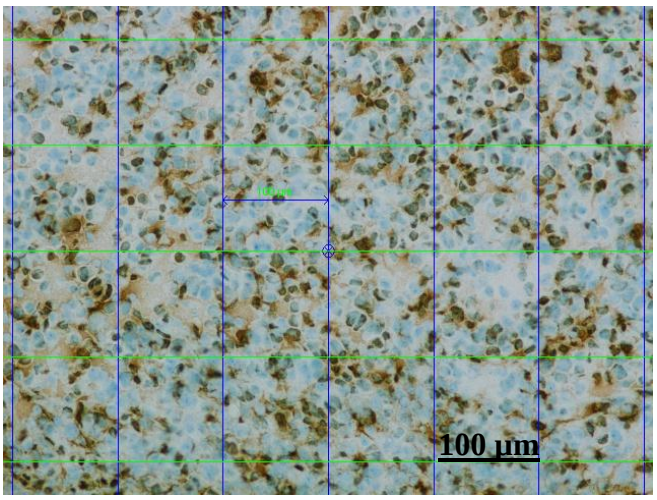

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Front page picture:

Cross section of MDA-MB-231 tumor, immunostained with KI-67 for proliferating cells (x 100 magnification)

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Annette Garmann-Johnsen

2. Abstract

Background: Previous studies of hyperbaric oxygen (HBO) treatment on chemically induced and murine breast cancer models have shown promising results. This study was performed to elucidate the effects of HBO on a human breast cancer model, in an effort to make the results more clinically relevant. Our objective was to develop a human breast cancer model (MDA-MB-231) in NOD/SCID mice. This model was to be exposed to early or late hyperbaric oxygen therapy to evaluate effects concerning tumor growth, angiogenesis and proliferation. Finally, we wanted to elucidate if the effect of chemotherapy could be enhanced by HBO.

Methods: Immunodeficient NOD/SCID mice were injected with the MDA-MB-231 cells to form tumors. Mice were divided in six groups: controls (early and late), HBO (early and late), chemotherapy (5FU) and HBO/5FU. Controls were exposed to normal ambient pressure air throughout the experiment, while HBO treatment was performed with pure oxygen 4 times (each 90 min) at 2.5 bar. 5FU was given immediately prior to HBO treatment in the combined group. Tumor growth was measured by a caliper. Immunostaining was used to discover differences in blood vessels (CD-31) and proliferating cells (KI-67).

Results: The tumor model developed with 100 % take. Tumor growth was significantly inhibited in the early treated HBO group compared to controls, but not after late treatment. No differences were found in angiogenesis or proliferation between HBO and controls neither in the early nor the late group. Tumor size was not significantly different after the combined HBO/5FU treatment than after HBO alone.

Conclusion: We can conclude that MDA-MB-231 tumor establishment was successful. HBO inhibits tumor growth significantly if given early, but not when administered late. This inhibitory effect could not be explained by differences in blood vessels or proliferating cell densities. HBO did not potentiate the effect of 5FU in this tumor model. Further studies are needed.

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4. Introduction

4.1 Cancer

4.1.1 Cancer incidences

Yearly, there are an estimated 12.7 million new cancer cases on a worldwide basis. Breast cancer is one of the most common forms of cancer, with approximately 1.38 million new cases each year (approximately 11% of all cancer cases) (1). A total of 7.6 million people annually die of cancer (around 13% of all deaths). 458 000 deaths per year are caused by breast cancer, constituting 6% of cancer deaths (2). The reason death rates are lower in breast cancer than in general, are that the majority of cancer cases occur in developed countries (1) were good treatment options coincidentally are also more easily accessible to the general population. However, cancer, along with cardiac disease, represents the leading causes of mortality in developing and developed regions. Thus, finding new efficient treatment options is of great importance. The numbers presented are from 2008.

4.1.2 Cancer characteristics

Cancer is an abnormal cell growth. Cancer may progress into malignancy if oncogenes are activated or tumor suppressor genes are inhibited (3). Cancer cells proliferate at a higher rate and are eliminated at a lower rate than regular somatic cells. This disruption of homeostasis causes invasion and the following destruction of healthy tissues. The danger of cancer lies in the fact that a tumor will deplete the nutrient supply of nearby tissues, that gradually will starve to death (3). The illnesses known as cancer are genetically quite diverse, and the mutation rate is high. But cancer most often displays certain universal characteristics according to a review by Hanahan et Weinberg, 2011 (4) (Fig 1):

- Sustaining proliferative signaling: Growth factors affect the size of cells and changes normal cell cycle progression. In doing so, the growth factors are disrupting the homeostasis of cell number that exists in non-cancerous tissues. There are several ways by which cancer cells can maintain proliferative signaling: Cancer cells are provided these growth signals by themselves (5-8)

or by stromal cells (9, 10). Cancer cells can also increase the amount of receptors for growth factors, or bypass the need for growth factors by activating cellular mechanisms downstream of the receptors (4).

- Evading growth suppressors: Cancer cells must avoid the systems that reduce cell proliferation. Among these are the tumor suppressor genes. The tumor suppressors gets signals from outside and within the cell and this information decides whether cells should be allowed to proliferated or enter senescence or be eliminated by apoptosis. In malignancy, enough of the genes coding for tumor suppressor genes have been rendered dysfunctional. Normal cells are regulated by contact inhibition, a system that most cancer cells have escaped.

- Inducing angiogenesis: Tumor cells needs provision of nutrients and oxygen and removal of carbon monoxide and waste products like all other cells. In an adult, angiogenesis, formation of new blood vessels from existing vasculature, is almost non-existent. While in a tumor an "angiogenic switch" is almost constantly turned on to promote the tumor growth (11). Vascular endothelial growth factor (VEGF) is a a prominent example of a gene that promotes angiogenesis. Expression is increased by both hypoxia and oncogene signaling (12-14). The angiogenesis derived from such signaling does, however, produce abnormal and malfunctioning blood vessels (15, 16). The angiogenesis is induced early in cancer development (11, 16). There also exist angiogenesis inhibitors, several different have been discovered, that counteracts the angiogenetic program of tumors (17-20).

- Enabling replicative immortality: Normal cells have limited replicative potential. After a given number of mitosis, cells will either enter senescence or die. Cancer cells proliferate without limitations. Based on several studies, the telomeres that protect DNA during replication are associated with this mechanism (21, 22). In normal cells the telomeres are shortened by each replication, and this eventually exposes chromosomes to destruction that make further replications impossible. The enzyme telomerase, that continuously restores the telomeres, is highly prevalent in cancer cells. Though this mechanism is not always this straight forward.

- Resisting cell death: Apoptosis is controlled cell death that does not cause inflammations of the surrounding tissues. Apoptosis prevents normal cells from progressing into malignancy (23-25). Increased oncogene signalling and DNA damage acquired as a result of hyperproliferation, normally induces apoptosis in cells. However, cancer cells having reached a higher level of malignancy, and not responding predictably to therapy, can attenuate the process of apoptosis (23, 25). Commonly cancer cells escape apoptosis if the tumor suppressor qualities of TP53 are lost. Cancer cells may also downregulate pro-apoptotic factors, prevent the extrinsic death pathway or increase expression of antiapapoptotic genes or of survival signals. Autophagy is the process of cells disintegrating themselves by means of their lysosomes. Autophagy, another mechanism of eliminating abnormal cells, can be induced by cellular stress like nutrient deficiency (26, 27). In addition to being harmful for cancer cells, autophagy can also be of benefit because it makes cellular components readily available for surrounding surviving cancer cells. Autophagy represents another obstacle for tumor cells to overcome in order to progress towards malignancy. (28) Necrosis is externally caused cell death that leaves cell debris. When cells die by necrosis, in contrast to apoptosis, proinflammatory signals are released to the surroundings. This attracts inflammatory cells from the immune system (29-31). These cells normally functions by evaluating the tissue damage and removing waste products. However, in relation to cancer cells, immune inflammatory cells can be beneficial if they are able to induce angiogenesis, cancer cell proliferation and invasiveness. These cells can also emit factors that promote cell proliferation of surrounding cells (30). Thus, although a proportion of cancer cells will be eliminated, the tumor as a whole might benefit from a certain degree of necrosis.

- Activating invasion and metastasis: When tumors form metastasis and invade other tissues, the cancer malignancy increases notably. Cells change shape, and their attachment to adjacent cells and the extracellular matrix are altered, a process associated, amongst others, with the loss of an important cell-to-cell adhesion molecule called E-cadherin in cancer cells. Hence, a functional E-

cadherin suppresses invasion and metastasis significantly (32, 33). In addition, other genes expressing similar qualities along with genes promoting cytostasis are generally downregulated. Invasion and metastasis occurs as a cascade of events (34, 35). First the cancer cells invade the immediate surroundings. Then the cells intravasate through the basal membrane and into the blood and lymph stream before they exit the vessels in a new organ and enters its parenchyma. Micrometastases are formed, and finally, as proposed by several studies, macroscopic metastasis emerge in the step called "colonization". Responsible for this process, except for the final step of colonization, is a program termed "epithelial-mesenchymal transition" (EMT) that alters cells, enabling them to go through this process (36-40). Communicative signaling, previously shown between cancer and stromal cells, reinforces the invasion and metastasis (41-44).

In the last years there have been emerging new hallmarks due to improved insight into the complexity of tumor biology (4):

- Deregulating cellular energetics: In order for cancer cells to perform all actions leading to increased malignancy, they require not only a certain signaling, but also an altered energy metabolism to support the high level of proliferation and growth. Normally in aerobic cells, glucose is metabolized to pyruvate by glycolysis. Then pyruvate is metabolized to carbon dioxide and water in the mitocondria in the oxidative phosphorylation, using a lot of oxygen in the process. This last step optimizes the amount of ATP produced in metabolism. For anaerobic tissues, in contrast, glycolysis alone is preferred. However, studies have shown that cancer cells do not follow these regulations (45-47). Cancer cells are able to switch to only glycolysis even in an oxygen-rich environment, the "aerobic glycolysis". The amount of ATP gained here is relatively low. Therefore the cancer cells compensates, for instance by upregulating glucose transporters, especially GLUT1, resulting in higher glucose uptake (48-50). A connection has been discovered between glycolysis and activation of oncogenes as well as mutations of tumor suppressor genes. (e.g., RAS, MYC) and mutant tumor suppressors (e.g., TP53) (48-50). In the

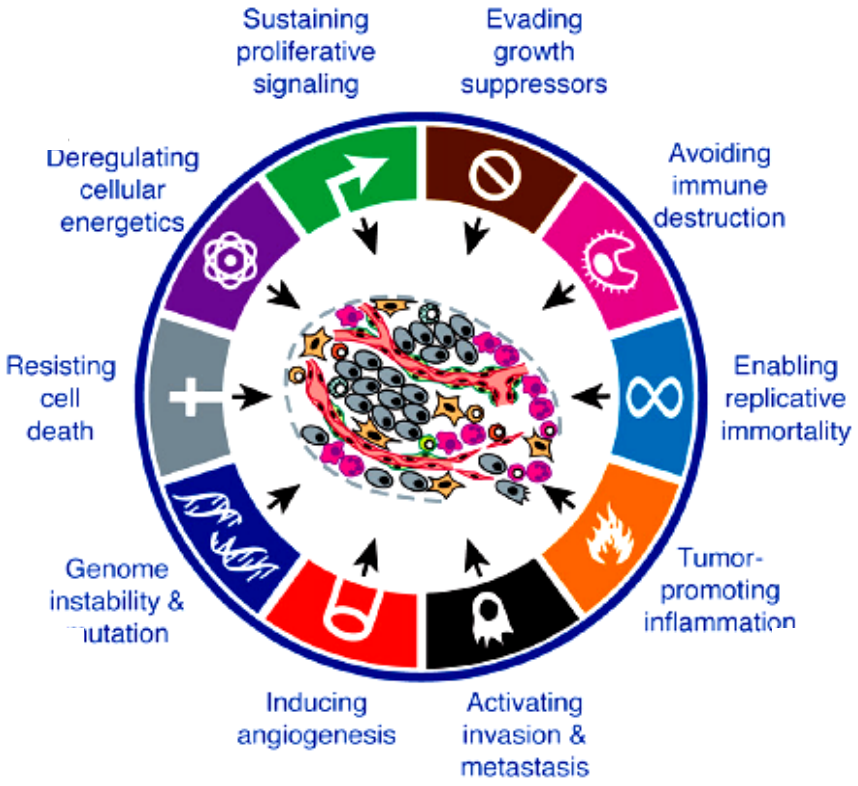
situation of hypoxia, glucose transporters are upregulated in addition to enzymes involved in the glycolytic pathway (48, 50, 51). Like the oncoprotein Ras, hypoxia can elevate HIF1 and HIF2 transcription factor levels, aiding glycolysis (51-53). When trying to understand why cancer cells prefer glycolysis, there has been made a hypothesis stating that the increased glycolysis releases glycolytic compounds to mechanisms that generates cell materials (54, 55). This in turn facilitates new cell generation. Additionally, tumors may have two distinct metabolisms. The first type already mentioned produces lactate as a byproduct, and the other cell type uses lactate as fuel mediated by parts of the citric cycle (56-58). This mechanism can also be found in muscle tissue (56-58). The cancer metabolism is programmed by many of the same genes that induce the other hallmarks.

- Avoiding immune destruction: The immune system presents a notable barrier to cancer progression, demonstrated by various clinical studies. Studies using animals lacking different immune system components, revealed a heightened risk of cancer. Based on this, it was concluded that both the innate and acquired immune system protect against cancer (59, 60). In transplantation studies cancer cells from immunodeficient mice are not able to form new tumors in mice with a functional immune system (59-61). But in the opposite situation, the cells could metastasize. The assumption derived from this is that caner cells prevailing in a normal animal model, were weakly immunogenic, meaning they create a small response in the immune system. Having this characteristic enables cancer cells to successfully form tumors. However, tumors with highly immunogenic cells can also survive by disabling certain immune system processes (62, 63). In addition, there is the recruitment of immunosuppressive inflammatory cells that benefit cancer progression (64, 65).

There are also enabling characteristics that allow cancer cells to acquire the above-mentioned hallmarks (4):

- Genome instability and mutation**:** Mutated genomes will sometimes gain competitive advantages over the unaltered cells. They will proceed to eventually dominate in number compared to other genome types. These mutations results in certain cases to a more cancerous cell. The changes are not always mutations. Instead epigenetic mechanisms might be responsible (66-68). Although mutations occur regularly, the genome maintenance systems correct most of the errors in the DNA, keeping the mutation rate fairly low. However, the genomes of cancerous cells often mutates at a higher rate to achieve all the genes required for successful cancer development (69, 70). This occurs as a result of diminished resistance against the mutagenic agents, of a malfunctioning in the genomic maintenance machinery or through inadequacies of the DNA surveillance systems normally operating, especially by the tumor suppressor TP53 (71-73). Interestingly, since a cancerous cell without telomeres will gain DNA damage, telomerase seems to function both as a tumor promoter as well as a tumor suppressor (74).

- Tumor-promoting inflammation**:** It has long been known that certain tumors contain cells from the immune system, much like in tissues with inflammation (75). Now, through analysis with more sophisticated techniques, it appears that virtually every tumor contains such cells (76). Traditionally this gathering of immune cells was assumed to occur to eliminate the cancer tissue. However, for a while now, research has been indicating the opposite. The inflammatory response increases cancer malignancy, as many studies have shown (30, 44, 77, 78). Cells of the immune system are able to release mutagenic chemicals and reactive oxygen species that affect surrounding cancer cells (30).



Figur 1: Hallmarks of cancer, modified from Hanahan and Weinberg, 2011 (4)

4.2 Hypoxia

Hypoxia is a common feature in solid tumors. Tumors are dependent upon removal of carbon dioxide and waste products and of being supplied by oxygen and nutrients to grow. Initially during tumor growth, diffusion from the surroundings suffices to oxygenate the tumor. However, when the tumor reaches a certain size (about 2 mm), it needs to form new blood vessels to get enough oxygen for the cells to survive, a process called angiogenesis. The blood vessels often grow in an inadequate manner and are usually leaky (4). Due to improper blood vessel formation there are perfusion limitations in the tumor. Furthermore, the diffusion distances greatly limits oxygen saturation of the tumor. This leaves tumor cells in a hypoxic state. Thus, there are often large hypoxic regions in the center of tumors of moderate (4-10 µm) or larger sizes (79, 80). The oxygen concentration in tumors ranges from the normoxic periphery, through the hypoxic middle regions to the inner anoxic centre (81, 82). The low oxygen concentration centrally leads on to necrosis (82). The tumor regions

depleted of oxygen, but not to the degree of necrosis, enters a static phase without mitosis.

Despite the fact that the hypoxia of solid tumors can have negative consequences, it has been shown that hypoxia promotes tumor growth, angiogenesis and metastasis and reduces the efficacy of cancer therapies in several different fashions. Thus, cells that thrive in the hypoxic environment will harbour an aggressive phenotype leaving patients with poor prognosis (83-88)

Since hypoxia leads to more cancerous cells, hypoxia might therefore be an important target in cancer therapy. We therefore predict that targeting hypoxia might reduce or even remove some of the cancer characteristics discussed in section 4.1.2, resulting in a less malignant tumor. There are several ways of opposing the hypoxia, hyperbaric oxygen was chosen in this study.

4.3 Hyperbaric oxygen (HBO) therapy

Hyperbaric oxygen (HBO) therapy is to intermittently expose a subject to 100% oxygen at a higher than normal ambient pressure. Under normobar and normoxic conditions haemoglobin is approximately 97% saturated with oxygen, which equals 19.5 vol% The blood plasma, however, only has 0.32 vol% dissolved oxygen. HBO does not significantly alter the O_2 binding to haemoglobin, but increase the amount of O_2 dissolved in the blood. At a pressure of 3 atmosphere (atm) combined with 100% oxygen, plasma oxygen levels are raised to 6 vol%, a 95% increase. HBO treatment also markedly increases the diffusion range for oxygen in tissues (89). After HBO treatment, the oxygen partial pressure (pO_2) will remain elevated for up to an hour (90). Partial pressure equals the pressure exerted by each of the constituents of a mixture of gasses.

There are several laws explaining why hyperbaric oxygen therapy is a successful method for creating a hyperoxic tumor environment:

Henry's law: $\boldsymbol{C = kP_{gass}}$

Where C is the concentration of a gass in solution, k is the constant for the solution and P_{gass} is the partial pressure of the gass above the liquid. This law explains the balance between partial pressure of a gass in a gass mixture and the concentration of the same gas in a liquid when the liquid and gass mixture are in direct contact.

The relevance of Henry's law in this study is to understand how much of the oxygen in the lungs of the mice that will dissolve in blood plasma during HBO treatment.

Boyle's law: $\boldsymbol{PV = K}$

Where P er absolute pressure, V is volume og K is a constant. In a gas with a fixed temperature and amount, volume and pressure will be inversely proportional. When pressure is elevated, volume is reduced and vice versa. This law is important when considering how to compress and decompress an oxygen chamber with living animals inside.

Fick's principle: $\boldsymbol{VdC_i = F(C_a - C_v)\, dt}$

Where C_i, C_a and C_v are gass concentrations in tissue, arterial and venous blood respectively. F is perfusion, t is time and V is volume. This equation is used to find the amount of substance uptake, in this study a gas, for an organ or a tissue in a given time period. Uptake equals the gass concentration in arterial blood minus its concentration in venous blood multiplied by perfusion. Tissues will continue to receive the dissolved gass until equilibrium is reached between blood plasma and tissue. Fick's principle explains the rate of tissue oxygenation during HBO treatment if all oxygen is provided by the blood vessels. The inadequate blood vessel structure of tumors will of course influence the tissue oxygen saturation as well.

Hyperbaric oxygen is used in non-cancerous tissues for treating several diseases like decompression sickness, carbon monoxide poisoning, wounds and necrotic ulcers, as defined by the Undersea and Hyperbaric Medical Society (UHMS). HBO is also known to induce angiogenesis in areas lacking proper perfusion.

4.3.1 HBO and breast cancer

HBO in combination with cancer therapy, has for many years been studied with the main objective to evaluate if HBO had a tumor promoting effect. Two reviews have concluded that HBO does not enhance tumor growth (91, 92).

Feldmeier et al. (92) compared animal studies on HBO and all types of cancer from1966 to 2001 (93-108). There was no primary or metastatic tumor growth in 15 of 17 studies. Two of 17 studies showed growth, one model of lung cancer (105) and a chemically induced cancer model (103), although these two may not necessarily be reliable because of the experimental conditions. Tumor inhibitory results were gained in 4 studies (93, 94, 101, 108), and 2 studies had mixed results (103, 104). These diverging results probably reflect the many different variables, like the number of treatments and treatment pressure. Furthermore, Some tumors were induced by tumor transplants, others by cell injections or chemical induction,and many different tumor types were studied (in many different animals as well).

An unpublished review by Stuhr and Moen (2012) evaluated HBO studies from 2004-2012 and suggested that tumor types should be an important factor to look at when evaluating if HBO treatment works, since we know that no common treatment has been shown to be efficient in all types of cancer. They divided the research after cancer type and found that breast and head and neck cancers showed tumor inhibition when treated with HBO while cervical and bladder and prostate cancers were non-responders.

So several studies by our laboratory (109-115) and others (114) have shown that HBO inhibits different types of breast cancer *in vivo* and *in vitro*. They all used clinically relevant HBO protocols.

An anti-angiogenetic effect was often correlated with the tumor reduction of HBO (111, 112, 115, 116). Reactive oxygen species (ROS) were measured to be neutral indicating that differences in ROS concentration do probably not cause the tumor inhibition (111). HBO as an adjuvant for chemotherapy had a positive effect both *in vivo* and *in vitro* in several studies (111, 113, 117), however no effect was found in one study (115). The effect of HBO on metastases is still unsure. Studies of DMBA induced breast tumors exposed to HBO, induced increased cell death, reduced

proliferation and changed histology (110, 112). Most interesting was probably the shift in an entire gene program from epithelial to mesenchymal transition (EMT) to mesenchymal to epithelial transition (MET), indicating oxygen to be an important key in this switch to less malignant tumor.

When treating DMBA induced rat tumors with HBO and 5FU both separately and in combination, a significant inhibition of tumor growth was measured between controls and HBO (for both alone or combined) treated tumors. In addition the combined treatment inhibited tumor growth significantly more than HBO alone (113). Furthermore, the inhibitory effect was still found 12 days post treatment. Previous studies have discovered the same, although these particular studies used higher pressure or more exposures (94, 101, 104). The effect of HBO is, by all evidences, caused by the elevated oxygen pressure and not by the high pressure (94). The main HBO effect on the DMBA induced tumors is summarized in Fig. 1.

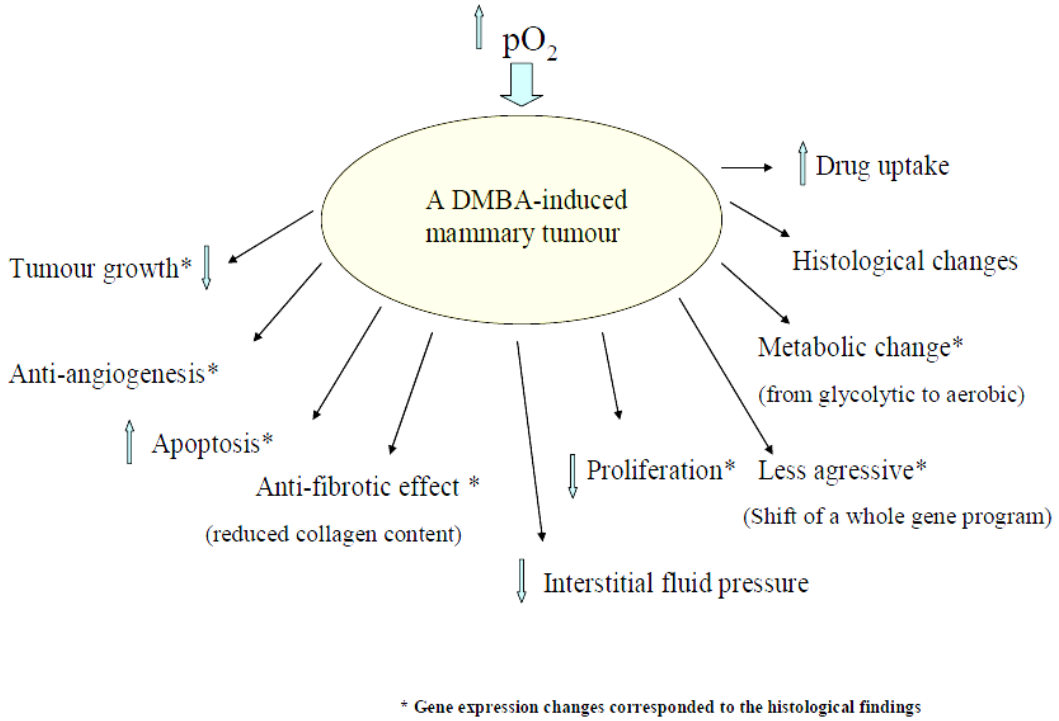


Figure 2: A summary of the HBO effects previously shown on DMBA (chemically) induced mammary tumors (118)

A similar inhibitory tumor growth response was found in a 4T1 murine mammary tumor model (109, 115), although not as pronounced as in the DMBA model.

Thus, HBO has shown positive effects against chemically induced and murine breast cancer models in previous studies at our laboratory. On this background the next step was to use a human breast cancer cell line to be more clinically relevant. It would be of interest to see if hyperbaric oxygen treatment could be used as adjuvant therapy in the future.

4.4 Aims

This study had the following aims:

1) To develop a human breast tumor (MDA-MB-231) in NOD/SCID mice after injecting human breast cancer cells into the groin area

2) To investigate if HBO (early or late treatment) have an effect on angiogenesis, proliferation and growth in the present mammary tumor model

3) To investigate if HBO potentiate the tumor inhibitory effect of chemotherapy in the same mammary tumor model

5. Methods and materials

5.1 Mice

A total of 44 female NOD/SCID (non-obese diabetic/severe combined immunodeficiency) mice (Taconic farms Inc, Denmark), weighing between 20 and 25 g were used. NOD/SCID mice have a reduced ability to produce T- and B-lymphocytes because of chromosomal abnormalities. They have less resistance against infections, and transplantations are more likely to be accepted by their bodies. (119) The NOD mice are used as model animals for type 1 diabetes and other autoimmune diseases (120)

The mice were kept in individually ventilated, pathogen-free cages (Makrolon IV, Techniplast Gazzada S.a.r.l., Buggugiate, Italia) at the animal facility at the Department of Biomedicine, Bergen, Norway. They had access to food (RMI pellets from Special Diets Service, Essex, UK) and water *ad libitum*, with a room temperature of 21 º C and air humidity of 40-60 %. The light/dark cycle was 12/12 hours.

All the experiments were approved by the Norwegian Committee for Animal Research (the Norwegian State Commission for Laboratory Animals and experimental procedures).

5.2 Anaesthesia

During cell injections and tumor size measurements, the mice were shortly anesthetized with Isofluran (Isobal®vet, Schering-Plough Animal Health) and N_2O gas. The anesthetic was added at a rate of 2-5 l/min. The mouse was first placed in a plexiglas anesthetic chamber flushed with O_2 (1.0 l/min) combined with compressed air (1.0 l/min). During the experiments the mice got anesthesia form a nozzle to the nose/mouth area. A heating pad kept the body temperature at 37 º C ± 0.5 º C. All mice were sacrificed by CO_2.

5.3 The cell-line

A human breast cancer cell line, MDA-MB-231, was used. This breast cancer cell line was obtained from American type Culture Collection, Rockville, MD, USA and was originally isolated from a 51 year old Caucasian female in 1973 by pleural effusions. MDA-MB-231 is a well-established cell line that is efficient in forming colonies. The cells have epithelial-like (spindle shaped) morphology.

5.4 Culturing of cells

The MDA-MB-231 cells were cultured in F12K medium (Bio-Whittaker, Verviers, Belgium) supplemented with 100 ml/L of fetal calf serum, 100 U/L of penicillin and 100 mg/L of streptomycin (Sigma-Aldrich, Steinheim, Germany). The cells were amplified as a monolayer in plastic tissue culture flasks 75 cm^2 (NUNC, Roskilde, Denmark) in a humidified incubator set at 37 º C with 5 % CO_2 and 95 % air, and were seeded until approximately 80 % confluence. All cell culture work was performed in a laminar flow bench with a HEPA filter (Thermo Scientific, USA) under sterile conditions.

5.5 Establishing tumors

We performed pilot studies to evaluate the amount of cells needed to develop tumors. In order to identify the number of cells/ml solution, the cells were trypsinized into a single cell suspension and counted using a cell nucleocounter.

200 µl of the single cell suspension were mixed with 200 µl of both buffer A and B (Bergman-Nucleocounter-chemometech, Allerød, Denmark) as described by the manufacturer.

The lysis Reagent A disrupts the plasma membranes of the cells, allowing the fluorescent dye propidium iodide (PI) to stain the nuclei. The Reagent A has a pH of about 1.25 and also contributes to disaggregate cell clusters. The stabilizing Reagent B was added after Reagent A in order to raise the pH value, because the PI is able to stain the DNA in the cells' nuclei more efficiently in an alkaline environment.

The solution was then centrifuged (LABINCO L46, Breda, The Netherlands) directly before being loaded into the nucleocassette (Bergman-Nucleocounter-chemometech, Allerød, Denmark). The nucleocassette is a disposable cassette filled with PI. The cassette automatically loads 50 µl of the cell solution. The PI stains the nuclei of the cell, making it possible for the cell nucleocounter to count the number of cells per ml. The total amount of cells/ml had to be multiplied by three due to the dilution caused by the added buffers.

The rest of the cell suspension was centrifuged (Heraeus instruments, Megafuge 1,0 R, Hanau, Germany) at 900 rpm for 4 min. The F12 medium was then removed and the PBS (Sigma-Aldrich-Company, Steinheim, Germany) was added to the cell pellet at an amount calculated as follows:

PBS (added):

$$\frac{\text{total amount of cells} * 0{,}15\ \text{ml}}{5\ \text{mill cells}}$$

The mice were then injected with 5 million cells in 0.15 ml MDA-231 cell solution in the fat pad on each side of their mammary crest in the groin area. By day 8, 100% of the mice had developed tumors ranging in sizes from 5-8 mm^3.

The mice were divided into six groups (Table 1): Controls (both early and late), HBO (both early and late), 5FU (the chemotherapeutic drug Fluorouracil 50 mg/ml. iv. Hospira Nordic AB, Stockholm, Sweden) and combined HBO and 5FU. The control and HBO group each contained 17 mice over all, while the 5FU and combined HBO and 5FU group each contained 5 mice. To distinguish between the mice within each group, their tails were marked using a permanent marker.

Table 1: The different experimental groups:

Experimental groups		Number of mice	Gas	Ambient pressure (bar)	Days from injection to treatment	Days of treatment	Exposure time (min)	Number of exposures
Controls	Early	10	Air	1	7	11	-	-
	Late	7	Air	1	24	11	-	-
HBO	Early	10	100% O_2	2.5	7	11	90	4
	Late	7	100% O_2	2.5	24	11	90	4
5FU		5	Air	1	7	11	-	-
HBO/5FU		5	100% O_2	2.5	7	11	90	4

5.6 HBO treatment

The Hyperbaric Animal Research Chamber OXYCOM 250 ARC (HYPCOMOY, Tampere, Finland) was used (Fig 2). This is a cylindrical pressure chamber in which the inner diameter is 25 cm, the inner length is 55 cm and the volume is 27 liters. The chamber is equipped with a gas in- and out-let and a manometer monitoring pressure in the chamber. To prevent fire in a pure O_2 environment, no litter was allowed into the chamber.

First the chamber was flushed with pure O_2 (medical quality) until reaching 100 % O_2 (~15 minutes). Then the pressure was slowly increased from 1 to 2.5 bar (equivalent to 15 msw) over approximately 10 minutes. Over a period of 90 minutes, the atmosphere inside the chamber was kept at this level. To ensure an atmosphere containing >97 % O_2 at all times during the 2.5 bar period, the chamber was flushed with pure O_2 for 5 minutes every 10-15 minutes. After the treatment the chamber was slowly decompressed to 1 bar (~ 10-15 minutes). The mice underwent this treatment on days 1, 4, 7 and 10. Day 1 being either 7 days (early treatment) or 24 days (late treatment) after the injection of tumor cells.

Figure 2: The hyperbaric oxygen chamber

5.7 Chemotherapy

The chemotherapeutic drug Fluorouracil (5FU) (Hospira Nordic AB, Stockholm, Sweden) was given as 50 mg/ml. iv. in all the mice in the 5FU group and the 5FU/HBO group on identical days as the HBO treatments (Day 1, 4, 7 and 10). The preparing of 5FU injections was performed under an extractor hood. A dose of 1.5 mg/kg in 0.2 ml saline was injected intraperitoneally between the tumors, immediately prior to the HBO treatment in the combined group.

5.8 Measurements of tumor growth

The tumors were measured with a caliper on days 1, 4, 7 and 11 or day 1 and 11. The location of the tumor did only allow measurements in two dimensions. The best estimate of tumor volume given these restrictions was a calculation based on a cylindrically shaped tumor according to this equation:

$$(\pi/6) * a^2 * b$$

where a is the shortest and b is the longest transversal diameter.

On day 1 if the tumors had an abnormal shape, this was drawn on a schematic mouse to ensure that the tumor was measured equally along the same axis on the remaining

days. Additionally, body weights of mice were measured on the first and last day to get an indication of any treatment influence on their health.

5.9 Immunohistochemistry

On the last day of measurements, the tumors were quickly dissected out, snap frozen in liquid nitrogen and stored at –80ºC until further use. The frozen tumor tissue was embedded in Tissue Tek (Sakura Fintek Europe, Zoeterwounde, the Netherlands) and cut into 10-20 µm slices with a cryostat microtome (Leica CM 3050 S-Cryostat, Nussloch, Germany).

5.9.1 CD-31

To stain for tumor blood vessels, CD-31 was used. CD-31 is also called platelet endothelial cell adhesion molecule (PECAM-1). It's a protein that is present on the surface of platelets, monocytes, neutrophils and certain varieties of T-cells and targets blood vessels. The frozen slides of tumor (20 µm) were immunostained with rat anti-mouse CD-31 (AbD serotec, Morphosys UK Ltd, Oxford, UK) as primary antibody This is a monoclonal antibody, which means that the antibody was produced by cells that were clones of each other. Biotinylated rabbit-anti-rat (Vectastatin ABC kit, peroxidase Rat IgG PK 4004, Bioteam AS, Trondheim, Norway) was used as secondary antibody. Prior to applying the antibodies, rabbit serum was used as a blocking agent to avoid nonspecific binding of the secondary antibody. Further, H_2O_2 in methanol was used to quench endogenous peroxidases that the Diaminobenzidine tetrahydrochloride (3.3 DAB, Sigma-Aldrich, Germany) would otherwise bind to. An avidin biotin peroxidase complex was used to bind to the biotinylated secondary antibody. To visualize the blood vessels, the chromogen DAB was used. DAB functions as an electron donor, and when oxidized it gets a dark brown color and becomes insoluble. Richardssons stain was used to counterstain the rest of the tumor tissue. The cross-section of CD-31 positive structures was quantified per mm^2 using a counter grid with 100 x magnification. The entire tumor was measured for cross-sections. The blood vessel diameter was also measured. Within each tumor 100 adjacent blood vessels were measured and grouped in 2 µm range clusters. Both measurements were performed on a Nikon Eclipse E600 microscope (Nikon

Corporation, Tokyo, Japan), using a Nikon Digital Camera DXM 1200F (Nikon Corporation, Tokyo, Japan) on pictures of 100 x magnification. The Computer-software program NIS-Elements AR 3.2 64-bit (Laboratory Imaging Ltd, Prague, Czech Republic) was used.

5.9.2 KI-67

To be able to quantify proliferating cells, and hence how aggressive the tumor is, we used KI-67. The KI-67 protein is an antigen associated with cell proliferation because it is detectable in all active phases of the cell cycle, but absent in resting phase. By staining for KI-67, it's possible to determine how many of the tumor cells that were in a state of mitosis. It's also possible to find out where in the tumor most proliferation occurs. The 10 µm thin tumor tissue slides were fixed with acetone. Then the primary antibody was monoclonal mouse anti-human KI-67 Antigen (Dako Denmark A/S, Glostrup, Denmark). One slide was immersed in Mouse IgG1 (Dako Denmark A/S, Glostrup, Denmark) to serve as a negative control. The secondary antibody was labeled polymer-HRP anti-mouse (Dako Denmark A/S, Glostrup, Denmark). DAB (Dako Denmark A/S, Glostrup, Denmark) was used as a chromogen, with Richardson's stain as counterstaining. For each tumor, 4 cross-sections were quantified with a counter grid by counting proliferating and non-proliferating cells separately. The Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan) and a Nikon Digital Camera DXM 1200F (Nikon Corporation, Tokyo, Japan) were used to take pictures of x100 magnification. The Computer-software program NIS-Elements AR 3.2 64-bit (Laboratory Imaging Ltd, Prague, Czech Republic) was used.

5.10 Haematoxylin-Eosin staining

Two frozen section from each group was stained with Haematoxylin-Eosin (HE) to be able to visualize any changes in histology between the treatment groups. This was performed, but needs to be evaluated by a pathologist.

6. Results

6.1 Establishing tumors

Pilot studies showed that a total of five million cells were found to be efficient in forming tumors when injected into groin area of the mice. Thus, five million MDA-MB-231 mammary tumor cells were injected subcutaneously in the groin area in all our experiments. They formed tumors in the female NOD/SCID mice with a 100 % take within 7 days.

6.2 Body weight

Throughout the experimental period, the mice maintained stable body weights regardless of treatment, indicating no negative treatment effects.

6.3 Tumor growth

6.3.1 Early HBO treatment - 7 days post cell injection

A total of 60 MDA-MB-231 mammary tumors, divided between the four different groups, were measured as described under section 5.8 either day 1, 4, 7 and 11 or only day 1 and 11. The average tumor size day 1 for all groups was between 100 and 150 mm^3, and the control tumors increased by approximately 100% within the 11 day experimental period.

In the early HBO, 5FU and combined treatment group, tumor growth was significantly reduced compared to the controls (Fig 1). However, subjected to the same treatment, the tumors showed a wide range of responses, indicating the large heterogeneity within the tumors.

HBO induced a significantly greater inhibition of tumor growth compared to 5FU after 11 days. Furthermore, after 11 days, the combined treatment reduced tumor growth significantly compared to 5FU alone (Fig 3). There was, however, no significant difference between tumor size in the HBO and combined treatment group.

Thus, HBO did not potentiate the tumor growth reduction by 5FU in this tumor model.

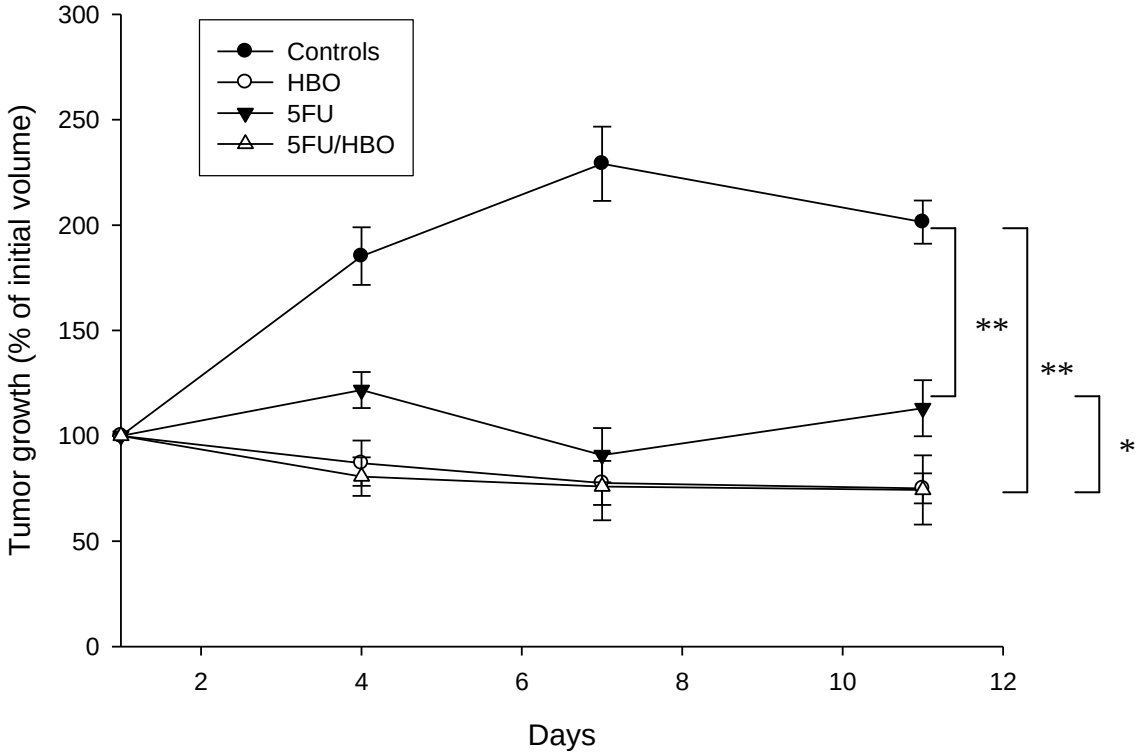


Figure 3: MDA-MB-231 human mammary tumor growth (% of initial volume) in controls (n=20), hyperbaric oxygen (HBO) (n=20), 5-Fluorouracil (5FU) (n=10) and combined HBO and 5FU (n=10) treated tumors over a period of 11 days. This early treatment was initiated 7 days post cell injection. Treatments were given day 1, 4, 7 and 10. Mean ± SEM. *$p<0.01$ **$p<0.0001$ compared to controls or 5FU.

6.3.2 Late HBO treatment - 24 days post cell injection

To compare an early treatment effect of HBO (Fig 4A) with a late treatment effect (Fig 4B), an additional 28 MDA-MB-231 mammary tumors, separated in early and late control and HBO groups, were measured.

Tumor size was significantly reduced in the early HBO treated tumors compared to controls during the 11 day period (Fig 4A), while exposing the mice to late HBO did not induce a significant reduction in tumor growth other than at the 4 day mark. (Fig 4B) Thus, early HBO treatment induced a statistically significant tumor inhibitory effect, while late HBO treatment did not.

A

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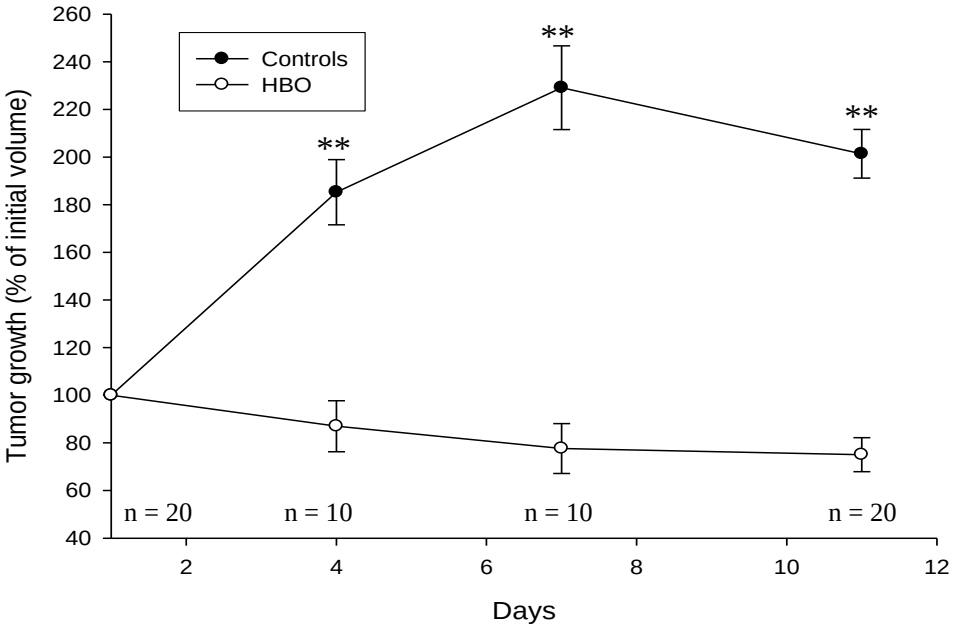


B.

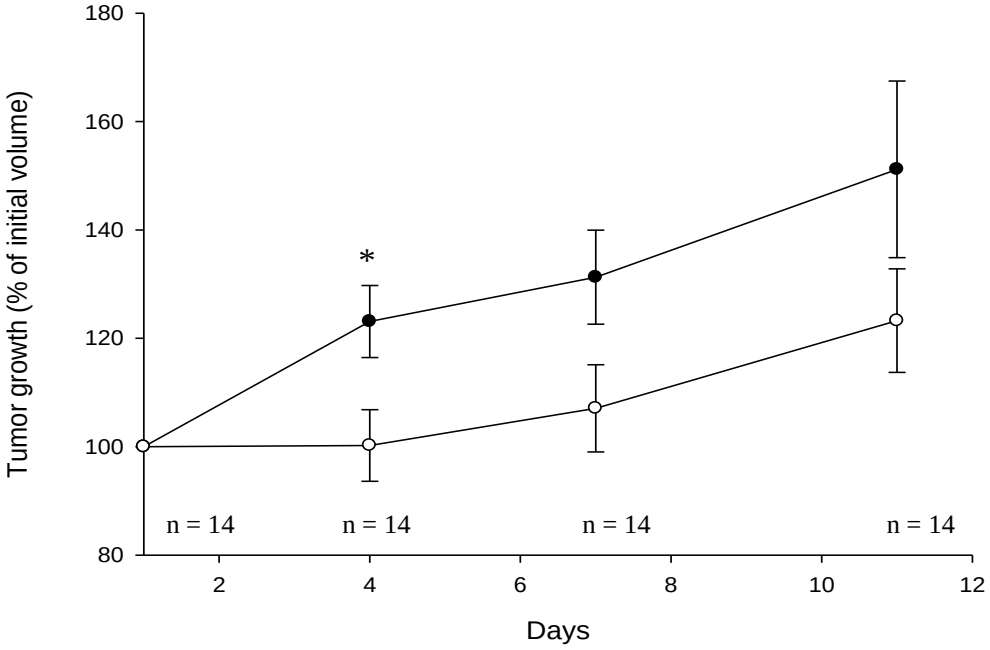


Figure 4: MDA-MB-231 human mammary tumor growth (% of initial volume) over a period of 11 days. A. Early treatment was initiated 7 days post cell injection. B. Late treatment was initiated 24 days post cell injection. Treatments were given days 1, 4, 7 and 10. Mean ± SEM. *p<0.01, ** p<0.0001 compared to controls.

6.4 Tumor blood vessels

Since angiogenesis is known to greatly influences tumor growth, blood vessels were stained using rat anti-mouse CD31 as primary antibody as described under section 5.9.1.

The average tumor blood vessel density, was approximately 13 vessels/mm^2 in all the treatment groups (Table 2). Thus, there was no statistically significant difference between the groups.

The average tumor blood vessel diameter was approximately 7 µm in controls and HBO regardless of early (Fig 5A) or late (Fig 5B) treatment. Thus, there was no significant difference between the groups (Table 1). In these MDA-MB-231 tumors there was a heterogeneous blood vessel diameter distribution within each tumor, as can be seen Fig 5.

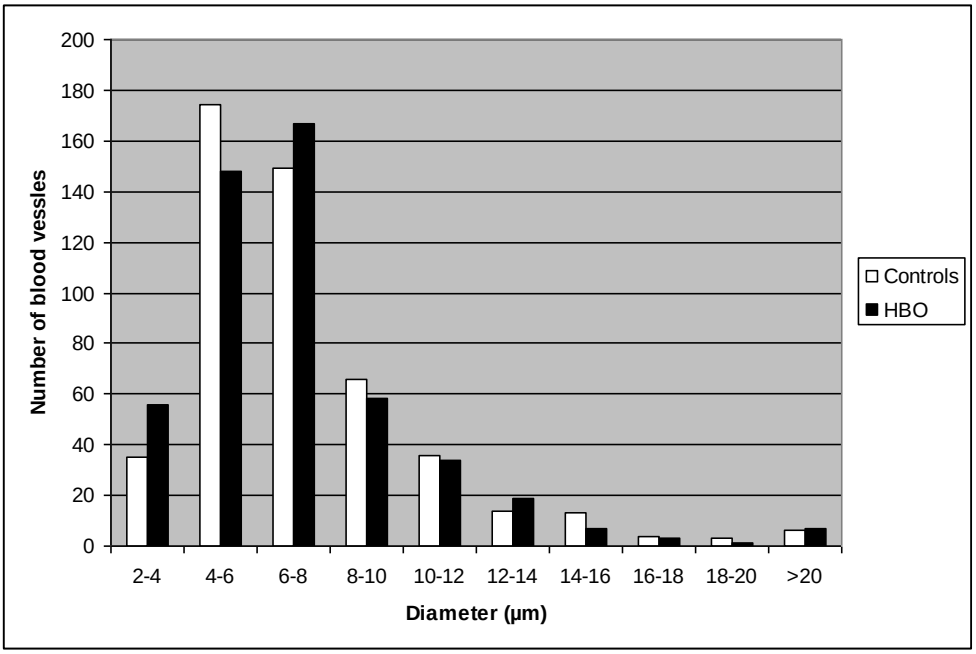
Based on this, the tumor growth inhibition in the early HBO treated tumors is probably not attributed to blood vessel density or size. HBO does not appear to affect angiogenesis in this study.

Table 2: Immunhistochemical analysis

Tumor blood vessel density and diameter (CD31) and proliferation (KI67) in MDA-MB-231 human mammary tumors from controls and hyperbaric oxygen (HBO) treated tumors. n = 5 for all groups. Mean ± SEM.

	Early treatment **7 days post cell injection**		**Late treatment** **24 days post cell injection**	
	Controls	**HBO**	**Controls**	**HBO**
Pressure pO_2	**1 bar**	**2.5 bar**	**1 bar**	**2.5 bar**
% O_2	**20**	**100**	**20**	**100**
Blood vessels density (number/mm^2)	13.5 ± 1.59	12.4 ± 1.75	13.5 ± 0.79	13.4 ± 0.65
Blood vessel diameter (µm)	7.3 ± 1.65	7.2 ± 1.83	7.4 ± 1.35	7.6 ± 1.48
Proliferation (% of Ki67 positive cells)	0.31 ± 0.03	0.28 ± 0.01	0.36 ± 0.02	0.4 ± 0.04

A



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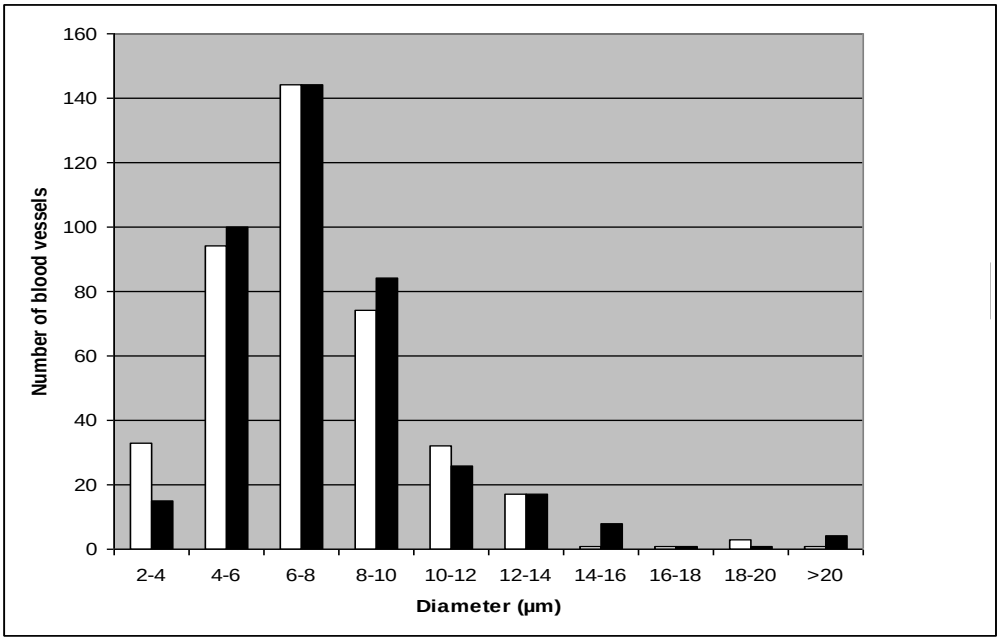

Figure 5: Blood vessel diameters in MDA-MB-231 human mammary tumors in controls and hyperbaric oxygen (HBO) treated tumors after 11 days of treatment. A. Early treatment was initiated 7 days post cell injection (n=5) B. Late treatment was initiated 24 days post cell injection (n=4-5).

6.5 Proliferation

The proportion of a tumor's cells that are in an active stage of the cell cycle could possibly contribute to or indicate the degree of malignancy of the tumor. For this reason, immunostaining was performed for proliferating cells using the Ki67 staining method as described under section 5.9.2.

In the early treated group, the average proportion of proliferating in relation to total number of cells, were approximately 0.30. (Table 2)The corresponding value for the late treatment group was between 0.35 and 0.40. However, there was no statistically significant measurable difference between the HBO treated and the control tumors for neither the early nor the late treatment group.

Based on this, the tumor growth inhibition observed in the early HBO treated tumors is probably not attributed to reduced density of proliferating cells. HBO does not appear to affect proliferation in this study.

7. Discussion

The methods and results will be discussed in this section before ending up in a conclusion:

7.1 Methodological aspects

7.1.1 The cell line

MDA-MB-231 was used because it is a well-established cell line used for both *in vitro* and *in vivo* studies. The intent of using a human cell line was to make the study more clinically relevant, and being an addition to the two other mammary tumor models (one murine and one chemically induced) already studied using hyperbaric oxygen (109-112, 115, 116) at our laboratory.

7.1.2 NOD/SCID mice

Mice are suitable model animals because they breed well in a lab environment, they do not require large housing facilities and are easy to handle. The cons are that their fur and urine contains allergens, putting people working in the lab at risk of allergies. Weighing ethical considerations against body similarities, mice make one of the best compromises for human studies. Immunodeficient mice were used because they are less likely to reject foreign tissues in their bodies, an advantage when human cells are injected, as performed in our study. They are disease-free and genetically very similar, which makes our experiments more accurate with fewer variables to take into consideration. In this study, the cells were injected in animal models. *In vivo* method is preferred over *in vitro* studies because the conditions required to investigate are mostly unattainable in vitro. This is due to the tumor complexity, since the surrounding tissues (stroma) will infiltrate the tumor and is shown to strongly influence tumor growth. As a measure to prevent using more model animals than necessary, complying with the guidelines from the local ethical committee, each mouse was injected with two tumors. Female mice were used because they serve as an ideal hormonal microenvironment, since mamma tumors will increase much faster in size when implanted in female mice.

7.1.3 The anesthesia

Isofluran was used because the animals were supposed to wake up quickly after procedures. N_2O gas ensures a faster isofluran uptake. Although these substances can be harmful in high doses or during long term exposure, little to no side effects are expected when administered as in this study. Thus, this was considered the best method of anaesthesia for the present study.

7.1.4 Culturing of cells

F12K medium was used because it ensures the best growth conditions for this cell line and was originally developed primarily for human primary cells. Sterile conditions are to prevent contamination, so as to make sure the cells injected are all MDA-MB-231. With each cell split, the cell line is marked as a higher passage. The lower passages, that we used, are preferred because the likelihood of unknown mutations is less. The cell line has nevertheless regularly been tested to confirm that it still is MDA-MB-231.

7.1.5 Establishing of tumors

Cell injections were chosen over direct tumor implantation because previous studies have shown that if pieces of a tumor of foreign origin is to be implanted in a model animal in order to get tumor formation, success rates are very low (118). The take rate is, however, higher for cell injections. The 100% take rate indicates that the MDA-MB-231 cell line is a suitable human cell line for tumor experiments in the NOD/SCID mice. Although advantages of implanting bits of tumor, is that you would get part of human tumor stroma, making it more like a human cancer case. The mice were under 3 months old when used in these experiments to avoid them regaining their immune system.

7.1.6 HBO-treatment

The oxygen chamber can catch fire because of the pure oxygen interior. This is avoided by keeping it litter and oil free. The ambient pressure needed to be a compromise between effect on tumor inhibition and avoiding toxicity, and a 2.5 bar

ambient pressure fits these criteria. This evaluation is based on previous studies from our laboratory. A prolonged exposure of higher oxygen partial pressure might have resulted in oxygen toxicity, inducing seizures, short-sightedness, inner ear trauma or lung problems. The experimental setup, including number of days from cell injection to sacrifice, and exposure time to HBO and time in between was the same as previous studies from our group so as to compare. The late treatment started where the longest previous studies had cut off (17 days) to evaluate what would happen if treatment started at this later stage. We had a conservative decompress and compress rate to avoid volume expansion or compression of the gas-filled cavities in the bodies of the mice, according to Boyle's law.

7.1.7 Chemotherapy

The drug 5-Fluoruracil was used because it is a commonly used chemotherapeutic drug that is readily available. We injected between the tumors to ensure that the drug would be able to affect both of them. The chosen dosage is a compromise between effect and toxity and is perhaps a bit lower than anticipated since HBO reduced tumor sizes more than 5FU alone.

7.1.8 Tumor growth

The tumors were measured externally with a calliper. This was considered the best way to do it since the mice needed to be alive for the rest of the test period. Measurements were performed in two dimensions. The formula assumed a cylindrical shape and this is not exactly correct but the best option under the circumstances. Since the same person measured all tumors, the inaccuracies would be minimal between controls and HBO tumors.

7.1.9 Immunohistochemistry and quantification techniques

CD31 is commonly used for staining blood vessels, and our primary and secondary antibodies are well established. CD31 could possibly also stain lymph tissues, since it contains the same target molecules, thus creating faulty results. However, for a tumor it is suitable, since tumors generally do not contain lymph tissue.

The quantification of blood vessels involved manually counting blood vessels. This method is vulnerable to human error, but the same person did all measurements, so inaccuracies will ideally be equally big between groups. This was nevertheless considered a good method, because a person can better use their judgement to distinguish what is a vessel than a computer software due to the non-uniform tumor blood vessels. The entire cross-section area was quantified, making data more reliable.

However, the diameters were measured by a computer to increase accuracy. Five representable areas (x100 magnification) for each tumor was chosen. This is a reliable estimate for the blood vessel diameters for the tumor as a whole.

The KI67 method is also a common immunostaining procedure. The stained cells were counted manually in addition to non-proliferating cells. This tedious method is more accurate than a computer measuring areas specifically colored vs uncoloured areas of the tumor. A possible source of error is poor staining, which makes distinguishing proliferating cells from non-proliferating cells difficult. Four representative areas (x100 magnification) from the tumor cross-sections were quantified as an estimate for the entire tumor. We alternated between HBO and control slides when counting to even out subjective bias.

A possible source of error concerning immunohistochemistry, is that in some cases two tumors deriving from the same individual, were used, while other mice were just represented by a single tumor. Since different genetic processes occurred in the different mice, despite their over all very similar gentic profile, this could be a problem. The variation would be much less between two from the same mouse compared to two from different mice, affecting the statistical significance of the data.

7.2 Evaluation of results

7.2.1 Body weight

Weight changes can be a good indicator of health and disease in the model animals. A significant weight loss could be unethical. However, the body weight remained stable

throughout the experiment, indicating no influence of HBO, chemotherapy or cancer progression.

7.2.2 Tumor growth

These tumors did not grow in an exponential manner, possibly because the cells were obtained from a different species.

Tumors responded in general relatively heterogenic to the same treatment. This strengthens the hypothesis that the genome of cancer cells is unstable and prone to mutations.

Early treatment: The present study showed a significant MDA-MB-231 mammary tumor growth inhibition after early HBO treatment. The hyperoxia has thus executed some type of anti-cancer function. This corresponds to what is previously found in our lab in both chemically (110-112, 116) and murine (109, 111, 115) mammary tumors. Kluft et al. showed an inhibition of transplanted mammary carcinomas in C57 black mice after HBO treatment (114). Granowitz et al. demonstrated strong anti-proliferative effect of HBO on different mamma cancer cells in vitro (117). Thus, HBO seem to have an inhibitory effect on mammary tumors in general.

Since angiogenesis is important for tumor growth, differences in tumor blood vessel density is one possible cause for the differences in size between the controls and HBO measured in the MDA-MB-231 tumors. However, blood vessel density and size was unchanged. Thus, hyperoxia did not affect blood vessels in these MDA-MB-231 mammary tumors. This is in contrast to what was found in intermittently HBO exposed DMBA and 4TI tumors, and might be due to the difference in tumor type.

The anti-tumor effect found after HBO could also be due to reduced proliferation of tumor cells. However, the anti-tumor effects were not caused by a change of density of proliferating cells as shown in our study. This is in contrast to the anti-proliferation effect of HBO found in DMBA tumors *in vivo* and in different mammary tumor cells *in vitro* (117).

However, the inhibitory effects of HBO on the tumors might be due to enhanced density of apoptotic cells and/or different levels of ROS.

ROS: Reactive oxygen species, or free radicals, occurs in all eukaryotic cells during aerobic metabolism. Normal tissues experiences lower levels of ROS that are furthermore properly controlled by antioxidants (121, 122). ROS can be induced by hypoxia, reoxygenation (reperfusion) or hyperoxia (as in our experiment). Cancer tissue, in contrast to non-cancerous tissues, fluctuates between periods of hypoxia and reperfusion due to poor vasculature. This is a major cause of oxidative stress in tumors, which increases ROS production (123). ROS can be harmful for lipids, proteins, carbohydrates and nucleic acids (124). Heightened levels of ROS, causes breaks of the DNA. The mutations that ensue may promote oncogenic transformation and apoptosis malfunction (125, 126). Studies strongly indicate a connection between ROS and carcinogenesis (121, 127, 128).

When ROS levels are lower, by tumor standards, initially in cancer development, it has tumorigenic properties. However, when reaching sufficiently high levels, ROS has a toxic effect on tumors, as demonstrated in various experiments (122, 129). The HBO might ensure that ROS reaches this limit and cancer cells are destroyed, which several studies can attest to (130, 131).

The added oxidative stress of ROS induces apoptosis at first. Interestingly though, as cancer progresses to a highly malignant stage, apoptosis is down-regulated and antioxidants activated, and the cancer cells can form a colony (121). However, if treatment is continued long enough, the antioxidant system will weaken and lose its ability to counteract ROS. This is called the “threshold effect” Apoptosis and permanent damage is inflicted upon cancer cells as a result (122, 132-134). This might be how HBO inhibits tumor growth, in the cancer cases were HBO has this effect. Nevertheless, if ROS was enhanced after HBO, we would expect a similar inhibitory effect after late HBO treatment. Furthermore, ROS was unchanges in the DMBA study (111) after HBO treatment, indicating this to less likely be the reason for tumor growth inhibition in our study.

Another explanatory mechanism is the Hypoxia inducible factor-1 (HIF-1). It regulates cellular processes and is strongly influenced by oxygen (135). If HIF-1 is exposed to a normoxic environment, the HIF-1α subunit is degraded. In contrast, hypoxia will stabilize HIF-1α. A functional HIF-1α causes transcription of genes for

proteins promoting O_2 delivery (VEGF-vascular endothelial growth factor, erythropoietin). A downregulation of HIF during HBO would nevertheless also induce an anti-angiogenic effect we could not demonstrate However, it improves the metabolism in oxygen-poor surroundings by various adaptations. (136) making them less aggressive.

Late treatment: In the late treatment group there was a trend towards reduced tumor growth after HBO treatment. However, it was not significant because of the big deviation in responses. Thus, giving HBO at a late stage does not have an effect on this human mammary tumor model, indicating that elevated oxygen influences the internal genetics and or metabolic factors involved in the early development of these tumors. It seems that to treat this type of tumor, an early treatment is necessary to obtain desirable results.

HBO and chemotherapy:

HBO has previously been shown to enhance the chemotherapeutic effect in different solid tumors (111). However, the combined HBO/5FU therapy caused approximately the same degree of tumor inhibition as HBO alone. This might tells us that HBO did not potentiate the uptake or enhance the effect of chemotherapy in the present human MDA-321 tumor model, that our metod of giving the drug ip between the two tumors was inefficient, or that the drug amount was not enough. The interstitial fluid pressure might have been too high for the drug to reach the tumor cells.

There were several reasons we expected HBO to enhance the chemotherapeutic effect as a tumor inhibitor. In previous studies by our group, they've demonstrated HBO as an adjuvant to chemotherapy (111, 113). Underlying reasons are further discussed. Hypoxic cells enter a static phase of the cell cycle. This presents a dilemma for radiotherapy and chemotherapy that selectively targets cells undergoing high levels of mitosis, as shown in experiments(137). Hypoxic tumors are generally denser in tissue composition than their surroundings, and the tumors present interstitial hypertension (138).

An attempt to understand the underlying mechanisms for the tumor inhibitory effect of HBO, my speculations: It is possible that an oxygen deprived tumor that is exposed

to a rich supply of oxygen, will perceive this as a sign that it did not just get more oxygen, but also more nutrients and removal of waste products and carbon monoxide. This seems legit, because in nature, more oxygen is always associated with blood vessels. In this artificial setting, it only gets the oxygen and nothing else. So the tumor "thinks" it has all these other benefits and turn off genetic mechanisms that it "believes" to be unnecessary. The result: tumor inhibiton.

7.3 Conclusion

Aim 1: To develop a human breast tumor (MDA-MB-231) in NOD/SCID mice after injecting human breast cancer cells into the groin area

We successfully developed a human breast cancer model in NOD/SCID mice.

Aim 2: To investigate if HBO (early or late treatment) have an effect on angiogenesis, proliferation and growth in the present mammary tumor model

Hyperoxia had a strong inhibitory effect on MDA-231 mammary tumors if HBO was administered early, but not if HBO was given late. Hyperoxia did not influence angiogenesis or proliferation in the present model.

Aim 3: To investigate if HBO potentiate the tumor inhibitory effect of chemotherapy in the same mammary tumor model

HBO did not potentiate the effect of chemotherapy on the present mammary model.

7.4 Further studies

In the future, studies aimed at elucidating the genetic mechanisms underlying the tumor inhibitory effect of HBO, by use of proteomic and genetic analysis could be of value, in order to investigating exactly how this effect occurs and what particular mechanisms are involved.

Detailed studies of tumor pathology after HBO exposure could reveal morphological changes.

It would also be of interest to combine HBO with other treatments to measure the combined effect, in addition to understand the results obtained at a genetic level.

Since different tumor types respond radically different to the same treatment (HBO), it would be interesting to pin point the exact qualities of each tumor type that make them either responders or non-responders.

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