A reduction in the interstitial fluid pressure per se, does not enhance the uptake of the small molecule weight compound 5-fluorouracil into 4T1 mammary tumours

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Abstract

The tumour interstitium represents a major barrier to drug delivery and modification of the tumour extracellular matrix (ECM) is one strategy that could promote better delivery. We have focused upon three factors in the tumour interstitium that could influence drug uptake into the tumour tissue; the interstitial fluid pressure (P_{if}), collagen content and the tumour blood vessel density (TBVD). Two treatment groups were used: repeated hyperbaric oxygen (HBO) and single HBO (both to 2.5 bar, 100% O_2, à 90 min). The controls were exposed to normal atmosphere (1 bar, 21% O_2). P_{if}, angiogenesis, collagen content and uptake of [H^3]-5FU ([5-fluorouracil) was investigated. P_{if} and TBVD significantly decreased after hyperoxic treatment, without any change in collagen content. Uptake of 5FU was not affected by hyperoxic treatment. Thus, a reduction in P_{if} *per se* does not enhance the uptake of 5FU in 4T1 mammary tumours. The fibrotic ECM (unaltered collagen content) together with a less dense microvasculature might help explain this.

Introduction

One of the major challenges in cancer therapy is tumour resistance to chemotherapy. Access to the solid tumour, by systemic administration of chemotherapeutic agents, is gained via the blood supply. The drug must then penetrate through the tumour microenvironment to reach the cancer cells in sufficient amount to reach a concentration resulting in lethal toxicity. Thus, factors in the tumour microenvironment like interstitial hypertension (elevated P_{if}), a dense ECM and abnormal tumour blood vessels have been shown to contribute to resistance to chemotherapy.[1-6]

In order to grow and expand the tumour has to develop new blood vessels from pre-existing vasculature, by angiogenesis. The induction of angiogenesis is a consequence of an imbalance between multiple inhibitor and stimulator molecules, referred to as the *angiogenic switch*.[7] The microvasculature in tumours has severe structural and functional abnormalities as well as being surrounded by a high density of tumour cells compressing the blood vessel. These factors cause inadequate blood flow in the tissue which again might impede the delivery of chemotherapeutic agents.[8]

The tumour microenvironment is further constituted by a pathologically increased P_{if}, assigned to form an obstacle to the uptake of chemotherapy.[9] The elevated P_{if} leads to decreased transcapillary transport, thereby, altering the influx of anticancer drugs into the tumour tissue.[10] The mechanisms behind the elevated P_{if} in tumours is not yet fully understood, but high vascular permeability, irregular vascular architecture, non-functional tumour lymphatic's, a high collagen content and increased contractibility of fibroblasts has been pointed to as contributing factors.[9,11-13]

After the drug molecule has entered the interstitium, the drug transport and penetration are dependent on the composition and conductivity of the interstitium.[14] The remodelling of the extracellular matrix (ECM) during tumorigenesis, involve an increase in collagen content as well as increased contractility of fibroblasts, resulting in a denser and more rigid tumour microenvironment. Netti *et al*. showed that tumours with a more extended collagen network were more penetration-resistant.[3] Furthermore, a study degrading collagen enhanced the interstitial diffusion-rate and intra-tumoural delivery of drugs.[4] Thus, a dense ECM will impede the transport of macromolecules in the tumour interstitium, making transport of chemotherapeutic agents more difficult.

Previous studies have shown that hyperbaric oxygen treatment (HBO) potentiates the effect of chemotherapeutic agents like doxorubicin, alkylating agents and 5FU.[15-18] HBO involves the administration of pure oxygen at higher than normal atmospheric pressure. Thus, increased pressure, coupled with the inspiration of 100% oxygen, substantially increase the amount of oxygen dissolved in the plasma, independently of the haemoglobin and the pO_2 is elevated for a substantial time after decompression.[19] It has previously been shown that hyperoxia significantly reduces the number of blood vessels in DMBA-induced mammary tumours as well as in gliomas.[20,21] We have also shown that hyperoxia induce a reduction in P_{if}, in addition to inducing an increase in the uptake of 5FU into the DMBA induced mammary tumours in rats, when measured directly post-treatment.[22] The present study is an extension of these studies and performed to elucidate if this was applicable also for other mammary tumor models. We wanted to determine if hyperoxia alters P_{if}, blood vessel density and collagen content in the 4T1 murine mammary tumour model, and, if such ECM changes would enhance the uptake of 5FU into the tumour tissue.

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Materials and Methods

Mice

Female NOD/Scid mice (Taconic farms Inc, Denmark), weighing on average 20 g, were used. The animals were housed in individually ventilated pathogen-free cages at the animal facility at Department of Biomedicine, Bergen, Norway, and had access to food and water *ad libitium*. All animals were anesthetized by Isofluran (Isobal®vet, Schering-Plough Animal Health) and N_2O gas during the experiments, the only exception being the microdialysis protocol. The latter procedure was performed with a subcutaneous injection of Midazolam (Dormicum, F. Hoffmann-La Roche AG, Basel, Switzerland) in combination with Fentanyl/Fluanison (Hypnorm, Janssen Pharmaceutical, Beerse, Belgium) (Hypnorm-Dormicum) due to the need for immobilization of the animal over an extended time period. All animals were

sacrificed with saturated KCl during anaesthesia. All experiments were performed in accordance with recommendations of the Norwegian State Commission for Laboratory Animals, and were approved by the local ethical committee.

4T1 cell line and culture conditions

The murine 4T1 mammary cell-line was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in standard plastic tissue culture flasks 75 cm^2 with RPMI-1640 medium supplemented with 10% Foetal Calf Serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 % L-glutamine (all from Sigma-Aldrich, Steinheim, Germany). The cells were amplified as a monolayer at 37°C in a humidified incubator with 5 % CO_2 and 95 % air, and were seeded until ~ 80% confluence. Cells were harvested with Trypsin (Sigma-Aldrich, Steinheim, Germany) and washed in PBS before making a final cell suspension in PBS.

In vivo experiments

All mice were given 17β-estradiol in the form of a pellet (0.18 mg/pellet- 60 day release) (Innovative Research of America, Sarasota, FL, USA) s.c. in the neck area. $3.0x10^6$ cells dissolved in 0.15 mL PBS were injected s.c. into the mammary fatpad of the groin area, and developed tumors within 6 days of injection.

Experimental groups and treatment design

The different experimental groups and their treatment details are given in Table 1.

Hyperbaric oxygen treatment

A hyperbaric Animal Research Chamber OXYCOM 250 ARC (HYPCOM OY, Tampere, Finland) with an inner diameter of 25 cm, inner length of 55 cm and a volume of 27 L was used. The chamber is equipped with a gas in- and out-let, and a manometer for pressure chamber monitoring. 100% oxygen content was obtained over a time frame of 15 minutes by flushing the chamber with pure O_2. The pressure was raised over a period of 5 min to 2.5 bar (15 msw), maintaining this pressure level for 90 minutes. To ensure 100% oxygen saturation the chamber was flushed with pure O_2 for 3-5 minutes every 10-30 minutes depending on the number of mice in the chamber. The animals were slowly decompressed to normal atmospheric pressure over a period of approximately 10 minutes.

Interstitial fluid pressure (P_{if}) measurements

P_{if} was measured using the wick-in-needle (WIN) technique.[23,24] A standard 23-gauge needle filled with nylon floss and saline was placed in the central part of the tumour and connected to a transducer dome through a PE-50 catheter. The needle had no additional side hole, as originally used in WIN, due to small tumour volume and problems with stabilizing the pressure. The transducer dome was connected to pressure-measurement software (PoweLab/ssp ADinstruments, PowerLab chart 5, version 5.11). The fluid communication between the interstitium and the measuring system was ensured by compression and decompression of the catheter (clamping). This caused a transient rise and fall in the pressure. A measurement was accepted when the pressure returned to pre-clamp value (± 1 mmHg).

Immunohistochemistry-staining for CD31

After sacrificing the animals, tumours were immediately dissected out and snap frozen in liquid nitrogen and subsequently stored at -80°C. Frozen tumour sections (20 μm) were used for immunostaining of blood vessels using rat anti-mouse CD31 (AbD serotec, Morphosys UK Ltd, Oxford, UK) as primary antibody and rabbit anti-rat (Vectastatin ABC kit, peroxidise Rat IgG PK 4004, Bioteam AS, Trondheim, Norway) as secondary antibody. Diaminobenzidine tetrahydrochloride (3.3 DAB, Sigma-Aldrich, Germany) was used as a chromogen to visualize blood vessels, and Richardsons stain was used as nuclear counter stain. Image analysis and quantification was performed with a Nicon Elipse E600 microscope (Nicon, Japan) and NIS-Element AR 2.3 program (Laboratory Imaging Ltd, Praha, Czech Republic). The cross-sectional density of CD31 positive structures was quantified per mm^2 using a counter grid.

Determination of collagen content in the tumours

The amount of hydroxyproline (to estimate collagen content) was determined in acid hydrolysates of the tumour tissue by a colometric method adapted from Woessner *et al.*[25] The tumours were free of fat and freeze-dried before the analysis started. The tumour tissue was finely crushed and weighed before being hydrolysed, and subsequently diluted with ddH_2O. The volumes of ddH_2O added depended on the amount of freeze dried tissue in the test tube (mg), and were calculated according to the equation: (225/X mg freeze dried tissue/ 4 mL)x5. A standard reagents curve was made by adding a known amount of hydroxyproline stock. Chloramine T, Perchcloric acid and p-DABA were then added to both the standards and the test solute. Sample sizes of 250 μL were dispensed in duplicates in microplates with 96 wells (Maxisorp, NUNC, Denmark). The absorbance of the samples was read at 557nm using a spectrophotometrical microplate reader (Molecular Devices SpectraMax, Plus 384, GMI Inc., USA). The results were displayed through a computer (Pentium Processor with Windows XP), using software Softmax PRO (Molecular Devices, USA).

Microdialysis

To determine the uptake of radioactive labelled [^{3}H]-5FU (Nycomed Amersham, Buckinhamshire, UK) into the tumour tissue, a microdialysis technique,[26] further modified in our laboratory was used.[27] When anaesthetized, the femoral vein was cannulised for injection of [^{3}H]-5FU. One microdialysis probe (CMA/20 Microdialysis AB, Stockholm, Sweden) was placed in the jugular vein and one in the tumour. Both probes were connected to a pump (CMA-100, Microdialysis AB, Stockholm, Sweden) and the catheters were perfused with saline, first at 30 μL/min for 5 minutes to remove any bubbles from the system, and then at a rate of 1 μLmin throughout the rest of the experiment. The catheter and probes were left to stabilize and equilibrate for 15 min before sampling of dialyslate. Sampling of dialyslate from both tumour and plasma started immediately after injection of 0.2 mL 0.37 MBq ^{3}H-5FU and fractions were collected every 10 min for a total of 70 min. The area under the curve (AUC) for the plasma and tumour was calculated as the product of counts per 10 min (cpm) for a total measurement period of 70 min. Uptake of [^{3}H]-5FU was expressed as AUC tumour divided by AUC plasma.

Due to a narrow time window to sample the plasma while the pO_2 was still elevated in the in the single HBO treated tumour tissue, the mice were prepared for sampling prior to entering the hyperbaric chamber. Two HBO treated groups were included to clarify if a possible alteration in P_{if} was due to elevations in pO_2 (single HBO treatment) and/or to long term vascular changes (repeated HBO treatment).

Table 1. The experimental groups: control, repeated HBO (day 1, 4 and 7) and single HBO treatment.

Experimental groups	Gas	Ambient pressure	pO_2	Number of exposures	Exposure time
Control	Air	1 bar	0.2	-	-
Repeated HBO treatment	O_2	2.5 bar	2.5	3	90 min
Single HBO treatment	O_2	2.5 bar	2.5	1	90 min

Statistics

All data were tested for normality prior to the choice of statistical analysis. We used two-tailed unpaired Students t-test (normalized data) or one-way ANOVA (non-normalized data) for testing the statistical differences between the groups. Differences were accepted as statistically significant at $P<0.05$. Standard error of mean are indicated in the figures. The software program SPSS for Windows was used for statistical analysis.

Results

Hyperoxia lowers interstitial fluid pressure

As elevated P_{if} has been proposed to inhibit the transport and effect of chemotherapy, we wanted to elucidate if this was applicable also for the 4T1 murine mammary tumours. P_{if} is usually high in solid tumours, and as expected this was also the fact in these tumours. P_{if} was approximately 7 mmHg in our 4T1 control tumours. After treatment, the average P_{if} was significantly reduced (~50%, $P<0.05$) compared to control after both single and repeated HBO treatment (Figure 1).

Hyperoxia decrease tumour blood vessel density

The 4T1 control tumours had a dense vasculature, as shown by immunostaining CD31. However, the blood vessel density in the repeated HBO treated tumours was significantly decreased ($P<0.001$) compared to control (Figure 2), clearly demonstrating that repeated HBO treatment has an anti-angiogenic effect on the 4T1 mammary tumours.

Hyperoxia does not influence the collagen content in the tumours

A dense collagen network has been proposed to be a hindrance to efficient transport of chemotherapeutic drugs. Therefore, we have elucidated the collagen content in both control and HBO treated tumours, by quantifying the amount of hydroxyproline in the tumour tissue. The amount of hydroxyproline in the repeated HBO treated group was, however, not statistically significant different when compared to the control group (Figure 3).

Hyperoxia does not influence the uptake of 5FU

Microdialysis was used to examine if oxygenation of the tumour after HBO treatment influenced the uptake of the chemotherapeutic drug (5FU) into the tumour tissue. However, microdialysis analysis showed no increase in the uptake of 5FU into the tumour tissue 24 hours after the repeated HBO treatments, when the pO_2 in the tumour tissue was normalised again, nor after the single HBO treated group, when the pO_2 was still elevated in the tumour tissue (Figure 4).

Discussion

We present data showing that a reduction in P_{if} *per se* does not enhance the uptake of 5FU into 4T1 mammary tumors.

The tumour interstitium represents a major barrier to drug delivery[9] and modification of the tumour ECM is one strategy that could promote better delivery. We have focused on factors in the interstitium which could possibly contribute to this resistance to drug delivery; the interstitial fluid pressure (P_{if}), the collagen content and the tumour blood vessel density.

The rationale for using hyperoxia as a treatment form is based on previous studies within this field. Stuhr *et al.* showed a significant potentiated growth reduction in DMBA-induced mammary tumours when administering 5FU immediately before HBO treatments compared to HBO alone.[18] Additionally, Takiguchi *et al*. showed that concomitant HBO and 5FU administration enhanced the uptake in Sarcoma 180 implanted in mice.[15] Moen *et al*. showed an increase in the uptake of [^{3}H]-5FU into DMBA-induced rat mammary tumours as quantified by microdialysis, when the drug was administered immediately after one single hyperoxic treatment.[22]

Interstitial hypertension (elevated P_{if}) is commonly known in tumours and has been attributed to hinder an effective uptake of different chemotherapeutic drugs.[9] Several investigators have shown increased uptake of chemotherapeutic agents after a decrease in tumour P_{if} .[10,28,29] Being one of the smaller chemotherapeutic agents, 5FU might move by diffusion, and, therefore, remain less affected by a decrease in P_{if}. Nevertheless, previous results have shown an enhance uptake of the low molecular weight compound ^{51}Cr-EDTA after a lowering of P_{if} in both colonic carcinomas and DMBA induced mammary tumors.[30] A reduction in P_{if} would, therefore, be expected to enhance the uptake of 5FU, also in this study. The 4T1 mammary tumours exposed to

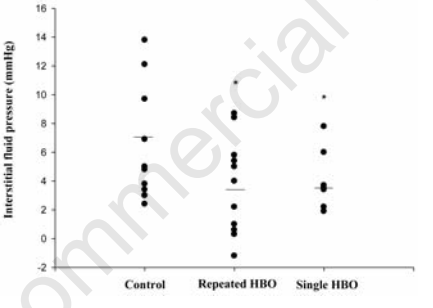

Figure 1. Interstitial fluid pressure measurements in control (n=11), repeated hyperbaric oxygen (HBO) treated (n=11) and single HBO treated (n=8) 4T1 mammary tumours. Average values are indicated as small, horizontal lines. Mean ± SEM * P<0.05 compared to control.

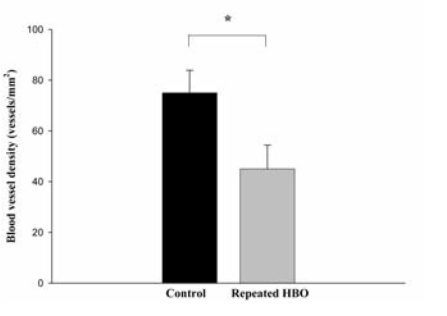

Figure 2. The average blood vessel density from 5-8 representative cross section pictures from 5 controls and 5 repeated hyperbaric oxygen (HBO) treated 4T1 mammary tumours. Mean ±SEM. * P<0.001 compared to control.

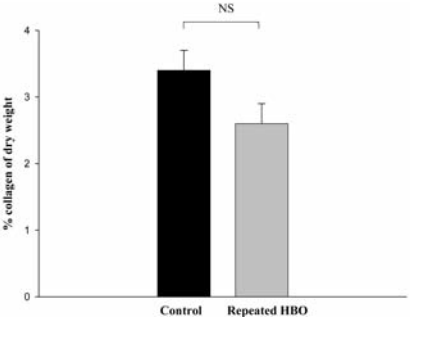

Figure 3. The average collagen content after 8 days, in control (n=8) and in repeated hyperbaric oxygen (HBO) treated 4T1 mammary tumours (n=8). Mean ± SEM.

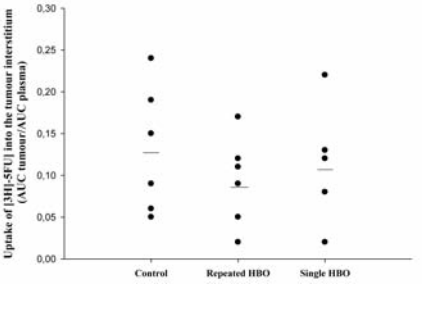

Figure 4. Uptake of radioactive labelled 5-fluoruracil ([^{3}H]-5FU) in control (n=6), in repeated hyperbaric oxygen (HBO) treated (n=6) and in single HBO treated (n=5) 4T1 mammary tumours. Average values are indicated by small, horizontal lines.

either single or repeated HBO exposure, both showed a significant reduction in P_{if}. Surprisingly, neither of the HBO treated groups showed an increase in drug uptake into the 4T1 mammary tumour tissue. A study by Moen *et al*. showed an enhanced uptake of [^{3}H]-5FU in combination with HBO treatment in DMBA induced mammary tumours.[22] However, the enhanced uptake could not be assigned to the decreased P_{if} found in the same study. This conclusion corresponds to Flessner *et al*. who despite a significant lowering effect of PGE on tumor P_{if} did not find enhanced penetration of ^{125}I labelled Trastuzumab into metastatic ovarian cancer.[31] Together, these findings fail to find a correlation between decreased P_{if} *per se* and increased uptake.

It is believed that collagen type 1 and its organization into fibrils have a significant role in limiting the diffusion of large molecules in the interstitium, as the narrow spacing between the collagen fibrils will exclude or hinder the migration of larger particles.[32] In accordance with this, treatment with collagenase has been shown to increase the diffusion and convective permeability of tumours.[3,4] Previous studies on DMBA induced mammary tumours in rats show that repeated HBO exposures decrease the collagen content in the tumours.[20,22] We, therefore, wanted to elucidate the effect of hyperoxia on collagen density in the 4T1 mammary model. However, 4T1 mammary tumours did not show a statistically significant decrease in collagen content after repeated hyperoxic treatments. The treatment protocol in the present study included only 3 hyperoxic treatments over a period of 8 days, instead of 4 hyperoxic treatments over a period of 11 days, the latter previously proven to be the most efficient treatment protocol.[18] The 11 day protocol was not possible to follow through in this study, as the 4T1 control tumours behaved very aggressively, growing rapidly too big. The procedure was, therefore, ended on day 8. With this in mind, one might speculate if there were too few treatments in the study and, hence, a too short time-frame to induce a statistically significant change in collagen content. Further, since there was no change in the dense tumour microenvironment after 3 HBO treatments this might have contributed to hindering the delivery of 5FU into the tumour tissue in the present mammary tumour model, despite a reduction in P_{if}.

Since chemotherapy is distributed systemically, it needs a functional vascular system to be able to reach the cancer cells. The vasculature in malignant tumours is formed by angiogenesis, is irregular, dilated, and tortuous and can have dead ends. The blood, therefore, flows irregularly, moving more slowly and, sometimes, even oscillating.[7] Furthermore, the tumour vasculature is very permeable to water and small proteins. Anti-angiogenic therapy has been suggested to induce normalization of the tumour blood vessels, leaving the vasculature with less leaky and tortuous vessels, with normalized basement membranes and better pericyte coverage.[33] The normalization of the tumour vasculature might result in a reduction in P_{if}, by reducing the leakiness of the vessels. Blood vessel density was significantly reduced in the hyperoxic 4T1 mammary tumours, implicating a possible normalization of the tumour vasculature which might, probably, partly be responsible for the reduction in tumour P_{if}. However, anti-angiogenic therapy can decrease the overall distribution of large molecules in the interstitium,[34,35] and decrease blood perfusion.[36] Since hyperoxic treatment has an anti-angiogenic effect on the 4T1 mammary tumours, this, together with a possible reduced permeability, will impede trans-endothelial transport of [^{3}H]-5FU, even though P_{if} is lowered. This could explain why the uptake of [^{3}H]-5FU was not increased in the 4T1 mammary tumours.

The conclusion of our study is, that a significant reduction in P_{if} *per se* does not influence the uptake of the low molecular weight compound 5FU in 4T1 mammary tumors. A possible explanation for this might be the unaltered collagen content together with the anti-angiogenesis found in the treated group. The underlying mechanisms behind these findings need to be further elucidated.

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