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Effect of hemoglobin solution on the response of intracranial and subcutaneous 9L tumors to antitumor alkylating agents

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Abstract. The 9L gliosarcoma growing subcutaneously in the hind leg of the Fisher 344 rat contains major areas of severe (<5 mmHg) hypoxia, making up about 49% of the tumor. Intravenous administration of an ultrapurified polymerized bovine hemoglobin solution (8 ml/kg) along with normal air breathing reduces the percentage of severe hypoxia to about 24% and increases oxygenation throughout the tumor. Coadministration of the hemoglobin solution increased the tumor growth delay of subcutaneously implanted 9L tumors treated with carmustine (BCNU), cyclophosphamide, or ifosfamide but did not significantly change the tumor growth delay produced by cisplatin (CDDP). Coadministration of the hemoglobin solution with each of the four antitumor alkylating agents resulted in a near doubling of the percentage of increase in life span in animals bearing intracranial tumors treated with the combination as compared with animals treated with the drugs alone. Increases in serum blood urea nitrogen (BUN) and creatinine levels in treated animals returned to normal by 11 days posttreatment. Major changes in liver enzymes occurred with the combination of cyclophosphamide and the hemoglobin solution at 4 days posttreatment; however, these values returned to the levels in the untreated control animals within 1 week thereafter. These results indicate that further exploration of the use of hemoglobin solutions in cancer therapy is warranted.

Introduction

Multimodality therapy for malignant brain tumors, including surgery, radiation therapy, and chemotherapy, has been investigated for over 20 years. Radiation therapy significantly prolongs patient survival [17, 21, 38]. BCNU (carmustine) also produces a statistically significant survival benefit. Procarbazine, methyl-lomustine (methyl-CCNU), CCNU, and streptozotocin are about as effective as BCNU [4, 21, 22]. Patient survival for longer than 18 months is specifically related to the addition of chemotherapy to radiation and surgery [4, 21, 22]. Recently, patients with brain tumors have been treated with cisplatin (CDDP) and vincristine or with CDDP, cyclophosphamide, and vincristine prior to or after radiation therapy [2, 10, 15–17, 24, 25].

Two of the major difficulties in the therapy of malignant brain tumors are the regional and clonal heterogeneity of human brain tumors within individual patients and the problems of the blood-brain barrier [11, 15, 19, 35]. The heterogeneity of brain tumors is responsible, at least in part, for the development of resistance to chemotherapeutic agents. Intrinsic variation in chemosensitivity has been demonstrated for human glioma cells, and low-dose nitrosourea therapy has been shown to result rapidly in resistant cell populations [11, 22]. Tumor masses are also very heterogeneous in oxygenation and contain regions of hypoxia [12, 13, 36, 37]. Preclinical studies conducted both in vitro and in vivo have established that hypoxia protects cells from the cytotoxic actions of radiation and many chemotherapeutic agents and thereby may be a significant factor in therapeutic resistance [1, 20, 33].

Several methods for increasing tumor oxygenation are under investigation, including the use of hyperbaric oxygen [29], the use of perfluorochemical oxygen-carrying emulsions along with breathing of a high-oxygen-content atmosphere [29], the use of pharmacologic manipulations to increase blood flow and/or oxygen off-loading [23], and the use of hemoglobin solutions with air or oxygen breathing [30, 31].

Bovine blood is a ready source of hemoglobin [5–8]. The molecular structure of bovine hemoglobin is similar to that of human hemoglobin [5]. The oxygen affinity of bovine hemoglobin is regulated by chloride ions, whereas that of human hemoglobin is influenced by the presence of 2,3-diphosphoglycerate [6, 14]. In addition, in contrast to

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human hemoglobin, bovine hemoglobin has low concentrations of organic phosphates [18] and has more pronounced Haldane (CO_2) and Bohr (pH) effects [5]. Ultrapurified bovine hemoglobin has been shown in preclinical studies to have low antigenicity among mammals [7, 8].

In the current report, an ultrapurified polymerized bovine hemoglobin solution was given i. v. to rats bearing 9L gliosarcoma. Oxygen measurements were made in subcutaneous tumors while therapeutic investigations were done in animals bearing intracranial and subcutaneous tumors [27, 32].

Materials and methods

Drugs. The hemoglobin solution (Biopure Corp., Boston, Mass.) is a polymerized form of a highly purified bovine hemoglobin solution that contains 13 ± 1 g bovine hemoglobin/dl. P_{50} measurements of the hemoglobin solution under conditions designed for testing human hemoglobin gave values of 34–37 mmHg. The hemoglobin component has a molecular mass ranging from 65 to 500 kDa (w/v). The hemoglobin solution also contains sodium (150 m*M*), chloride (110 m*M*), and potassium (4.0 m*M*) in a buffer solution (pH 7.6–7.9). The circulating half-life of the preparation is about 2 days [9]. Carbogen comprises 95% oxygen and 5% carbon dioxide. Cyclophosphamide and CDDP were purchased from Sigma Chemical Co. (St. Louis, Mo.). BCNU and ifosfamide were obtained from the Dana-Farber Cancer Institute pharmacy.

Tumor. 9L/SF gliosarcoma cells were obtained as a gift from Dr. Dennis Deen (University of California, San Francisco, Calif. [3]. These 9L cells were maintained in culture in our laboratory over the past 10 years in α-minimum essential medium (α-MEM; Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Sterile Systems, Inc., Logan, Utah) and antibiotics. For experiments, 9L cells (4×10^4) in 10 µl of media without serum were implanted intracranially in male Fischer 344 rats (Taconic Farms, Germantown, N.Y.) weighing 200–250 g on day 0. Each rat was anesthetized with sodium pentobarbital. A midline scalp incision was made and a hole was bored through the skull with a 23-gauge needle at a point located 2 mm behind the right coronal suture and 2 mm lateral to the midline. The cell suspension was injected into the right frontal lobe at a depth of 3 mm from the dural surface. The needle was removed and the hole was filled with dental cement. The scalp was sealed with a surgical clip [27, 32]. 9L cells (2×10^6) were implanted subcutaneously into the flank of each animal 2 days later.

Oxygen measurements. Tissue oxygen measurements were made using a pO_2-Histograph (Eppendorf, Inc., Hamburg, Germany). The polarographic needle microelectrode was calibrated in aqueous solutions saturated with air and 100% nitrogen. The electrode was used for tumor measurements if there was less than 1% variation in current measurements upon repetition of the calibration cycle. For tumor measurements, the rat was anesthetized by an i. p. injection of Ketaset (35 mg/kg) and xylazine (25 mg/kg) prepared in phosphate-buffered 0.9% saline. The animal was placed on a heating pad and covered with a blanket to maintain body temperature. The core temperature was measured with a rectal thermometer. The tumor site was shaved and tumor diameters were measured with calipers. A small patch of skin located about 4 cm from the tumor was shaved and an incision was made, allowing the reference electrode (Ag/AbCl-ECG) to be inserted subcutaneously and secured. The tumor was exposed by removing about 1 cm^2 of skin over the site. The tumor capsis was then perforated with a 20-gauge needle. The pO_2 microelectrode was positioned in the perforation.

The pO_2 microelectrode under computer control was placed 1 mm into the tissue and then retracted 0.3 mm. The probe current was then measured and after 1.4 s the probe was moved forward again. The total length of the measurement path was determined by the size of the tumor. After the probe had reached the end of its measurement path it automatically retracted. The probe was then repositioned in the same perforation at a different angle and stepwise measurements were again initiated. In all 3 diameters were measured in each tumor for a total of 40–60 measurements [34].

Tumor pO_2 measurements were made under four conditions: (1) during normal air breathing, (2) during carbogen (95% O_2/5% CO_2) breathing, (3) 10 min after intravenous hemoglobin solution (8 ml/kg) administration with normal air breathing, and (4) 15 min after the initiation of carbogen breathing following intravenous hemoglobin solution administration. Data collection through 3 tumor diameters accrued about 50 pO_2 measurements and took about 10 min. The pO_2 microelectrode was recalibrated in aqueous solutions saturated with air and 100% nitrogen after each data collection; therefore, the pO_2 microelectrode was recalibrated four times during the course of the experiment. Recalibration requires about 15 min. Therefore, the interval required for tumor pO_2 measurements under the four conditions tested was about 1 h and 40 min.

Tumor growth delay and increase in life span. Treatment was initiated when the subcutaneous tumors had reached 100 mm^3 in volume (7 days after tumor cell implantation). All of the anticancer drugs were given i. p. CDDP (8 mg/kg) was injected in a single dose on day 7. Cyclophosphamide (100 mg/kg) and ifosfamide (100 mg/kg) were given in three doses on alternate days (days 7, 9, and 11); the hemoglobin solution (8 ml/kg) was injected i. v. prior to each anticancer drug injection.

The survival of animals was monitored daily. Animals that were moribund or unable to reach food or water were killed by CO_2 inhalation. The data are presented as percentages of increase in life span (ILS) of treated as compared with untreated control animals.

The progress of each subcutaneous tumor was measured three times weekly until it reached a volume of 500 mm^3. Tumor growth delay was calculated as the number of days required for each individual tumor to reach a volume of 500 mm^3 as compared with the untreated controls. Each treatment group contained four animals and the experiment was repeated twice. Days of tumor growth delay are the mean values ± SE for the treatment group as compared with the controls [28].

Determination of serum blood urea nitrogen and creatinine concentrations. Blood samples (1 ml) were collected by cardiac puncture. Serum was prepared by centrifugation and stored frozen at –20° C. Serum blood urea nitrogen (BUN) and creatinine levels were determined by standard techniques using a Cobas Mira analyzer (Roche Diagnostic Systems, Nutley, N.J.).

Results

Tissue oxygen tensions were measured in the rat 9L gliosarcoma under conditions of normal air and carbogen breathing prior to and after i. v. administration of the hemoglobin solution (8 ml/kg). Table 1 shows parameters describing the oxygenation of the 9L tumors under various

Table 1. Oxygenation parameters for subcutaneously growing 9L gliosarcoma in the presence and absence of administration of the hemoglobin solution and/or carbogen breathing[a]

Measurement condition	% Readings	pO_2, mmHg		
	<5 mmHg	10th percentile	Median	90th percentile
Air	49	0.0	6.5	28
Carbogen	41	0.0	25	116
Hemoglobin solution:				
Air	24	2.7	18	29
Carbogen	0	14.5	55	152

[a] See Materials and methods for details

Table 2. Growth delay of subcutaneously implanted 9L gliosarcoma produced by antitumor alkylating agents with or without a hemoglobin solution

Treatment group	Tumor growth delay, days[a]	
	Alone	+ Hemo solution
–	–	1.5 ± 0.4
BCNU (3 × 15 mg/kg)[b]	5.8 ± 0.5	7.8 ± 0.6*
CTX (3 × 100 mg/kg)[b]	9.1 ± 0.7	14.0 ± 0.9*
CDDP (8 mg/kg)[b]	9.4 ± 0.8	10.1 ± 0.8
Ifos (3 × 100 mg/kg)[b]	10.4 ± 0.9	14.3 ± 1.0*

[a] Mean number of days required to reach a tumor volume of 500 mm^3 as compared with untreated controls. Control tumors reached 500 mm^3 in 19.5 ± 0.5 days

[b] BCNU, cyclophosphamide (CTX), and ifosfamide (Ifos) were given by i.p. injection on days 7, 9, and 11 after implantation of the tumor. CDDP was injected i.p. as single dose on day 7

* Significantly different from drug alone according to the Dunn multiple-comparisons test ($P < 0.01$)

conditions. Oxygen tensions of less than 5 mmHg are considered severely hypoxic and probably represent regions of therapeutic resistance. Oxygen tensions in normally oxygenated tissues range from about 15 to 30 mmHg. Under normal air-breathing conditions, nearly half of the 9L tumor is severely hypoxic. Under carbogen-breathing conditions, the severely hypoxic regions were reduced to about 40% of the tumor while the median pO_2 value increased about 4-fold to 25 mmHg. Administration of the hemoglobin solution (8 ml/kg) markedly altered the oxygen profile of the tumors. With air breathing after administration of the hemoglobin solution, the percentage of severe hypoxia in the tumors was reduced to 24% and the median pO_2 value reached 18 mmHg. The addition of carbogen breathing to administration of the hemoglobin solution markedly increased the oxygenation of the tumor such that severe hypoxia was eliminated and oxygenation through 90% of the tumor was normal or greater than normal.

One advantage of a hemoglobin solution in the clinical setting is that improved tumor oxygenation can be achieved under normal air-breathing conditions. For therapeutic investigations, rats were implanted with 9L tumors intracranially and subcutaneously such that the ILS and tumor growth delay could be assessed in the same animal. Administration of the hemoglobin solution alone did not alter the growth of subcutaneous tumors (Table 2). Administration of the hemoglobin solution (8 ml/kg) prior to treatment with BCNU (3 × 15 mg/kg) resulted in a 1.3-fold increase in tumor growth delay as compared with treatment with BCNU alone. Similarly, administration of the hemoglobin solution prior to each injection of cyclophosphamide (3 × 100 mg/kg) resulted in a 1.5-fold increase in tumor growth delay as compared with treatment with cyclophosphamide alone. There was no effect on the tumor growth delay produced by a single dose of CDDP (8 mg/kg) by administration of the hemoglobin solution prior to the drug. Like that produced by cyclophosphamide, the tumor growth delay resulting from treatment with ifosfamide was increased 1.4-fold by prior administration of the hemoglobin solution.

The effect of the treatments on the growth of intracranial tumors was determined by the ILS of the treated groups as compared with the untreated controls (Table 3). An ILS value of 25% or more is therapeutically significant. Although BCNU alone improved the survival of the animals, the ILS did not reach statistical significance; however, with administration of the hemoglobin solution prior to the BCNU, an ILS value of 38% was achieved. Cyclophosphamide was not as effective a treatment for the intracranial tumor as for the subcutaneous tumor, producing an ILS value of 20%; however, when the hemoglobin solution was injected prior to the cyclophosphamide, an ILS value of 43% was achieved. Although administration of the hemoglobin solution did not alter the response of the subcutaneous 9L tumor to CDDP, there was an increase in the survival of animals treated with the hemoglobin solution prior to CDDP as compared with animals treated with CDDP alone. Like cyclophosphamide, ifosfamide was not as effective a treatment for the intracranial tumor as for the subcutaneous tumor; however, administration of the hemoglobin solution prior to each injection of ifosfamide resulted in an ILS value of 38%.

Renal toxicity can be an important issue for several of the anticancer agents tested in this study. Renal toxicity can also be a factor in the administration of hemoglobin preparations. Serum levels of BUN and creatinine were determined at two time points, day 15 and day 22, after subcutaneous tumor implantation (Table 4). Day-15 measurements were made 4 days after the final hemoglobin and/or drug injections (except for CDDP, which was given in a

Table 3. Increase in life span of rats bearing intracranially implanted 9L gliosarcoma produced by antitumor alkylating agents with or without a hemoglobin solution

Treatment group	Survival (days)[a]			
	Alone	% ILS[b]	+ Hemo solution	% ILS[b]
Controls	26 ± 1.2	0	27.8 ± 1.4	7%
BCNU (3 × 15 mg/kg)[c]	31.6 ± 1.5	22%	35.9 ± 1.7*	38%
CTX (3 × 100 mg/kg)	31.3 ± 1.5	20%	37.2 ± 1.6*	43%
CDDP (8 mg/kg)	33.6 ± 1.7	29%	38 ± 1.8**	46%
Ifos (3 × 100 mg/kg)	30.9 ± 1.3	19%	35.9 ± 1.6*	38%

[a] The survival of untreated controls was 26 ± 2.2 days

[b] Percentage of increase in life span as compared with untreated controls

[c] Treatments were given as described in Table 2

*, **Significantly different from drug alone according to the Dunn multiple-comparisons test ($P < 0.01$, $^{**}P < 0.05$)

Table 4. Serum BUN and creatinine levels in rats bearing 9L gliosarcoma[a]

Treatment group	Days after tumor implantation					
	Day 15			Day 22		
	BUN	Crea	BUN/Crea	BUN	Crea	BUN/Crea
Controls	22.5	0.50	43	20.8	0.55	37.8
Hemoglobin solution	25.7	0.75	34.3	24.2	0.60	40.3
BCNU (3 × 15 mg/kg)	21.6	0.75	28.8	27.1	0.60	45.2
Hemoglobin solution/BCNU	23.0	0.75	30.7	21.2	0.55	38.5
CTX (3 × 100 mg/kg)	24.4	0.70	34.9	20.6	0.55	37.5
Hemoglobin solution/CTX	?8.6	0.90	?9.6	15.0	0.50	30.0
CDDP (8 mg/kg)	23.1	0.65	35.5	26.1	0.55	47.5
Hemoglobin/CDDP	30.9	0.75	41.2	25.9	0.50	56.8
Ifos (3 × 100 mg/kg)	21.3	0.50	42.6	16.2	0.45	36.0
Hemoglobin solution/Ifos	19.2	0.50	38.4	16.3	0.50	32.6

[a] Measurements of BUN and creatinine were done on serum samples obtained from blood collected by cardiac puncture

single dose on day 7) and day-22 measurements were made 1 week later. Administration of the hemoglobin did not alter the BUN or creatinine level or the BUN/creatinine ratio as compared with measurements made in untreated tumor-bearing animals. In animals treated with BCNU or the hemoglobin solution and BCNU, there was a 1.5-fold increase in creatinine at 4 days after the last drug injection that returned to baseline by day 22. Renal toxicity can be dose-limiting for CDDP; however, neither BUN nor creatinine levels measured in animals treated with CDDP alone or in combination with the hemoglobin solution were markedly altered from those determined in untreated tumor-bearing animals. Treatment with ifosfamide or with the hemoglobin solution and ifosfamide did not alter the serum BUN or creatinine levels determined in treated animals from those measured in untreated tumor-bearing animals.

Although animals treated with cyclophosphamide alone had a 1.4-fold increase in creatinine on day 15 and those treated with the hemoglobin solution and cyclophosphamide had a 1.8-fold increase in creatinine by day 22, creatinine levels returned to baseline in both of these groups (Table 5). The enzymes aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), and lactate dehydrogenase (LDH) were elevated in the untreated tumor-bearing animals as compared with the normal range for these enzymes. Although treatment with cyclophosphamide decreased AST and ALT serum levels relative to the control values, treatment with the hemoglobin solution and cyclophosphamide resulted in an AST level 12 times higher than normal and an ALT level 30 times greater than normal. Animals treated with this combination had decreases in serum protein and glucose. By day 22, levels of the serum components measured in animals receiving the hemoglobin solution along with cyclophosphamide returned to those determined in tumor-bearing control animals.

Discussion

The 9L gliosarcoma has been widely used as a model for brain tumors [3, 15, 27, 32]. When grown subcutaneously in the hind limb of the Fisher 344 rat, the 9L tumor showed major areas of hypoxia. This tumor was very responsive to administration of the hemoglobin solution. Under normal air-breathing conditions, the degree of hypoxia was markedly diminished after administration of the hemoglobin solution and hypoxia was eliminated with the addition of carbogen breathing to administration of the hemoglobin solution.

All of the chemotherapeutic agents examined in this study are relatively small molecules that should not be subject to blood-brain barrier exclusion on the basis of

Table 5. Serum levels of biochemical constituents and enzymes in 9L gliosarcoma-bearing rats at 4 days after completion of treatment with cyclophosphamide or the hemoglobin solution and cyclophosphamide

Serum content	Controls	CTX	Hemo/CTX	Normal range[a]
BUN	21.6	27.9	8.6	14.6–21.0 mg/dl
Creatinine	0.4	0.6	0.9	0.5–0.8 mg/dl
Transaminase (AST, SGOT)	1.224	358	2,571	126–209 U/l
Alanine aminotransferase (ALT)	528	74	1,713	25–56 U/l
Lactate dehydrogenase (LDH)	2,000	2,211	2,410	622–9,360 U/l
Phosphate	13.8	9.5	14.1	6.8–9.0 mg/dl
Total protein	6.1	7.1	4.4	5.4–6.9 g/dl
Albumin	3.8	4.0	1.7	2.8–4.6 g/dl
Glucose	177	197	34	97–155 g/dl

[a] Obtained from the Gossett Veterinary Clinical Chemistry Survey

molecular weight or charge [11]. Two of the drugs, cyclophosphamide and ifosfamide, are prodrugs that undergo metabolism in the liver to form short-lived active alkylating species. It is interesting that cyclophosphamide and ifosfamide, which were very effective treatments for subcutaneously growing 9L tumors, were the least effective treatments for intracranial 9L tumors. BCNU was least effective against subcutaneously growing 9L tumors but moderately effective against the intracranial 9L tumors. CDDP, which was the most stable and aqueously soluble drug studied, was very effective against both subcutaneous and intracranial 9L tumors.

The addition of the hemoglobin solution to treatment with the antitumor alkylating agents improved the overall therapeutic outcome of treatment of the intracranial tumors to a greater degree than it did treatment of the subcutaneous tumors. One possible reason for this differential effect may be that the intracranial tumors are much smaller (by necessity) than the subcutaneous tumors at the time of treatment. Another possible reason for the greater improvement achieved in the intracranial tumors by the combined treatment may be that the brain (and perhaps the brain tumor) is more highly vascularized than the subcutaneous tumor.

The greatest increases in therapeutic response upon addition of the hemoglobin solution to treatment with the antitumor alkylating agents were seen with cyclophosphamide and ifosfamide as evidenced by both increases in tumor growth delay and ILS values. The effectiveness of both BCNU and CDDP treatments was improved to a greater degree, as determined by the ILS, against the intracranial tumor than against the subcutaneous tumor. Although a control for the effect of hemodilution was not carried out in this study, controls injected with saline or a lipid emulsion did not show enhancements in the tumor response to chemotherapy or radiation therapy as compared with the cytotoxic treatments alone [26, 29].

Renal function in the rat is more resistant to disruption by toxic agents than is renal function in man. Although some increases in serum creatinine levels were observed at short times posttreatment, by 11 days posttreatment these values had returned to baseline. The combination of the hemoglobin solution and cyclophosphamide produced more systemic toxicity than did the other treatments as evidenced not only by an increase in serum creatinine values but also by an increase in levels of liver enzymes in the serum. Even in this case, however, the toxicity appeared to be self-limiting and to have resolved by day 22.

The antitumor alkylating agents examined in the current study are in use for the treatment of brain tumors in patients [2, 4, 10, 15–17, 21, 22]. The hemoglobin solution along with normal air breathing improved the oxygenation of the 9L tumor and improved the response of both the subcutaneously growing and the intracranial 9L tumors to treatment with the antitumor alkylating agents. These data provide some indication that the addition of the hemoglobin solution to treatment of malignant brain tumors in patients with antitumor alkylating agents may lead to an improved treatment outcome.

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