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SHORT REPORT

CD34+/CD45-dim stem cell mobilization by hyperbaric oxygen — Changes with oxygen dosage☆

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Abstract Because hyperbaric oxygen treatment mobilizes bone marrow derived-stem/progenitor cells by a free radical mediated mechanism, we hypothesized that there may be differences in mobilization efficiency based on exposure to different oxygen partial pressures. Blood from twenty consecutive patients was obtained before and after the 1st, 10th and 20th treatment at two clinical centers using protocols involving exposures to oxygen at either 2.0 or 2.5 atmospheres absolute (ATA). Post-treatment values of CD34+, CD45-dim leukocytes were always 2-fold greater than the pre-treatment values for both protocols. Values for those treated at 2.5 ATA were significantly greater than those treated at 2.0 ATA by factors of 1.9 to 3-fold after the 10th and before and after the 20th treatments. Intracellular content of hypoxia inducible factors −1, −2, and −3, thioredoxin-1 and poly-ADP-ribose polymerase assessed in permeabilized CD34+ cells with fluorophore-conjugated antibodies were twice as high in all post- *versus* pre-treatment samples with no significant differences between 2.0 and 2.5 ATA protocols. We conclude that putative progenitor cell mobilization is higher with 2.5 *versus* 2.0 ATA treatments, and all newly mobilized cells exhibit higher concentrations of an array of regulatory proteins.

Introduction

Stem/progenitor cells (SPCs) capable of multipotent differentiation can be mobilized from bone marrow and adipose tissue, enter the blood stream and migrate to peripheral sites where they may facilitate recovery from injuries (To et al., 1997; Gil-Ortega et al., 2013; Asahara et al., 1997). SPCs mobilization occurs after wounding, physical exertion and in response to a variety of chemical agents (Fiorina et al., 2010; Fukaya et al., 2013; Albanese et al., 2009;

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Table 1 Patient characteristics. Details for the Penn and Syracuse treatment centers show age/gender, cancer location, radiation dose (cGy), other health issues, tobacco and ethanol use. Penn-based patients were 62.1 ± 2.4 (mean ± SE) years old, 5 were female; and Syracuse-based patients were 62.0 ± 2.5 years old (NS), 5 were female. Radiation dosage was known in 14 Penn-based patients (6409 ± 133 cGy) and 12 Syracuse-based patients (6635 ± 345, NS). Abbreviations used are as follows: ND, not determined (not available from chart or the referring physicians); Brachy, brachytherapy; HTN, hypertension; HIV, human immunodeficiency virus infection; COPD, chronic obstructive pulmonary disease; DM-2, type 2 diabetes mellitus; Ca, cancer; DVT, deep venous thrombosis of a leg; CAD, coronary artery disease; CVA, cerebrovascular accident; GERD, gastro-esophageal reflux disease; Cholesterol, hypercholesterolemia; Esoph, esophageal; Afib, atrial fibrillation; Larengect., laryngectomy; aortic valve, history of aortic valve replacement, HCTZ, hydrochlorothiazide; and MVI, multivitamin pill.

	Age/ gender	Cancer	Radiat.	Other	Medications	Tobacco	Ethanol
Penn#							
1	43 M	Tongue	6000	HTN, HIV, COPD. Epilepsy, Asthma	Fosamprenavir, ritonavir, trimethoprim-sulfamethoxazole	None	None
2	67 M	Tongue	6600	HTN, DM-2, Adrenal CA	Amlodipine, terazosin, senna, MVI	None	Occasional
3	68 M	Prostate	Brachy, ND	Depression	Morphine, oxycodone, docusate sodium, omeprazole, citalipram, cyclobenzaprine HCl, gabapentin	Quit > 6 weeks	None
4	58 F	Sinus	5400	HTN, DVT, Cataracts, Glaucoma	Amlodipine, warfarin, alendronate, latanoprost gtts, ciprodex gtts	None	None
5	39 F	Cervix	ND	Depression	Paclitaxel, methadone, gabapentin, bupropion HCl fluoxetine, pentosan polysulfate, alprazolam, clonazepam	None	Occasional
6	55 M	Neck	6530	HTN, CAD, CVA, Cholesterol,	Ibuprofen, ranitidine, MVI	None	None
7	63 M	Larynx	ND	COPD, Hypothyroid	Gabapentin, levothyroxine	Quit > 3 years	Occasional
8	68 M	Tongue	ND	Lymphoma, Hypothyroid	Ramapril, levothyroxine	None	Occasional
9	57 M	Tonsil	6300	None	Pregabalin, oxycodone, lansoprazole, MVI, glycopyrrolate	Quit > 3 years	Occasional
10	67 M	Tongue	7000	GERD	Esomeprazole	None	None
11	48 M	Tonsil	6300	Asthma	Albuterol, gabapentin, glycopyrrolate, oxycodone	Quit > 5 years	None
12	65 F	Tongue	5580	Hypothyroid, Cataracts	Levothyroxine	None	Occasional
13	76 M	Prostate	ND	HTN, Cholesterol	Metoprolol, amlodipine, prevastatin	Quit > 25 years	Occasional
14	79 M	Tongue	6820	HTN, DM-2, COPD	Metoprolol, losartan, HCTZ, irbesartan, chlorpheniramine- hydrocodone syrup	Quit > 15 years	Occasional
15	68 F	Tongue	7000	HTN, GERD, Cholesterol, CAD	nebivolol, clopidogrel, rosuvastatin, metoclopramide	Quit > 3 years	Occasional
16	56 M	Tongue	ND	Cholesterol, CAD	Pentoxifylline, rosuvastatin, oxycodone	None	Occasional
17	78 M	Prostate	Brachy, ND	HTN, Crohn's	Atenolol	None	None
18	56 M	Neck	6600	HTN, Migraine, Cholesterol, Gout	Ezetimibe, pitavastatin, rizatriptan, allopurinal	Quit >15 years	Occasional
19	70 M	Tongue	7000	HTN, Hypothyroid,	Lisinopril, celecoxib, tramadol, trazodone, gabapentin, levothyroxine, MVI	None	Occasional
20	60 F	Tonsil	6300	None	Pentoxyfylline, oxycodone	None	Occasional

(continued on next page)

Table 1 (*continued*)

	Age/ gender	Cancer	Radiat.	Other	Medications	Tobacco	Ethanol
Syracuse#							
1	50 M	Tonsil	6996	ITP	Prednisone, oxycodone, xanax, fentanyl, sertraline	Chew	None
2	73 F	Tonsil	7000	COPD, Esoph Ca	Albuterol, fluticasone, esomeprazole, mometasone	Quit > 5 years	None
3	53 M	Mouth	7300	HTN	Morphine, oxycodone, colace, omeprazole, citalipram, cyclobenzaprine, gabapentin	Quit > 6 weeks	None
4	72 M	Prostate	6600	Cholesterol	Simvastatin	Quit > 5 years	None
5	55 M	Mouth	ND	AFib, Aortic valve	Coumadin, carvedilol, amlodipine, fluticasone, albuterol, acetaminophen-hydrocodone	None	None
6	61 M	Mouth	ND	HTN, Esoph strictures	Bisacodyl, lisinopril, omeprazole, ranitidine, sulfamethoxazole, trazadone	Quit > 8 weeks	Occas.
7	60 M	Mouth	6600	None	Ibuprofen, ranitidine, MVI	None	None
8	53 M	Palate	7000	Sinusitis	Pregabalin	Quit > 3 years	Occas.
9	57 M	Larynx	ND	Laryngect.	Gabapentin, cyclobenzaprine, oxycodone, nortriptyline, HCTZ	Quit > 3 years	None
10	68 M	Tongue	6400	Lung CA, COPD	Alendronate, travatan opthalmic sol, albuterol inh, levothyroxine, fluticasone inh, ferrous sulfate	Quit > 5 years	None
11	76 F	Breast	ND	Aortic valve	Bumetanide, atorvastatin omeprazole, ASA, levothyroxine, KCl, fluticasone, naproxen, acetaminophen-hydrocodone, pseudoephedrine, Vitamin D, MVI	None	None
12	79 M	Prostate	ND	HTN, Colon CA	Coumadin, amlodipine, atorvastatin, HCTZ, bicalutamide	Quit > 5 years	None
13	57 F	Larynx	6996	HTN	Lisinopril, methimazole, carvedilol	1 PPD	None
14	51 M	Neck	ND	HTN, Cholesterol, Hypothyroid	Levothyroxine, bisoprolol, pantoprazole, pravachol, MVI	None	Occas.
15	46 M	Mouth	ND	None	Acetaminophen	1 PPD	None
16	69 M	Prostate	7740	HTN, Cholesterol, Asthma	Albuterol inh, fluticasone inh, tiotropium inh, amlodipine, rosuvastatin, acetaminophen, fluticasone	None	None
17	82 F	Mouth	3000	HTN, CAD, Dialysis	Levothyroxine, isosorbide, vitamin b complex, donepezil, metoprolol, oxycodone, mesalamine, citalopram, nortriptyline, omeprazole, diphenoxylate, folate, MVI	None	None
18	69 F	Tongue	ND	HTN. Cholesterol, Asthma	Albuterol inh, amlodipine, atorvastatin	None	None
19	46 M	Palate	6996	Epilepsy	Lamictal, omeprazole, triamcinolone	None	None
20	62M	Tongue	6996	HTN	HCTZ	Quit > 5 years	None

Asahara et al., 1999; Rehman et al., 2004; Reyes et al., 2002; Takahashi et al., 1999). Exposure to hyperbaric oxygen (HBO_2) appears to be a reliable way to mobilize SPCs in humans and also has been shown in rodents and horses (Thom et al., 2006, 2011; Ma et al., 2011; Milovanova et al., 2009; Dhar et al., 2012). Animal studies indicate that one mechanism is based on activation of nitric oxide synthase type 3 (NOS-3) in bone marrow stromal cells with subsequent liberation of stem cell factor (Thom et al., 2006; Goldstein et al., 2006). Separate from mobilization, HBO_2 improves engraftment and differentiation of several progenitor cell types in organs such as the spleen, bone marrow, brain, peripheral nerve, pancreas, cartilage and heart (Aljitawi et al., 2014; Lee et al., 2013; Cherng et al., 2012; Khan et al., 2012; Zhang et al., 2010, 2011; Pan et al., 2009). One area of interest with circulating SPCs is the identification of the sub-set having propensity to form vascular endothelium, so-called endothelial progenitor cells (EPCs) (Hirschi et al.,

2008). Quantification of mobilized EPCs is based on flow cytometric detection of cell surface proteins and phenotypic manifestations of laboratory-grown clones (Hirschi et al., 2008; Mund et al., 2012). Cells mobilized by HBO_2 exhibit many of these surface markers and when cultured, some clones show lectin binding consistent with an endothelial phenotype (Thom et al., 2006, 2011). Animal studies have documented that HBO_2-mobilized SPCs form blood vessels *in vivo* and hasten wound healing (Milovanova et al., 2009; Goldstein et al., 2006; Gallagher et al., 2007).

HBO_2-mobilized SPCs have greater content of hypoxia inducible factors (HIFs) and thioredoxin-1 (Trx), which in the murine model confers improved neovascularization (Thom et al., 2011; Milovanova et al., 2008, 2009). Subsequent to HBO_2 treatments of refractory wounds and diabetic patients, the number of wound margin SPCs is increased and local HIFs and Trx appear to be within these localized SPCs (Thom et al., 2011; Ma et al., 2011). This suggests that SPCs play a role in supplying factors required for wound healing. Hence, evaluating intracellular proteins may have greater importance to assess SPCs function *versus ex vivo* manipulations. Assessment of intracellular regulatory proteins of cells selected based on surface markers precludes studying *ex vivo* cell growth because of need to permeabilize the cell membranes.

HBO_2 treatment involves breathing 100% O_2 at 2 to 3 atmospheres absolute (ATA) pressure for 1.5 to 2 h once or twice daily. HBO_2 has been shown to improve refractory diabetic wounds and delayed radiation injuries in randomized trials and use is supported by independent evidence-based reviews (Bennett et al., 2008; Clarke et al., 2008; Kranke et al., 2012; Goldman, 2009; Fife et al., 2007; Duzgun et al., 2008; Londahl et al., 2010). Several studies have failed to identify clinical efficacy (Annane et al., 2004; Margolis et al., 2013). Notably, these studies involved exposures to 2.0 ATA or use of face masks with questionable seals thus reducing the fraction of inspired O_2; whereas several prospective randomized trials documenting therapeutic benefit utilized pressures of 2.4 or 2.5 ATA in pure O_2-filled chambers or using head-covering hoods (Londahl et al., 2010; Marx et al., 1985). Whether clinical results may differ because of treatment protocols is unclear. The goal of this investigation was to evaluate whether mobilization of cells with surface markers considered consistent with SPCs (CD34+ and CD45-dim) and content of intracellular regulatory proteins differed between two commonly used HBO_2 protocols (Pober, 2012).

Methods

Patient management protocols

All procedures were approved by the Institutional Review Boards and patients signed informed consent. A consecutive series of patients was approached who had been referred for HBO_2 treatment because of complications from radiotherapy for cancer. On the basis of current standard of care, they were to receive at least 20 HBO_2 therapy sessions. Patient characteristics are shown in Table 1. Venous blood was collected prior to and after the 1st, 10th and 20th HBO_2 treatments into Cyto-Chex BCT test tubes (Streck, Inc., Omaha, NE) that contain a proprietary preservative. Samples from the same day of treatment (pre- and post-HBO_2) were analyzed concurrently within 3 days of collection.

The standard Penn-based practice for delivering O_2 involved placement of a balloon-cushioned face mask that is normally used for continuous positive airway pressure respiratory therapy. Treatments were conducted at 2.0 ATA for 2 h daily, 6 days/week. Intermittently the fractional inspired O_2 content in the mask was verified to be 100%. Syracuse-based treatments were conducted in an acrylic chamber pressurized with pure O_2 so that no special mask was required to assure 100% O_2 delivery. Treatments were at 2.5 ATA for 90 min daily, 6 days/week.

Flow cytometry

CD34+ and CD45-dim cells and relative concentrations of intracellular proteins were evaluated with a 10-color FACSCanto (Becton Dickinson, San Jose, CA) using standard acquisition software following published techniques (Thom et al., 2011; Milovanova et al., 2008, 2009). Briefly, nucleated cells were segregated from debris by DRAQ5 DNA staining and gates were based on true-negative controls according to fluorescence-minus-one analysis. Anti-actin fluorescence confirmed uniform cell permeabilization for intracellular protein analysis. Fluorescence/cell was determined and used to compare pre- *versus* post-HBO_2 cell populations.

Materials

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies were purchased from the following sources: From BD Pharmingen, San Jose, CA. R-phycoerythrin (PE)-conjugated mouse anti-human CD34 (Clone 581, a class III CD34 epitope; catalog number 555822), fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD45, catalog number 5558710 and allophycocyanin (APC)-conjugated mouse anti-human poly-ADP ribose polymerase (PARP) catalog number 558710; from R & D Systems, Minneapolis, MN, APC-conjugated anti-human hypoxia inducible factor (HIF)-1, catalog number IC1935P; from Novus Biologicals, Littleton, CO , PE-conjugated anti-human HIF-2 (catalog number NB100-122), FITC-conjugated anti-human HIF-3 (catalog number NB100-2529) and anti-human Trx catalog number EPR 6111 with secondary from Invitrogen, Grand Island, NY catalog number T-2769.

Statistical analysis

Statistical analysis of stem cell numbers and quantitative changes in wound protein markers were carried out by repeated measures analysis of variance followed by the Tukey test for multiple comparisons (SigmaStat, Jandel Scientific, San Rafael, CA). Statistical significance was taken as $p < 0.05$. Data sets were found to be normally distributed so results are displayed as mean ± SE, n = 20 for all groups. Pre- and post-treatment comparisons were made within each type (2.0 ATA and 2.5 ATA) and between the 2.0 and 2.5 ATA treatments for each number (1st, 10th and 20th) by two-tailed *t*-test.

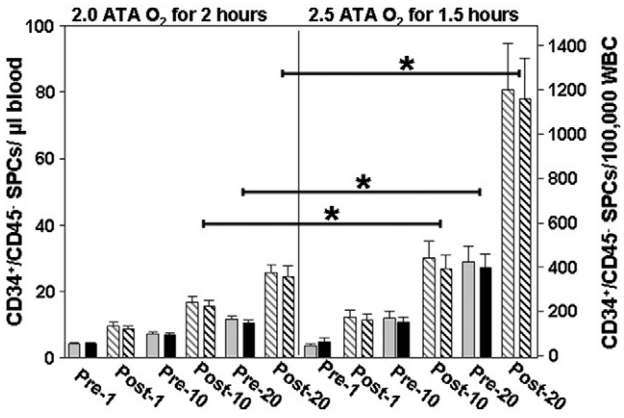

Figure 1 Leukocyte mobilization by HBO_2. The number of circulating CD34+, CD45-dim cells in blood before and after the 1st, 10th and 20th treatment of 20 patients exposed to at either 2.0 or 2.5 ATA. Data were normalized to blood volume (gray boxes quantified on the left ordinate axis) or to total circulating leukocyte count (black boxes quantified on the right ordinate axis) and are mean ± SE, * indicates significant difference between 2.0 and 2.5 ATA groups (ANOVA). All post-HBO_2 values are significantly different from pre-HBO_2 values at each treatment time in both groups (*t*-test).

Results

Circulating cells

Circulating CD34+ and CD45-dim leukocytes increased in blood from 20 consecutive patients undergoing HBO_2 therapy following a protocol of either 2.0 ATA or 2.5 ATA (Fig. 1). There were no significant differences in age, gender or radiation dose between groups (Table 1). Following the 10th as well as before and after the 20th treatment cell counts were significantly higher with the 2.5 ATA *versus* the 2.0 ATA protocol. Findings were essentially the same whether normalized to volume of blood (left axis of Fig. 1) or to total circulating leukocyte count (right axis) because total leukocyte counts for patients did not differ significantly over the course of the HBO_2 treatments (data not shown).

Intracellular protein concentrations

Significant elevations of intracellular regulatory proteins were found in permeabilized CD34+ cells after the 1st, 10th and 20th treatments with either protocol (Table 2). Because of variations in fluorescence intensity due to different lots of antibody and also flow cytometer laser intensities, only differences in cell fluorescence intensity for pre- and post-HBO_2 samples analyzed on the same day were compared and not intensity across a 20 treatment course.

Table 2 Intracellular protein content (fold-elevation post- *versus* prior to HBO_2).

Protein	Treatment #	2.0 ATA Protocol	2.5 ATA Protocol
HIF-1	1	2.35 ± 0.24	3.29 ± 0.55
	10	2.65 ± 0.21	2.67 ± 0.22
	20	2.54 ± 0.38	2.77 ± 0.26
HIF-2	1	2.33 ± 0.24	2.68 ± 0.30
	10	2.48 ± 0.15	2.54 ± 0.20
	20	2.54 ± 0.23	2.60 ± 0.21
HIF-3	1	2.27 ± 0.22	2.67 ± 0.31
	10	2.38 ± 0.24	2.29 ± 0.15
	20	2.43 ± 0.26	2.27 ± 0.15
Trx	1	2.34 ± 0.24	2.51 ± 0.26
	10	2.36 ± 0.22	2.28 ± 0.13
	20	2.44 ± 0.24	2.50 ± 0.29
PARP	1	2.36 ± 0.22	2.64 ± 0.26
	10	2.39 ± 0.22	2.42 ± 0.19
	20	2.57 ± 0.27	2.47 ± 0.22

Data show mean ± SE fold-differences in fluorescence of post-*versus* pre-HBO_2 permeabilized CD34+ cells using fluorophore-conjugated antibodies to proteins shown in column 1. All post-HBO_2 values are significantly different from pre-HBO_2 and there are no significant differences between the 2.0 and 2.5 ATA protocols.

Discussion

The results demonstrate that O_2 partial pressure influences SPCs mobilization with repetitive treatments. Whether this is due to augmented NOS-3 activation requires additional study. SPCs mobilization in response to a variety of drugs is compromised by older age, prior radiotherapy and use of several types of chemotherapy (*e.g.* platinum compounds, alkylating agents, purine analog and lenalidomide) (Jantunen and Kvalheim, 2010). None of these agents were being administered to patients during our study. We have reported previously that SPCs mobilization in response to a single 2.0 ATA O_2 exposure is the same between normal adults and those exposed to radiotherapy (Thom et al., 2006). Obviously, all patients in this study received radiotherapy but there was no significant difference in radiation dosage or patient age between the 2.0 and 2.5 ATA treatment groups (Table 1).

There were no notable deviations in the pattern of SPCs mobilization among the patients despite taking a variety of medications listed in Table 1. Some of these medications are known to have positive effects on SPCs mobilization (*e.g.* short term statin use, paclitaxel, certain β-blockers such as nebivolol and carvedilol); while others have a negative impact on mobilization (*e.g.* bisphosphonates, long-term use of statins and trimethoprim/sulfamethoxazole) (Hristov et al., 2007; Sorrentino et al., 2011; Besler et al., 2008; Wu et al., 2010; Fuchs et al., 2000; Xu et al., 2013; Fernandez et al., 2008). None of the medications listed in Table 1 had been started in the time frame while patients were receiving HBO_2 and all had been prescribed for over 2 months prior to patient enrollment. One patient in the 2.0 ATA group had HIV and one in the 2.5 ATA group had renal failure and was undergoing dialysis. HIV does not impede the efficacy of chemotherapeutic agent-mediated SPCs mobilization and renal failure may modify mobilization by some drugs but does not completely abrogate responses (Badros et al., 2001; Hill et al., 2012; Re et al., 2013; Gabarre et al., 2000). Whether these disorders influence HBO_2-mediated mobilization will require additional study. Clearly, there are differences in mobilization mechanisms between chemotherapeutic agents and HBO_2. Contrary to many of the stem cell mobilization drugs HBO_2 does not activate platelets or elevate leukocyte counts which can be thrombogenic (Ma et al., 2011; Powell et al., 2005; Thom, 2006, 2009).

Intracellular regulatory protein contents were elevated in all post-HBO_2 samples with no significant differences between protocols. Elevations are likely a characteristic of the bone marrow SPCs population primed for mobilization and a higher percentage is released with higher O_2 dose. Lower protein levels in pre-HBO_2 samples at the 10th and 20th treatments may reflect preferential perivascular sequestration of newly mobilized cells and/or protein degradation in cells remaining in the circulation for many hours. The difference in protein contents of newly mobilized SPCs has not been appreciated in mobilization studies involving chemotherapeutic agents. This is probably because responses to chemical agents proceed over a much longer time course.

A weakness of this investigation is that perhaps alternative or additional surface markers should be used to better characterize the mobilized cells. With regard to neovascularization potential, this is difficult to determine given the ongoing debate over EPCs characterization (Pober, 2012). Elevated intracellular proteins of HBO_2-mobilized cells suggest they may have improved propensity for growth/differentiation based on animal studies (Milovanova et al., 2008, 2009). HIF-3 and PARP were probed because they provide evidence that cells were not merely circulating endothelial cells or cells undergoing apoptosis. PARP levels would be expected to be quite low in apoptotic cells (Gajdusek et al., 2001). EPCs can be distinguished from mature CECs by determining 'clonogenic' proliferative capacity, but not by flow cytometric evaluation of surface markers (Pober, 2012). Our approach for assessing intracellular markers after membrane permeabilization precludes *ex vivo* growth analysis, which is why we probed for HIF-3. In animals we have found HBO_2-mobilized SPCs that form new blood vessels and hence not CECs are well endowed with HIF-3, whereas HIF-3 normally is highly tissue restricted (to thymus, lung and a lesser extent in brain, heart and kidney) (Milovanova et al., 2009; Gu et al., 1998). Therefore, we conclude that the cells mobilized by hyperoxia are SPCs and that treatment pressure influences mobilization efficiency. Functional consequences of this response require further study.

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