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Expression of Endothelial Selectin Ligands on Leukocytes Following Repeated Dives in SCUBA Divers

VEDRANA ČIKEŠ ČULIĆ[1,*]
ROKO MARTINIĆ[2]
ANITA MARKOTIĆ[1]
MARKO LJUBKOVIĆ[3]
TONI BREŠKOVIĆ[3]
JASNA MARINOVIĆ[3]
ŽELJKO DUJIĆ[3]

[1] Department of Medical Chemistry and Biochemistry, University of Split School of Medicine, Šoltanska 2, 21000 Split, Croatia

[2] Department of Pathophysiology, Laboratory for Tissue Typing, University of Split Hospital Center, Šoltanska 1, 21000 Split, Croatia

[3] Department of Physiology, University of Split School of Medicine, Šoltanska 2, 21000 Split, Croatia

[*] These authors contributed equally to this work

Correspondence:
Vedrana Čikeš Čulić, PhD, Assistant professor
Department of Medical Chemistry and Biochemistry
University of Split School of Medicine
Šoltanska 2, 21000 Split, Croatia
E-mail: vedrana.cikes.culic@mefst.hr

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Abstract

Background and Purpose: *Leukocyte cell surface adhesion molecule CD11b, decorated with CD15s, plays a critical role in the regulation of β_2 integrin function during neutrophile endothelial transmigration. Hyperbaric oxygenation reduces neutrophil-endothelial cell adhesion, which is mediated by Mac-1 (CD11b/CD18) β_2-integrin.*

Materials and Methods: *This study investigated the expression of CD15 and CD15s, on leukocytes following repeated trimix dives in two series: in the first series 7 divers performed 6 consecutive dives from 55-80 m, while in the second series 7 divers performed 3 consecutive dives from 63-65 m. A more intense dive profile was used in the second series, as can be seen from the longer total dive time. Five divers took part in each of the two series. CD15 and CD15s were determined before and after the 1st and the last dive.*

Results and Conclusions: *Leukocyte subpopulations were not elevated after either the first or last dives in series I. Only CD15+CD15s+ granulocytes were significantly decreased after the 1st dive. In the second series the monocyte proportion was increased and lymphocytes decreased within the total leukocyte population, while CD15s+ monocytes and CD14+CD15s+ granulocytes were elevated after the 1st dive. CD15+CD14+ granulocytes were decreased after the 1st and the last dive in the second series, while CD15s+ granulocytes were decreased only after the last dive in the second series. The current findings of decreased endothelial selectin ligand CD15s expression on CD15+ granulocytes after certain dives point to the role of this subpopulation in the endothelial damage prevention.*

INTRODUCTION

Immunological and inflammatory changes associated with self contained underwater breathing apparatus (SCUBA) diving could contribute to the complex pathophysiology of decompression sickness (DCS) (*1, 2*). SCUBA diving was shown to induce a significant increase in the total number of white blood cells and neutrophils (*3, 4*). Recently, we demonstrated that a single air SCUBA dive, induced a significant increase in the number of monocytes expressing the CD15 as well as the increase in the small CD15s+ monocyte subpopulation, al-

though it did not affect the absolute number of monocytes *(4).* These subpopulations of monocytes are expected to play an important role in host inflammatory responses through production of reactive oxygen species. On the other hand, granulocytes CD15+ and CD15s were slightly decreased (*4*). Repeated hyperbaric oxygen exposure during nine days did not change polymorphonuclear phagocytosis and oxidative burst but decrease lymphocyte proliferation (*5*). Moreover, a later study demonstrated leukocyte adhesion inhibition by hyperbaric oxygenation (*6*). The mechanism for adhesion inhibition by hyperbaric oxygenation is attributed to decreased granulocyte Mac-1 (CD11b/CD18) β_2-integrin expression. Following adhesion to the endothelium, primarily *via* CD62L and CD11b, the leukocytes migrate through endothelial intercellular junctions (*7, 8*). In addition to CD62L and CD11b, several polymorphonuclear leukocyte ligands for endothelial receptors have been identified, including P-selectin glycoprotein ligand-1 (PSGL-1) (*9*), E-selectin ligand-1 (ESL-1) (*10*), CD66-non-specific cross-reacting antigens (*11*), CD43 (*12*) and recently CD44 (*13*). These ligands are similar in that all express the sialylated fucosylated glycans (sialyl Lewis x-type glycans) or CD15s, which appear to be involved in recognition of E-selectin (*7, 10, 11, 12*). Zen et al. showed directly that human leukocyte CD11b is a major membrane protein decorated with CD15s and that CD15s related moieties mediate the binding of CD11b with E-selectin (*14*).

In this study we investigated expression of CD15 (or Lewis X, a precursor of CD15s, being shorter by one sialic acid residue) and CD15s on leukocytes during repetitive deep SCUBA divs by two different field diving profiles (one dive per day) with trimix mixtures (a mixture of oxygen, helium and nitrogen). Due to our recent finding of increased monocyte CD15+ and CD15s+ proportion after a single air dive (*4*), in this study we were interested to investigate changes in this population of monocytes after consecutive deep dives. Data about changes in physiological variables before and after consecutive dives are very sparse and it is presently unknown whether the later dives in a series are associated with worsened cellular homeostasis. Since during deep trimix dives individuals are exposed to higher and longer lasting hyperoxia than during air dives, increased oxidative stress could modify the leukocyte response. Therefore, we performed flow cytometry analysis of CD14, CD15 and CD15s antigens on peripheral blood mononuclear cells in blood samples collected 30 min before and 45 min after the dive by two different field diving protocols.

MATERIALS AND METHODS

STUDY POPULATION. All experimental procedures were conducted in accordance with the Declaration of Helsinki and were approved by the Ethics Committee of the University of Split School of Medicine. Each method and its potential risks were explained to the participants in detail and they gave written informed consent before the experiments, with the opportunity to withdraw at any point with no consequences.[e]In the first series seven experienced SCUBA divers aged 38.4 ± 7.4 years (range 31–48) were employed. Their height and body mass index were 1.8 ± 0.1 m and 25.8 ± 2.3 kg m^{-2}, respectively. In the second series seven divers aged 40.2±9 years old, with an average height of 1.8±0.1cm and 84.5±11.1 kg weight participated. Five divers took part in both studies. At the time of the study, none had any symptom of acute or chronic illness and all were non-smokers.

Field Diving Protocols: Series I. All dives were equipped with dry suits and a Galileo dive computer (Uwatec, Johnson Outdoors Inc., Racine, WI, USA) interfaced with a personal computer for later verification of the dive profile, sea temperature and heart rate (HR) (Polar Belt, Polar, Oulu, Finland). Sea temperature at the bottom and at the decompression stop was 17°C for all dives, while outside temperature varied between 15-18°C. The participating divers were members of the Croatian Search and Rescue Unit and the first study was performed during their regular exercise in technical diving with nitrox and trimix. The divers performed six dives in six consecutive days (one dive per day) with the diving depth ranging from 55 to 80 m. The breathing gas mixtures were trimix 14/45 (14% oxygen, 45% helium and 41% nitrogen), and nitrox 50 (50% oxygen and 50% nitrogen) to which divers switched during decompression at 21 m depth. In the last two dives the divers also used the "travel" trimix 30/20 mixture (30% oxygen, 20% helium and 50% nitrogen) during their decompression at depths between 39 m and 21 m. Decompression profiles were determined using V-planner according to Varying Permeability Model (VPM-B) (*15*). The bottom time for all the dives was short with all the dives resembling bounce dives.

In the second series divers performed three successive field SCUBA trimix dives in three consecutive days (one dive per day) with the diving depth ranging from 63 to 65 m. The bottom times varied between 13.5 and 16.5 min and total dive time including decompression ranged between 59 and 83 min. The water temperature was 19-20°C at the surface and 14-15°C at the bottom. Decompression profiles were also determined using VPM-B model.

The divers refrained from exercise 24 hours before diving, during decompression stop or after dives in both studies since these conditions have been reported to reduce venous gas bubbles (*16, 17, 18*). Subjects did not perform other dives between pre-dive testing and our post-dive measurement.

Timeline of measurements. The echocardiographic parameters of bubble grade were assessed in all divers within 1 h after resurfacing. The blood samples were col-

TABLE 1

Proportions of leukocyte subpopulations (lymphocyte, monocyte and granulocyte) in blood samples before and after the first and the last dive

Study I or II / Day	Lymphocyte		Monocyte		Granulocyte	
	Before dive	After dive	Before dive	After dive	Before dive	After dive
I / 1st day	82,73±6,42	81,69±5,68	8,84±3,37	10,94±3,83	8,46±5,65	7,35±3,06
I / last day	79,82±2,83	79,32±5,09	6,87±2,44	7,57±3,73	10,16±4,21	13,12±3,13
II / 1stday	74,37±4,71	68,20±4,77*	20,37±3,02	26,20±3,89*	5,28±2,64	5,57±1,15
II / last day	83,70±4,00	81,69±4,55	7,04±3,16	10,00±1,30	9,24±3,44	8,29±4,37

Values are means of percentages ± SD.
*p<0,05

TABLE 2

Proportions of CD14+CD15s+, CD15+CD15s+ and CD15+CD14+ leukocyte subpopulations in blood samples before and after the first dive in the first study.

	CD14+CD15s		CD15+CD15s		CD15+CD14	
	Before dive	After dive	Before dive	After dive	Before dive	After dive
Lymphocytes	0,79±0,54	0,99±0,97	0,34±0,15	0,33±0,14	0,11±0,09	0,14±0,13
Monocytes	66,11±14,89	68,08±15,05	29,55±13,34	28,71±15,81	18,96±11,69	25,49±16,62
Granulocytes	44,76±10,26	43,97±12,36	50,71±15,22	31,92±7,41*	23,35±8,71	21,39±10,33

Values are means of percentages ± SD
*p<0,01

TABLE 3

Proportions of CD14+CD15s+, CD15+CD15s+ and CD15+CD14+ leukocyte subpopulations in blood samples before and after the last dive in the first study.

	CD14+CD15s		CD15+CD15s		CD15+CD14	
	Before dive	After dive	Before dive	After dive	Before dive	After dive
Lymphocytes	0,76±0,38	0,54±0,27	0,27±0,12	0,27±0,05	0,09±0,05	0,09±0,02
Monocytes	71,92±8,66	63,11±25,65	25,79±10,59	35,63±8,05	24,06±12,05	29,08±9,59
Granulocytes	42,31±18,04	44,13±23,91	41,53±16,57	32,70±10,79	19,02±8,24	22,91±6,83

Values are means of percentages ± SD

lected from the antecubital vein with BD Vacutainer Systems (Becton Dickinson, UK Ltd, Cowley, Oxford, England) 30 min before and 45 min after the 1st and the last dive in the first series and the 1st and the last dive in the second series, with subjects in a relaxed supine position.

Detection of Venous Gas Bubbles. The subjects were placed in the supine position, and a phase array ultrasonic probe (1.5–3.3 MHz) was placed in a position to obtain a clear view of the right and left ventricles and atria. The transducer was connected to a Vivid 3 Expert ultrasonic scanner (GE, Milwaukee, WI, USA). The same experienced cardiologist performed all echocardiographic investigations.

Gas bubbles were observed in the right ventricle as high-intensity echoes. The cardiac images were recorded on S-VHS videotape for 60s at rest and after two coughs. The bubbles were graded using the method described by Eftedal and Brubakk (*19*). After grading, the values were

TABLE 4

Proportions of CD14+CD15s+, CD15+CD15s+ and CD15+CD14+ leukocyte subpopulations in blood samples before and after the first dive in the second study.

	CD14+CD15s		CD15+CD15s		CD15+CD14	
	Before dive	After dive	Before dive	After dive	Before dive	After dive
Lymphocytes	0,53±0,13	0,71±0,29	0,13±0,04	0,17±0,15	0,01±0,01	0,01±0,02
Monocytes	79,98±4,56	85,44±3,67*	8,31±7,68	4,83±4,55	5,17±5,21	2,98±2,92
Granulocytes	49,50±5,99	65,97±11,40*	26,04±19,55	9,52±6,32	13,44±10,13	4,17±2,64*

Values are means of percentages ± SD
*p<0,05

TABLE 5

Proportions of CD14+CD15s+, CD15+CD15s+ and CD15+CD14+ leukocyte subpopulations in blood samples before and after the last dive in the second study.

	CD14+CD15s		CD15+CD15s		CD15+CD14	
	Before dive	After dive	Before dive	After dive	Before dive	After dive
Lymphocytes	0,43±0,13	0,69±0,59	0,13±0,10	0,14±0,12	0,02±0,03	0,02±0,03
Monocytes	57,85±6,81	66,25±5,00	12,54±8,52	11,16±5,48	5,54±5,00	6,23±3,58
Granulocytes	28,94±9,99	31,87±12,33	39,87±20,57	10,45±7,23*	13,10±7,56	4,41±3,38**

Values are means of percentages ± SD
* p<0,01
** p<0,05

transferred to a linear scale (bubbles/cm^2) as previously described (*20*).

Antibodies. CD15s was detected with mouse anti-human CD15s antibody of the IgM isotype (Pharmingen, San Diego, CA, USA) and visualized using secondary fluorescein- isothiocyanate (FITC)-conjugated rat anti-mouse IgM antibody (Pharmingen, San Diego, CA, USA). Unlabelled mouse IgM (Caltag, Burlingame, CA, USA) was used as an isotype control. Monoclonal mouse anti-human CD15 conjugated with allophycocyanin (APC) (Pharmingen, San Diego, CA, USA) was used to detect CD15. Monoclonal anti-human CD14 antibody conjugated with phycoerythrin (PE) was used for the monocyte labeling (Pharmingen, San Diego, CA, USA).

Flow cytometry. Peripheral blood cells were isolated by density gradient centrifugation (Histopaque 1,077, Sigma-Aldrich, St. Luis, MO, USA). Cells (1x10^6) were suspended in 100 µL PBS with 0.1% NaN_3 and incubated in the dark for 30 minutes on ice with 0.5 µg of primary anti-CD15s antibody. After two washes in 0.1M PBS, containing 0.1% sodium azide, 0.5 µg of secondary FITC-conjugated affinity chromatography-purified rabbit anti-mouse was added to cells previously incubated with anti-CD15s and incubated on ice for 30 min. For triple lymphocyte labeling, cells were incubated with 1 µg of APC-conjugated antibody reactive to human CD15, and PE-conjugated antibody reactive to human CD14. Finally, cells were resuspended in 0.3 ml of 0.1M PBS containing 0.1% sodium azide.

Two-color fluorescence was measured at the excitation wavelength of 496 nm using a FACSCalibur (Becton-Dickinson, San Jose, CA, USA). Fluorescence was further quantified on the forward scatter/side scatter (FSC/SSC) dot plots. A total of 5x10^5 cells were acquired. Nonspecific binding of secondary antibody was excluded by incubating the cells only with the FITC-labeled secondary antibody.

Statistical analysis. Data in the text and tables are presented as mean ± standard deviation (SD). Normality of the distribution was confirmed for all parameters using Kolmogorov-Smirnov test. Differences in pre and post dive values were determined using the Student t-test for paired samples. Bubble grades were compared using the Friedman test. Statistical significance was set at P<0.05. All analyses were done using Statistica 7.0 software (Statsoft, Inc., Tulsa, USA).

RESULTS

All subjects completed both dive series without reporting any symptoms of decompression sickness (DCS). Venous gas bubbling was found after each dive in the first series divers, with somewhat higher bubbling in the 1st and the 3rd dive compared to the last dive, i.e. 6th dive (2 (1.0 – 2.5), 3 (2.5 – 3.5), 1(1.0 –1.8), respectively). Venous gas bubbling was also found after each dive in each of the divers in the second series (3 (3 – 4), 3 (2 – 3) and 3 (0.5 – 3) for the first, second and the third dive, respectively). Higher bubble grades were found during the 1st and the last dive in the second series when compared to the 1st and the last dive in the first series (3 (3-3.75) vs. 1 (1-2) p=0.016). Heart rate (HR) increased from baseline to about 30-35% of maximal HR (HRmax, calculated as 220 – age) at the bottom phase of each dive in both dive series and to about 20% of the HRmax during the decompression stops.

Proportions of leukocyte subpopulations (lymphocyte, monocyte and granulocyte) in the first series were not different before and after the 1st and the last dive based on R1-R3 gates on dot plot with scatter parameters (Table I). The proportion of granulocytes in all samples was low due to the method of leukocyte isolation which favors the isolation of mononuclear cells.

The proportion of CD15s+ granulocytes in the first series decreased significantly in blood samples after the 1st dive. CD15s+ granulocyte proportion was 50.7 ± 15.2% before the dive, whereas after the dive it decreased to 31.9 ± 7.4% ($p<0.01$) (Tab II2). CD15s+ granulocyte proportion was also somewhat decreased after the last dive, but this decrease was not statistically significant (TabIII3).

The expression of CD15 and CD15s was continuously low in lymphocytes and unaltered after the dives in the monocyte fraction (Tab II2 and TabIII3) in the first series.

Monocyte proportion was increased (p=0.014) and lymphocyte decreased (p=0.020) within the total leukocyte population, whand CD15s+ monocytes and CD14+CD15s+ granulocytes were elevated (p=0.019, and p=0.018, respectively) after the first dive in the second series (Tab IV4). Similarly to the first series, the proportion of CD15s+ granulocytes decreased after the first dive, but this decrease was not statistically significant (p=0.052). However, a statistically significant decrease in CD15s+ granulocytes (p=0.006) was found after the last dive in the second series (Tabe V5). CD15+CD14+ granulocytes were decreased after both dives in the second series (p=0.048 and p=0.017, respectively; Tab IV4 ad V5).

DISCUSSION

SCUBA diving exposes the human body to extreme physiological and environmental conditions (*21*) that could induce extensive immunological and inflammatory changes. During dives with compressed air as the breathing gas, the inert gas nitrogen is taken up by the tissues. Upon return of the diver to the surface, if the ambient pressure is reduced faster than gas can be eliminated, the partial pressure of gas in the tissue will be higher than the environmental pressure. This supersaturation can lead to gas coming out of solution, forming bubbles. In addition, the use of compressed air as the breathing as, brings depth restrictions due to the risk of developing nitrogen narcosis and oxygen toxicity at greater depths. These limitations are substantially reduced and the maximum safe depths are significantly increased with the use of gas mixtures such as trimix of oxygen, helium and nitrogen, which has become a method of choice for dives deeper than 50-60 meters (*22*).

In this study, the divers had a moderate level of venous gas bubbling following the 1st and the 3rd dive in the first series. However, although the number of these bubbles was lower in the last dive, there was no significant difference between the various dives. The lower number of bubbles in the last dive could be attributed to the use of the "travel" trimix gas mixture with the higher proportion of oxygen, which enabled a greater elimination of nitrogen compared to the previous dives. Dive profiles used in the second series resulted in significantly higher bubble grades. Venous gas bubbles play an important role in the pathophysiology of decompression sickness by altering endothelial cell phenotypes (*23*). Particularly important is the role of endothelial selectin (E-selectin), which mediates leukocyte adhesion and rolling along the vascular wall. E-selectin is specifically located in the lipid microdomains, so called lipid rafts (*24*).We have recently shown that E-selectin monocyte ligand CD15s is significantly increased following a single dive *(4),* while granulocyte CD15s was only slightly decreased. Granulocyte-endothelial cell adhesion tests indicate that CD11b, the major membrane protein decorated with CD15s (*14*) is decreased after hyperbaric oxygen treatment (*5*).

In the first field diving protocol employed in this study we investigated the effects of six repetitive dives on the proportion of CD15+CD15s+ leukocytes. Compared to our previous study (*4*) in which the dive depth was 54 m with 20 min bottom tme, and with compressed air as the breathing gas, in the current study the maximum depths were greater but with shorter bottom times. Additionally, in the current study the breathing gas mixtures were trimix and nitrox, which resulted in a smaller nitrogen and bubble load. We found a statistically significant decrease in CD15s+ granulocytes after the 1st dive and a slight, but non-significant, decrease after the last dive. Finding of the smaller effect of the last dive on the CD15s+ granulocytes could be related to the smaller number of vascular gas bubbles on that day, or even the possible acclimatization to these repetitive dives. However, this needs further investigation. As a contrast to our previous study, where an increase in CD15+ and CD15s+ subpopulations of mono-

cytes was detected (*4*), the monocyte fraction was unaffected in the first study series. This could result from different diving profiles (depth and bottom times), different gas mixtures used, as well as the experience and overall fitness of the participants. In the current study, all participants had considerable diving experience and were physically fit, suggesting the possibility that they are already somewhat acclimatized and resistant to such stress. Since these subjects are frequently exposed to a variety of different environmental stresses such as high and low pressure, hypoxia and hyperoxia, cold and warm temperatures, extreme exercise or breathing of dense air mixtures, it is possible that they are non-specifically preconditioned to any stress such as a combination of diving and moderate exercise and consequently do not represent the average diving population.

However, after the first dive of the second series, with the longer bottom time, a longer total divetime, and with higher grade of bubbles postdive, we detected an elevated proportion of both the total and the CD15s+ monocyte. Elevation of the CD14+CD15s+ granulocytes after the first dive in the second series was obviously achieved by elongation of the CD15 antigen at CD14+CD15+ granulocytes with sialic acid. This is indicated by the concomitant and a corresponding reduction in the fraction of CD14+CD15+ granulocytes.

After the last dive of the second series the monocyte and CD14+CD15s+ granulocyte populations were unaffected. Similarly to the first series, we found a significant decrease in CD15+CD15s+ granulocytes after the last dive of the second series. CD15s is the terminal glycosidic residue that plays a crucial role in the interaction between granulocytes and endothelium. Zen et al. presented direct evidence that both CD11b and CD18 subunits of β_2 integrin Mac-1 purified from normal human granulocytes are decorated with CD15s (*14*). Western blot analysis and double labeling of granulocytes, using anti-CD15s and anti-Mac-1 antibodies, further implies that CD11b and CD18 are the major granulocyte membrane proteins decorated with CD15s moieties. The expression of atherogenic adhesion molecule CD11b was found to be decreased after high frequency and long duration exercise (*25*). It has also been shown that a competitive marathon race can decrease neutrophile functions (oxidative burst activity and phagocytic activity) in athletes (*26*).

In contrast to monocytes, granulocytes produce considerably less pro-inflammatory cytokines and more quantities of cytokine inhibitors such as the TNF soluble receptor type 2 and the IL-1 receptor antagonist (*27*). The granulocytes are principally responsible for production of anti-inflammatory cytokines, while the monocytes preferentially produce pro-inflammatory cytokines. Therefore, our findings of increased CD14+CD15s granulocytes only after the first dive in the second sries, and the unaltered or decreased CD15s+ granulocytes after all repeated ives, indicate the possibility of increased production of anti-inflammatory cytokines by repeated dives. It has been suggested that the immune system may play an important role in adaptation to physical stress (*28*). Granulocytes are considered the classic acute inflammatory cell, since they are the first to arrive on location following local injury (*27*). Our findings of increased CD14+CD15s granulocytes only after the first dive in the second series is in accordance with their reported role in the acute inflammatory process. However, the precise role of the granulocytes in a mixed inflammatory cell response is not well defined (*27*). The findings of unaltered or decreased CD15s+ granulocytes after all repeated dives in our study support the attribution by Xing and Remick (*27*) to granulocytes. They compared granulocytes to firemen who bring the resources necessary to put out the flame of inflammation.

In conclusion, in the current study we detected inverse expression of endothelial selectin ligand CD15s on CD14+ and CD15+ leukocyte subpopulations following dives. CD14+CD15s+ leukocytes (within monocyte and granulocyte subpopulation) were increased while CD15+CD15s+ granulocytes were decreased after certain dives. Therefore, CD15 + CD15s+ granulocytes, which could represent granulocytes described earlier as intelligent cells critical for the regulation of the inflammatory process (*27*), deserve attention in further research.

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