Hypoxia and reoxygenation do not upregulate adhesion molecules and natural killer cell adhesion on human endothelial cells in vitro

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Abstract

Objectives: Ischemia/reperfusion injury is characterized by endothelial cell activation leading to increased expression of adhesion molecules such as inter-cellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, endothelial- and platelet-selectin (E- and P-selectin), and to the subsequent recruitment of leukocytes. The aim of the present study was to investigate the respective effects of a proinflammatory cytokine (tumor necrosis factor alpha, TNF-α), hypoxia and/or reoxygenation on adhesion molecule expression and natural killer (NK) cell adhesion in an in vitro model of I/R. Methods: Human aortic endothelial cells (HAEC) were stimulated in vitro for 8h with TNF-α (1000 U/ml) and exposed to hypoxia (1% O2), reoxygenation (21% O2) or different combinations thereof. Cell surface expression of ICAM-1, VCAM-1 and E-/P-selectin on HAEC was analyzed by flow cytometry, and culture supernatants were tested for soluble adhesion molecules by ELISA. Rolling adhesion of NK cells on HAEC was determined using a rotating assay. Results: Untreated HAEC constitutively expressed ICAM-1 on their surface but neither expressed E-/P-selectin, VCAM-1, nor shedded soluble adhesion molecules. Exposure of HAEC to hypoxia or hypoxia and reoxygenation did not upregulate cell surface expression or shedding of adhesion molecules. In contrast, TNF-α significantly upregulated cell surface expression of ICAM-1, VCAM-1, and E-/P-selectin and led to the shedding of ICAM-1 and E-selectin. Combined treatment of HAEC with TNF-α, hypoxia and reoxygenation reduced E-/P-selectin surface expression and enhanced E-selectin shedding, but did not further influence ICAM-1 and VCAM-1. Soluble VCAM-1 was not detected. NK cell adhesion on HAEC increased 4-fold after TNF-α stimulation, but was not affected by hypoxia or hypoxia and reoxygenation. Conclusions: Both the expression of endothelial adhesion molecules and rolling NK cell adhesion was upregulated by TNF-α but not by hypoxia alone or hypoxia followed by reoxygenation supporting the view that anti-inflammatory treatment may reduce ischemia/reperfusion injury.

Keywords: Ischemia/reperfusion injury; Hypoxia/reoxygenation; Human aortic endothelial cells; Adhesion molecules; Natural killer cells; Tumor necrosis factor alpha

1. Introduction

Ischemia/reperfusion injury occurs in several clinical situations including revascularization of coronary or peripheral arteries, solid organ transplantation, severe hypotension, and cardiac surgery employing extracorporeal circulation [1]. Reperfusion following ischemia represents a double edged sword: on one hand it is crucial to re-establish blood supply and salvage tissues from life threatening damage, on the other hand it leads to pathophysiological changes resembling inflammatory responses in many regards. One of the earliest events during ischemia/reperfusion injury is activation and dysfunction of the endothelium. Endothelial cells exposed to hypoxia in vitro release inflammatory mediators such as tumor necrosis factor alpha (TNF-α) during reperfusion, apparently due to a dysbalance between nitric oxid (NO) and free radical production [2]. Nevertheless, the role of O2-pressure changes as a cause of endothelial activation and/or damage independently from

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cytokine-mediated cellular inflammatory reactions is still not well defined. Ischemia/reperfusion-induced endothelial changes are characterized by a dysbalance of the coagulation system leading to obstruction of the microvascular perfusion, complement activation, and a release of von Willebrand factor. Moreover, several adhesion molecules are upregulated. Endothelial- and platelet-selectin (E- and P-selectin) bind to sugar residues on leukocytes. Members of the immunoglobulin superfamily, namely inter-cellular adhesion molecule (ICAM-1) and vascular adhesion molecule (VCAM-1), bind to their ligands lymphocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4), respectively (reviewed in ref. [3]). On a functional level, these adhesion molecules lead to the recruitment of various leukocyte subpopulations, such as neutrophiles and natural killer cells (NK cells), to the endothelium and their subsequent migration into the tissue [4,5]. Cellular infiltration into ischemic tissue and the subsequent enhancement of local inflammatory reactions result in the release of oxygen-derived free radicals, subsequent enhancement of local inflammatory reactions [4,5]. Cellular infiltration into ischemic tissue and the subsequent enhancement of local inflammatory reactions result in the release of oxygen-derived free radicals, proteolytic enzymes and inflammatory cytokines, all having deleterious effects on endothelial and myocardial function [4]. Furthermore, several clinical studies have demonstrated the release of biologically active soluble adhesion molecules into the plasma of patients with coronary artery surgery [8] and transplantation [9]. However, the functional role of soluble adhesion molecules in ischemia/reperfusion remains to be elucidated.

Using an in vitro model of ischemia/reperfusion the aim of the present study was to investigate the respective effects of the proinflammatory cytokine TNF-α, hypoxia, and reoxygenation on human aortic endothelial cells (HAEC) with special regard to the surface expression and shedding of adhesion molecules and the rolling adhesion of NK cells. We found that neither hypoxia nor reoxygenation increased the expression of adhesion molecules or rolling NK cell adhesion in the absence of TNF-α. These findings indicate: (1) that the induction of proinflammatory cytokines rather than hypoxia itself is responsible for leukocyte recruitment during ischemia/reperfusion; and (2) that therapeutic approaches to limit leukocyte-mediated endothelial damage during ischemia/reperfusion should aim at inhibiting the upregulation and/or the function of adhesion molecules.

2. Materials and methods

2.1. Cell culture

2.1.1. Endothelial cells

HAEC were isolated from pieces of human aorta. Adventitial tissue was roughly excised, the aortic lumen cut open, and the endothelial side was incubated with M199 (Life Technologies, Basel, Switzerland) supplemented with collagenase I (Worthington Biochemical Corporation, Lakewood, NJ), 20 mM HEPES (Life Technologies) and 2% penicillin/streptomycin (Life Technologies) for 15 min at 37°C. Then the tissue was rinsed with HANKS balanced salt solution (Kantonsapotheke, Zürich, Switzerland), and the resulting solution was filtered through a 22 μm cell strainer to remove tissue debris. After centrifugation, the cells were resuspended in 2 ml complete medium, M199 (Life Technologies) supplemented with 15% FCS (PAA Laboratories GmbH, Linz, Austria), 20 mM HEPES, 100 U/ml Penicillin, 100 μg/ml Streptomycin, 1% MEM non-essential amino acids (all Life Technologies), 100 μg/ml heparin (Sigma, Buchs, Switzerland) and 50 μg/ml endothelial cell growth supplement (BD Biosciences, Bedford, MA) and seeded on fibronectin-coated (Fisher Scientific AG, Wohlen, Switzerland) six-well-dishes (Nunc A/S, Roskilde, Denmark). HAEC were used up to passage 10 and their purity was regularly determined by phase contrast microscopy, LDL-uptake (Biomedical Technologies Inc., Stoughton, MA/USA), and CD31 expression.

2.1.2. Natural killer cells (NK cells)

NK cell lines were generated as previously described [10]. In brief, peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral blood of healthy donors by Ficoll (Amersham Pharmacia Biotech, Upsala, Sweden) density gradient centrifugation. NK cells were separated on a magnetic column by negative selection (NK Cell MACS Isolation Kit, Miltenyi Biotech, Bergisch Gladbach, Germany) and cultured in AIM-V medium (Life Technologies) supplemented with 20% human plasma obtained from healthy volunteers, MEM non-essential amino acids, 1% penicillin/streptomycin, 1% HEPES (Life Technologies) and 200 U/ml interleukin 2 (Cetus, Boston, MA). Lethally irradiated PBMC (3000 rad) were added as feeder cells during the 1st week. The purity of NK cell populations was defined by a CD3+/CD16+/CD56+ phenotype as determined by flow cytometry and was routinely >95%.

2.1.3. Hypoxia/reoxygenation (H/R)

HAEC were exposed to hypoxia (1% O2, 5% CO2 and 94% N2) for 8 h in a commercially available hypoxia chamber (Coy Laboratory Products, Grass Lake, MI) at 37°C in the presence or absence of TNF-α (1000 U/ml, Sigma). Thereafter, the cells were washed once with HANKS, normal cell culture medium was added, and reoxygenation was performed in a humidified cell culture incubator (21% O2, 5% CO2) at 37°C for another 8 h. Culture supernatants were collected and stored at -20°C for further analysis.

2.1.4. Antibodies

The following antibodies were used for flow cytometry: MHC class I (DX17, kindly provided by L. Lanier, University of California, San Francisco, CA), PECAM-1 (CD31, LCI-4 -6 and -7, kindly provided by P. Kilshaw, The
Babraham Institute, Cambridge, UK), ICAM-1 (BBIG-11, R&D Systems, Minneapolis, MN), VCAM-1 (1G11.B1, Neomarkers, CA) and E-/P-selectin (1.2B6 recognizing both human E- and P-selectin, Serotec, Oxford, UK). Negative controls were performed using irrelevant isotype-matched antibodies (MOPC-21, Sigma, Saint Louis, MI).

2.2. Flow cytometry (FACS)

HAEC were detached by trypsinization (0.05% trypsin, 0.5 mM EDTA, Life Technologies), resuspended in FACS buffer (HANKS, 0.1% bovine serum albumine) at a maximum of 10^6 cells/tube and incubated for 30 min on ice with saturating amounts of the indicated primary mouse antibodies. After washing with FACS buffer, the cells were incubated with secondary fluorescein isothiocyanate-conjugated goat anti-mouse antibodies (Boehringer Mannheim, Mannheim, Germany) for 30 min on ice, washed again, and analyzed on a FACScan (Becton Dickinson, Sunnyvale, CA). Irrelevant isotype-matched antibodies were used as negative controls and propidium iodide gating to exclude dead cells. Low density lipoprotein (LDL)-uptake was determined using a fluorescent probe (Dil-Ac-LDL, Bio-medical Technologies Inc., Stoughton, MA), 2 µg/ml of which was added to adherent HAEC for 4 h at 37°C before removal of the cells by trypsinization and analysis of LDL uptake by flow cytometry.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Cell free culture supernatants were analyzed in duplicate for their content of soluble ICAM-1, VCAM-1 and E-selectin by commercially available ELISA kits according to the instructions of the manufacturer (R&D Systems, Abdingdon, UK). To determine the protein concentration, the test sample absorbances at OD450 nm were plotted against a standard curve and expressed in ng/ml. The detection limits were 0.35 ng/ml for soluble ICAM-1, 2.0 ng/ml for VCAM-1, and 0.1 ng/ml for E-selectin, respectively.

2.4. Rotating adhesion assay

Rolling adhesion of NK cells on HAEC was analyzed using a modified Stamper-Woodruff assay [11]. Briefly, HAEC were grown to confluence in 30 mm culture dishes within the limits of a 20 mm circle administered with a nontoxic silicon oil coat (dimethylpolysiloxane; Sigma). Monolayers were treated as indicated, carefully washed with Weissman glucose buffer containing 5.55 mM glucose (Sigma), and overlayed with 10^6 NK cells in 100 µl Weissman glucose buffer supplemented with human albumin (5 mg/ml, Fluka, Deisenhofen, Germany). The dishes were rotated at 64 rpm in a shaker-incubator (Kühner AG, Birsfelden, Switzerland) at 37°C, inducing a shear stress of 0.7 dynes/cm² at a radius of 0.6 cm from the center on the endothelial monolayer. This shear stress corresponds to the lower range of shear present in post capillary venules in vivo. After 10 min the assay was stopped by prefixing all cells for 2 min with 1% paraformaldehyde. The monolayers were then gently washed with Weissman glucose buffer to remove non-adherent NK cells, fixed for additional 20 min and washed again. For quantification, five fields of 0.16 mm² were defined at a distance of 0.6 cm from the center of rotation and the number of adhering cells was counted by light microscopy.

3. Results

3.1. Neither hypoxia nor hypoxia/reoxygenation induce upregulation of adhesion molecules on HAEC

Resting HAEC constitutively expressed the adhesion molecule ICAM-1, but not VCAM-1 or E-/P-selectin (Fig. 1). Stimulation with TNF-α (1000 U/ml) significantly upregulated all adhesion molecules which were determined. In contrast, neither hypoxia alone nor hypoxia followed by reoxygenation had an influence on the cell surface expression of adhesion molecules. Moreover, exposure of HAEC to TNF-α in combination with hypoxia alone or hypoxia/reoxygenation did not induce further upregulation of adhesion molecules as compared with TNF-α alone. In contrast, the combination of TNF-α and hypoxia/reoxygenation led to a significant downregulation of E-/P-selectin (P < 0.01) and a trend towards a decrease of VCAM-1 expression (P = 0.058), whereas ICAM-1 expression was unchanged.

3.2. Hypoxia and reoxygenation enhance TNF-α induced shedding of ICAM-1 and E-selectin from HAEC

Next, we investigated whether adhesion molecules were shed from the surface of HAEC upon treatment with TNF-α, hypoxia or hypoxia/reoxygenation. Therefore, supernatants obtained from in vitro cultures of HAEC were analyzed by ELISA. Soluble adhesion molecules were not detected in supernatants of resting HAEC. Accordingly, soluble ICAM-1 was also absent in supernatants of hypoxia or hypoxia/reoxygenation-treated cell cultures. In contrast, as shown in Fig. 2, the mean concentration of soluble ICAM-1 was 3.31 ± 0.25 ng/ml after activation of HAEC with TNF-α, and even higher concentrations were found after treatment with TNF-α and hypoxia (4.09 ± 0.14 ng/ml) or hypoxia/reoxygenation (4.32 ± 0.08 ng/ml). E-selectin displayed a shedding pattern similar to ICAM-1. Soluble E-selectin was found after exposure of HAEC to TNF-α alone (0.88 ± 0.12 ng/ml) or in combination with hypoxia (0.90 ± 0.28 ng/ml), and a combined treatment with TNF-α and hypoxia/reoxygenation further increased the concentration of soluble E-selectin in HAEC supernatants (1.47 ± 0.51 ng/ml). None of the treatments induced shedding of VCAM-1.
**Fig. 1. Cell surface expression of adhesion molecules.** HAEC were exposed to TNF-α, hypoxia, hypoxia/reoxygenation (H/R) and different combinations thereof. Expression of ICAM-1, VCAM-1 and E-/P-selectin was determined by flow cytometry. Numbers indicate mean fluorescence intensity. Negative controls were performed with isotype-matches irrelevant antibodies (black histograms). Data are representative for at least four independent experiments.
3.3. Rolling adhesion of NK cells on HAEC is not increased by exposure to hypoxia or reoxygenation

To test whether hypoxia and/or reoxygenation have a direct impact on leukocyte adhesion, rolling adhesion assays using allogeneic human NK cells, which readily adhere to resting HAEC, were performed (Fig. 3). Rolling NK-cell adhesion to resting HAEC (median 19.25, range 10.25–24.0) was increased following TNF-α stimulation (median 94.0, range 33.3–226.0), which is in line with the enhanced surface expression of ICAM-1, VCAM-1 and E-/P-selectin described above. On the contrary, exposure of HAEC to hypoxia (median 18.0, range 6.5–25.85) even decreased NK cell adhesion, and subsequent reoxygenation (median 21.5, range 12.9–36.75) did not lead to higher numbers of adhering NK cells as compared with resting HAEC. In addition, NK cell adhesion was not further increased on HAEC exposed to a combination of TNF-α and hypoxia (median 89.8, range 41.6–166.0) or TNF-α and hypoxia/reoxygenation (median 105.25, range 22.9–179.0), as compared with HAEC treated with TNF-α alone. In summary, hypoxia alone does not display any additional or synergistic effects on NK cell adhesion to HAEC in relation to stimulation of HAEC with TNF-α alone.

4. Discussion

Endothelial activation and subsequent upregulation of adhesion molecules is a well known phenomenon during ischemia/reperfusion. However, there are contradictory reports on the initial factor triggering endothelial activation; hypoxia versus proinflammatory cytokines. This issue is of importance for clinical decision making regarding ischemia times and the design of therapeutical strategies to prevent ischemia/reperfusion. Since the debate is still open, we designed a study to evaluate the different impacts of oxygen pressure changes and of the inflammatory stimulus TNF-α using an in vitro model for ischemia/reperfusion.

Upregulation of cell surface adhesion molecules on HAEC clearly depended on stimulation with TNF-α whereas hypoxia and hypoxia/reoxygenation failed to increase the expression of the adhesion molecules ICAM-1, VCAM-1 and E-/P-selectin independently. These results confirm and expand our previous findings showing that hypoxia does not upregulate adhesion molecules on human umbilical vein endothelial cells, but enhances lipopolysaccharide-induced E-selectin expression on porcine aortic endothelial cells [12]. In the present study, reoxygenation additional to hypoxia was analyzed. This treatment did not upregulate adhesion molecules on HAEC, but rather decreased the cell surface expression of E-/P-selectin confirming an earlier report on E-selectin expression on umbilical vein endothelial cells [13]. One possible explanation for this decrease consisted in shedding of the adhesion molecules, as suggested by a previous report showing that E-selectin shedding from umbilical vein endothelial cells was indeed significantly enhanced by a combination of TNF-α and hypoxia/reoxygenation [14]. Indeed, we also found that soluble E-selectin was present in
supernatants of HAEC following stimulation with TNF-α alone or in combination with exposure to hypoxia. Additional reoxygenation further increased the shedding of E-selectin. Therefore, E-selectin shedding may explain, at least in part, the reduced amount of E-selectin detected on the surface of HAEC after TNF-α and hypoxia/reoxygenation. In contrast, there was no evidence for a similar mechanism regulating the expression of the two other adhesion molecules tested, ICAM-1 or VCAM-1.

The effect of adhesion molecule upregulation on leukocyte recruitment to HAEC was tested in functional NK cell adhesion experiments. Whereas neutrophil-mediated tissue injury after adhesion and infiltration has been clearly documented during ischemia/reperfusion [15], little is known on a potentially additional role of NK cells. NK cells are part of the leukocyte population infiltrating inflammatory tissue and transplanted organs [5]. Since NK cells are able to lyse endothelial cells [5], they might as well mediate endothelial damage during ischemia/reperfusion. In our experiments, a low number of NK cells adhered to resting HAEC. Stimulation with TNF-α strongly increased NK cell adhesion on HAEC, whereas hypoxia with or without subsequent reoxygenation failed to increase NK cell adhesion. In contrast, there was rather a trend towards decreased NK cell adhesion on hypoxic endothelium. Since TNF-α treatment of HAEC in combination with hypoxia/reoxygenation reduced the surface levels of E-selectin and VCAM-1, NK cell adhesion on HAEC, which was not reduced by TNF-α and hypoxia/reoxygenation, seems to depend primarily on ICAM-1/LFA-1 interactions. However, this indirect evidence needs to be confirmed with blocking assays using monoclonal antibodies specific for ICAM-1, VCAM-1 and E-/P-selectin, respectively. In addition, since allogeneic NK cells were used in this report future experiments have to assess the susceptibility of ischemic endothelial cells to autologous NK cell adhesion and lysis.

Taken together, our data support the notion that endothelial activation in the setting of hypoxia/reoxygenation, used as model for ischemia/reperfusion injury in vitro, strongly depends on proinflammatory mediators such as TNF-α. Several animal and clinical studies already tested the feasibility of blocking inflammation and leukocyte adhesion to prevent cardiovascular disease. It was suggested to specifically block the nuclear factor-κB, a transcription factor regulating the expression of ICAM-1 and VCAM-1 in response to TNF-α [16], alternative approaches consist of anti-TNF-α therapy [17] or inhibitors of cellular signalling pathways such as Poly-(ADP)-Ribose-Polymerase [18]. Direct blocking of ICAM-1 or its ligands may represent at least a potential effective clinical treatment of ischemia/reperfusion since in animal models the cerebral infarction size was reduced by anti-ICAM-1 antibodies administered during reperfusion [19]. However, in patients with ischemic stroke, anti-ICAM-1 therapy was not successful [19,20]. Accordingly, clinical trials of leukocyte-adhesion blockade in myocardial infarction and hemorrhagic shock were disappointing [21]. The reason for the discrepancy between the positive results obtained in animal models and the negative clinical trials are not fully understood and currently under investigation. Another perspective lies in the development of synthetic adhesion molecule blockers, which may be less immunogenic than antibodies and may be tolerated better.

The role of soluble adhesion molecules during
ischemia/reperfusion is not well defined. Theoretically, they may contribute mechanistically to the disease and/or serve as disease markers. However, preliminary clinical studies revealed contradictory results on the latter. Soluble adhesion molecules were evaluated after coronary angioplasty and acute myocardial infarction [22], and in individuals who developed peripheral arteriosclerosis, baseline levels of soluble ICAM were significantly higher compared to those who remained healthy. However, the ranges were very tight and therefore did not allow the use of soluble ICAM-1 as a predictive marker [23]. In another study, soluble VCAM-1 was strongly elevated in patients with coronary artery disease and death related to cardiovascular diseases, whereas the correlations for E-selectin and ICAM-1 were less stringent [24]. A meta analysis analyzing the value of soluble adhesion molecules as predictor for coronary heart disease came to the conclusion that measurements of soluble adhesion molecules did not further add information for diagnosis after adjustment for other cardiovascular risk factors [25]. Thus, it seems that soluble adhesion molecule concentrations in patient serum are only increased in a state when cardiovascular damage is already clinically obvious.

Overall, the in vitro data obtained in the present study indicate that the initial steps of ischemia/reperfusion leading to endothelial cell activation mainly depend on inflammatory responses and are not directly influenced by oxygen pressure changes. Future studies are necessary to further explore the potential role of soluble adhesion molecules shed from activated endothelial cells and NK cell-mediated damage during ischemia/reperfusion. Nevertheless, successful therapy of ischemia/reperfusion may focus on limiting the inflammatory reactions of the organism. To date, adhesion molecule blockade did not show any clinical benefit in treating ischemia/reperfusion, possibly because leukocyte-induced tissue damage depends on parallel mechanisms. Isolated blocking of only one arm seems to deviate the system to alternative pathways, therefore combined approaches of preventing endothelial activation and blocking leukocyte adhesion will be necessary to avoid inflammation and tissue damage during ischemia/reperfusion injury.

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References


Appendix A. Conference discussion

Dr C. Yankah (Berlin, Germany): You are presenting adhesion molecules as a marker for atherosclerotic formation. Did you find out other markers which are competitive to the adhesion molecules as a control mechanism and therefore can be used to prevent the process of atherosclerosis on your model?

Dr Schmidt: The most investigated adhesion molecules in the context of ischemia/reperfusion injury are indeed ICAM-1, the selectins and VCAM-1. They are the ones responsible for adhesion of various kinds of leukocytes which is a prerequisite to infiltration into the tissue. There are several multi-centre studies under way to assess an association between adhesion molecule serum levels and severity of atherosclerosis; one study aiming at blocking ICAM-1 in order to attenuate the ischemia/reperfusion injury subsequent to stroke was disappointing so far.

Dr M. Deja (Katowice, Poland): I just wanted to know what was the protocol for achieving hypoxia and reoxygenation in your situation? How long was the hypoxia and what was the real method of insuring that this was a strong enough hypoxic insult?

Dr Schmidt: Hypoxia and reoxygenation were performed for 8 h each in a defined, closed hypoxia chamber equipped with an oxygen sensor assuring constant hypoxia of 1%. The time period of 8 h was chosen according to previous studies demonstrating the upregulation of the three adhesion molecules investigated during this time.

Dr A. Haverich (Hannover, Germany): Not a question, but a comment. I only rise to compliment the authors on this very nice study, for two reasons, one, they had a reductionary approach in taking the question from the cellular level into this tissue culture approach. It also shows that surgical research can actually be done without going all the way down to molecular genetics and still have a very clear and clean model as to study effects. They’re very appropriate for the clinics.

Dr J. Vaage (Stockholm, Sweden): Let me just make a comment. Did you find this to be a surprising result that you didn’t find any increase in adhesion molecules in this model with hypoxia or in reoxygenation alone?

Dr Schmidt: Yes, we were surprised but in line with previously published data. But this finding underlines the importance of dissecting a complex in vivo process into its different components in vitro and also demonstrated the high impact of secondary inflammatory reactions.

Dr Vaage: Do you believe that the findings would be different if you had ischemia-reperfusion situation with blood and blood cells?

Dr Schmidt: Definitely, we were using an artificial in vitro system which served to investigate hypoxia and reoxygenation effects rather than ischemia/reperfusion injury. However, it will be interesting to establish an experimental approach which is more closely related to the in vivo situation.