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ORIGINAL ARTICLE

Hypoxia increases membranal and secreted HLA-DR in endothelial cells, rendering them T-cell activators

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Summary

Transplantation involves preoperative ischemic periods that contribute to endothelial cell (EC) dysfunction and T-cell activation, leading to graft rejection. As hypoxia is a major constituent of ischemia, we evaluated its effect on the ability of ECs to express HLA-DR, which is required for presentation of antigens to T cells, and by itself serves as an important target for allogeneic T cells. Primary human umbilical vein ECs (HUVEC) and the human endothelial cell line EaHy926 were incubated in normoxia or hypoxia ($PO_2 < 0.3\%$). Hypoxia increased the membranal expression (by 4–6 fold, $P < 0.01$) and secretion (by sixfold, $P < 0.05$) of HLA-DR protein, without influencing the accumulation of its mRNA. Alternative splicing, attenuated trafficking, or shedding from the plasma membrane were not observed, but the lysosomal inhibitor bafilomycin A1 reduced HLA-DR secretion. Hypoxia-induced endothelial HLA-DR elevated and diminished the secretion of IL-2 and IL-10, respectively, from co-cultured allogeneic $CD4^+$ T cells in a HLA-DR-dependent manner, as demonstrated by the use of monoclonal anti-HLA-DR. Our results indicate a yet not fully understood post-translational mechanism(s), which elevate both membranal and soluble HLA-DR expression. This elevation is involved in allogeneic T-cell activation, highlighting the pivotal role of ECs in ischemia/hypoxia-associated injury and graft rejection.

Introduction

In transplanted organs, donor endothelial cells (ECs) initiate an allogeneic immune response, directly presenting nonself HLA-DR molecules to memory $CD4^+$ T cells [1]. Although different T-cell subsets require diverse surface and soluble co-stimulatory molecules to mediate ECs injury and exert graft rejection [2,3], they all recognize MHC class II molecules, mostly HLA-DR, which is constitutively expressed *in vivo* on ECs [4], and up-regulated during transplant rejection [5].

The process of organ transplantation necessarily involves ischemia/reperfusion that is associated with acute and chronic graft rejection [6,7], where inflammation, endothelial dysfunction and increased cytokine production play a major role [8–10]. Many aspects of endothelial dysfunction have been studied, but the effect of ischemia/hypoxia on MHC class II expression, which may contribute to rejection, was hardly explored. Although hypoxia is a major constituent of ischemic events, only one report [11] showed increased MHC class II expression on brain ECs following hypobaric hypoxia. The hypoxic regulation of HLA-DR and its relevance to allogeneic T-cell activation were not evaluated.

The most potent stimulator of MHC class II expression is IFNγ, which functions mainly at the transcriptional level. The details of the transcriptional regulation were comprehensively studied in professional antigen presenting cells (APCs) [12,13]. Although constitutively expressed *in vivo*, HLA-DR expression on ECs is lost

during *in vitro* culture, and can be restored by addition of IFNγ [1,9]. Here we used this loss to evaluate the isolated effects of hypoxia, without exogenous IFNγ, on the expression of HLA-DR in ECs. As we detected elevated membranal HLA-DR on hypoxic ECs, and soluble HLA-DR only in hypoxic ECs supernatants, we further demonstrated that hypoxia-induced HLA-DR in ECs lead to activation of allogeneic T cells in T/ECs co-cultures, as manifested by cytokine secretion.

Materials and methods

Endothelial cells

The human endothelial cell line EaHy926 was cultured as described before [14], and the medium was replaced with serum-free medium containing 0.1% BSA before beginning of the experiments. Human umbilical vein ECs (HUVEC) were isolated from different donors, cultured as before [15], and used on passages 3–5. Before the beginning of the experiments, media was replaced with serum-free Bio-MPM1 medium containing 0.1% BSA, 1% glutamine and 1% endothelial cell growth factor (ECGF). This part of the study was approved by the Carmel Medical Center Helsinki committee. Viability of cells was determined using XTT (Biological Industries, Beit-Ha'emek, Israel).

$CD4^+$ T lymphocytes

Twenty-five milliliters of fresh whole blood donated by healthy volunteers was incubated with 1.25 ml of the RosetteSep human $CD4^+$ T-cell enrichment Cocktail (StemCell Technologies, Vancouver, Canada) to deplete unwanted cells. After 20 min incubation, the cells were separated on a Lymphocyte Separation Medium (LSM; ICN-Chappel, Aurora, OH, USA), centrifuged and washed several times. The pellet was re-suspended in RPMI-1640 medium with 10% FCS, and 5×10^4 $CD4^+$ T cells (purity was >84 ± 3.7% as determined by dual labeling with CD4 and CD3) were plated in 96-well plates with phytohemagglutinin (PHA) (3 μg/ml) and incubated alone, with allogeneic non-$CD4^+$ T cells (PBMC), or with 5×10^4 EaHy926 cells for 48 h, with or without addition of the mouse monoclonal anti-HLA-DR. T-cell activation was estimated by the amounts of secreted cytokines. This part of the study was also approved by the Carmel Medical Center Helsinki committee.

Normoxic and hypoxic conditions

For normoxia, the cells were incubated in a regular incubator (21% O_2, 5% CO_2, 74% N_2). Hypoxic incubation was performed in a sealed anaerobic workstation (Concept 400; Ruskin Technologies, Leeds, UK), where the hypoxic environment (O_2 < 0.3%, 5% CO_2, 95% N_2), the temperature (37 °C), and humidity (>90%) are kept constant. Supernatant samples were taken at the end of the exposure to hypoxia and the partial pressures of O_2 (mean of 31 ± 0.4 mmHg), CO_2 (mean of 32.6 ± 0.5), and pH (mean values of 7.3 ± 0.01) were determined using a blood gas analyzer ABL510 (Radiometer, Copenhagen, Denmark).

Flow cytometry

After incubation in normoxia or hypoxia for 48 h, 10^6 EaHy926 cells were scraped with addition of cold PBS (pH 7.4) containing EDTA and labeled with FITC-conjugated mouse monoclonal anti-HLA-DR (clone BRA30; IQ Products, Groningen, the Netherlands). After washing, the cells were fixed in 0.1% formaldehyde and analyzed by a Coulter-XL flow cytometer (Coulter Electronics, Bedfordshire, UK). Dead cells were excluded from the analysis by their forward and sideway light-scattering properties. To determine intracellular HLA-DR, the cells were permeabilized after fixation (2% FCS, 0.1% sodium azide, 0.1% saponin in PBS) and labeled with the antibody.

Radioimmunoassay

1.5×10^4 HUVEC cells were plated in a 96-well plate and incubated in normoxia or hypoxia. At the end of the experiment, the cells were washed (1% FCS in PBS) and labeled with 50 μl of the diluted (1:100) mouse monoclonal anti-HLA-DR for 40 min at 4 °C. 200 000 cpm/well of the secondary antibody (^{125}I-conjugated sheep anti-mouse-Ig (Amersham Pharmacia Biotech, Piscataway, NJ, USA) were added and incubated for additional 40 min in 4 °C. The cells were washed, trypsinized, collected, and their radioactivity was measured by a γ-counter.

Real time PCR analyses

Total RNA and cDNA were prepared as described before [16]. Expression of different regions in the HLA-DRα mRNA molecule (the external region coded by exons 1/2 and the transmembranal region coded by exons 4/5) were quantified by real-time PCR using the TaqMan© assay on demand kit with the ABI-PRISM 7000 (Applied Biosystems, Foster City, CA, USA). Analysis was carried out in triplicates in a volume of 20 μl (2 min at 50 °C, 10 min at 95 °C, and a total of 40 cycles, each of 15 s at 95 °C and 1 min at 60 °C) for both HLA-DRα and the endogenous reference gene RPLP0, which does not change in hypoxia, and the normoxic nonstimulated sample was

used as a calibrator to allow comparison of relative quantity between the samples by the comparative ΔC_T method. Average Ct values for both exons were 28 and those of the RPLP0 reference gene were 18, ruling out background noise.

Western blot

Supernatants from 10^6 EaHy926 cells cultures were concentrated (×20, Vivaspin; VivaScience, Hannover, Germany) and loaded onto a 10% SDS-PAGE (30 μl/lane). Analysis was performed [16], using diluted (1:1000) mouse monoclonal anti-HLA-DR (clone BRA30) and 1:5000 diluted HRP-conjugated donkey anti-mouse IgG (Jackson ImmunoReasearch Laboratories, West Grove, PA, USA).

Isolation of exosomes

After exposure of 10^6 EaHy926 cells to normoxia or hypoxia, supernatants were collected and centrifuged at 800 *g* for 10 min and then at 12 000 *g* for 30 min to sediment suspended cells. The resulting supernatants were ultra-centrifuged at 110 000 *g* (Micro-Ultracentrifuge RC-M150, rotor S120AT2-0200; Thermo Scientific, Sorvall, Suwanee, GA, USA) for 1.5 h at 4 °C to pellet the exosomes. Both pellets and concentrated supernatants (×50, Vivaspin) were loaded onto 10% SDS-PAGE followed by Western blot analysis.

Immunofluorescence

EaHy926 cells were stained after fixation [16] using the primary antibodies mouse monoclonal anti-HLA-DR or rabbit polyclonal anti-Rab11 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the secondary antibodies Rhodamine Red-X-conjugated donkey anti-mouse IgG and Cy2-conjugated donkey anti-rabbit IgG (Jackson). Images were acquired by confocal microscopy, using the Bio-Rad MRC 1000 confocal system (Bio-Rad Laboratories, Hercules, CA, USA).

ELISA

Human IL-2 and IL-10 DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA) were performed according to the manufacturer's instructions.

Statistical analyses

All values are presented as mean ± SE. Comparisons of two experimental groups were carried out using two-tailed *t*-test. Data consisting of more than two groups were analyzed using analysis of variance (ANOVA) with the Student–Newman–Keuls multiple comparisons test to evaluate significance between experimental groups. *P* values exceeding 0.05 were not considered significant.

Results

Hypoxia increases the membranal expression of HLA-DR in ECs

Incubation in hypoxia for up to 48 h did not significantly change ECs viability, and did not cause death relative to the normoxic control (Fig. 1a and b). Therefore, experiments were conducted for 48 h.

Because of the limited number of HUVEC, membranal expression of HLA-DR was evaluated using radioimmunoassay. A basal expression of HLA-DR was observed in normoxia, fourfold above the background, which was determined by the nonspecific binding of the secondary antibody. In hypoxia, a fourfold increase in membranal HLA-DR occurred ($P < 0.05$, Fig. 1c). A similar sixfold increase in both the percentage of EaHy926 cells positive for HLA-DR ($P < 0.01$, Fig. 1d) and in their mean fluorescence ($P < 0.001$, data not shown) was revealed by flow cytometry. No change was observed for the binding of the isotype control when the cells were exposed to normoxia or hypoxia.

Hypoxia-induced up-regulation of membranal HLA-DR is post-transcriptional

Total RNA at 48 h was extracted and the relative amounts of HLA-DRα mRNA were determined by amplifying two distinct regions of the transcript (explained in the next section) using real-time PCR (Fig. 2a and b). No difference in the levels of the HLA-DRα transcript in hypoxic versus normoxic EaHy926 cells was measured, suggesting a post-transcriptional regulation.

To explore a post-transcriptional mechanism, we first evaluated HLA-DR cellular amounts. Flow cytometry demonstrated a small nonsignificant increase in the cellular accumulation of HLA-DR protein in hypoxic EaHy926 cells [Fig. 2c (right panel) and d], while membranal expression was increased ($P < 0.01$). Confocal microscopy also showed a hypoxia-induced small increase in intracellular expression of HLA-DR in some cells, which was partly co-localized with Rab5, the marker for early endosomes (data not shown).

Involvement of the actin cytoskeleton, relevant to the intracellular trafficking of HLA-DR [17], was evaluated by incubation of ECs with increasing concentrations of cytochalazin B, the inhibitor of actin polymerization. In normoxic cells, cytochalazin B dose-dependently increased the membranal expression of HLA-DR ($P < 0.001$ relative

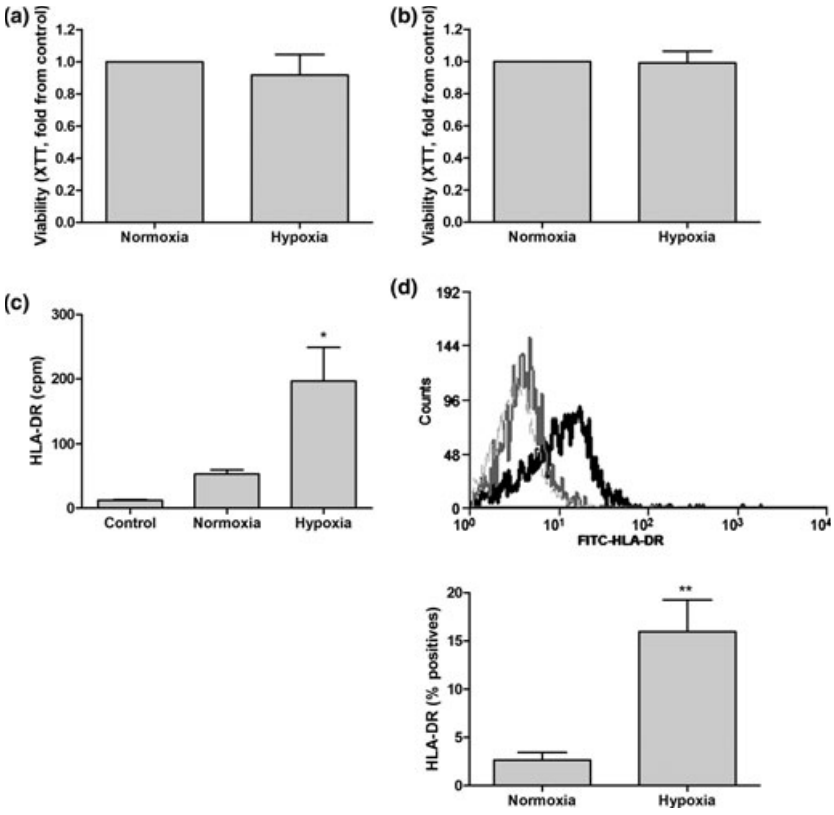

Figure 1 Hypoxia does not induce endothelial cell death and enhance surface expression of HLA-DR; 1.5×10^4 HUVEC or 10^6 EaHy926 cells were incubated for 48 h in normoxia or hypoxia. Cell viability was determined using the XTT kit for (a) HUVEC ($n = 6$) and (b) EaHy926 cells ($n = 4$). Membranal expression of HLA-DR at 48 h was determined using (c) RIA for HUVEC ($n = 4$) or using (d) flow cytometry for EaHy926 cells ($n = 9$). In panel (c), the control is the radioactively labeled secondary antibody; In panel (d), dashed light gray line is the isotype control; solid gray line is the EaHy926 cells in normoxia; black line is the EaHy926 cells in hypoxia. $*P < 0.05$, $**P < 0.01$ relative to normoxia.

to untreated normoxic cells, Fig. 2e), reaching the hypoxic levels of expression.

Hypoxia increases the secretion of soluble HLA-DR via lysosomal cleavage

Secretion of HLA-DR from ECs was studied by Western blot analysis of concentrated supernatants (×20), and revealed a sixfold increase ($P < 0.05$) in the alpha and beta chains of HLA-DR following hypoxia (Fig. 3a) relative to the almost undetectable protein in the normoxic supernatants.

Alternative splicing was checked using real-time PCR. The extracellular region that includes exons 1 and 2 of the HLA-DR transcript and the transmembranal region that includes exons 4 and 5 were amplified. No difference between normoxia and hypoxia was observed in both regions (Fig. 2a and b), ruling out alternative splicing.

HLA-DR secretion by exosomes was investigated by ultracentrifugating the supernatants [18] and examining HLA-DR in the exosomal pellets by Western blots. HLA-DR was found only in the supernatants, before and after ultracentrifugation, indicating that it does not reside in ECs-derived exosomes (Fig. 3b). This was further confirmed by confocal microscopy (Fig. 3c), which demonstrated that HLA-DR was not co-localized to Rab11, a marker for exosomes and multi-vesicular bodies (MVB) [19].

Protease cleavage of HLA-DR, either on the cell surface or in the lysosome, was investigated by incubating EaHy926 cells with wide-range protease inhibitors and with the lysosomal inhibitor bafilomycin A1, which prevents lysosomal acidification. The final concentrations chosen were first determined not to cause cell death by the XTT assay (data not shown). Inhibitors of serine, cysteine, and aspartic proteases or metalloproteinases, that collectively inhibit many proteases with access to membranal HLA-DR, did not change the hypoxia-induced secretion of HLA-DR, but bafilomycin A1 inhibited it (Table 1), suggesting cleavage of HLA-DR in the lysosome.

Hypoxia-induced HLA-DR expression stimulates $CD4^+$ T cells to secrete cytokines

The ability of EaHy926 to stimulate enriched $CD4^+$ T cells was studied in the presence of the lectin PHA, which clusters TCR complexes and bypasses the requirement for antigen recognition by TCR. In preliminary experiments (Fig. 4a), the optimal concentration of PHA (3 μg/ml) was determined, which by itself did not induce expression of HLA-DR on EaHy926 cells (data not

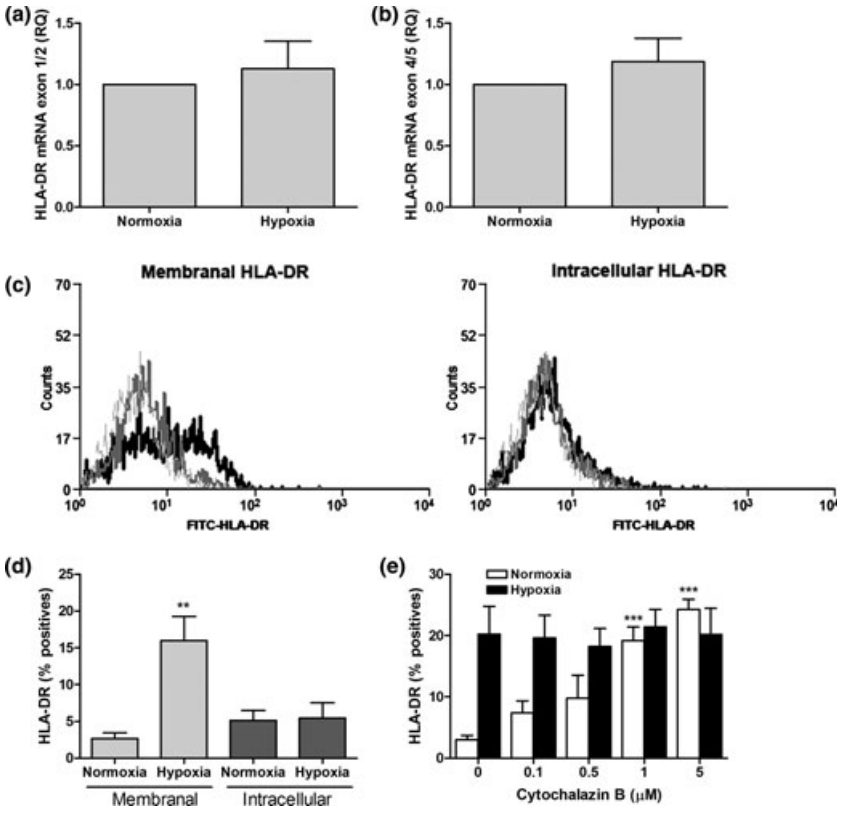

Figure 2 Hypoxia up-regulates surface HLA-DR post-translationally. (a,b) Total RNA was extracted from 10^6 EaHy926 cells and HLA-DR mRNA was quantified using real-time PCR for the extracellular and he transmembranal domains (n = 8) using the ΔCt method. RQ, relative quantity. (c) Representative histograms and (d) analysis (n = 6) of surface and intracellular expression of HLA-DR by flow cytometry. Dashed gray line, isotype control; solid gray line, EaHy926 cells in normoxia; black line, EaHy926 cells in hypoxia. (e) Effects of increasing amounts of cytochalazin B on surface expression of HLA-DR, analyzed using flow cytometry (n = 9).

shown) or IL-2 secretion from the enriched $CD4^+$ T cells after 48 h of incubation. However, PHA was needed and in its absence IL-2 was not secreted even from the co-culture of allogeneic $CD4^+$ T cells and EaHy926 ECs. After setting the conditions, we examined whether EaHy926 ECs stimulated T cells. As a positive control we used non-T cells (PBMC) cultured with allogeneic $CD4^+$ T cells, which exhibited a 10-fold increase in IL-2 secretion ($P < 0.05$ relative to $CD4^+$ T cells alone), that was not changed in hypoxia. Co-culturing EaHy926 cells with $CD4^+$ T cells in normoxia increased IL-2 secretion by 17-fold ($P < 0.001$ relative to $CD4^+$ T cells alone, Fig. 4 b). Hypoxia, or addition of anti-HLA-DR to normoxic cultures, further increased IL-2 secretion by 50% ($P < 0.05$).

Conversely, although co-culturing of EaHy926 cells and $CD4^+$ T cells resulted in a sixfold enhancement in secretion of IL-10 ($P < 0.001$ relative to the negative controls of EaHy926 or $CD4^+$ T cells alone, Fig. 4c), hypoxia or anti-HLA-DR significantly reduced IL-10 by 1.8 and 3.7 folds, respectively ($P < 0.001$).

Discussion

Allo-MHC class II presentation by ECs to T cells is critical in transplanted organ rejection, as ECs are the first cells encountered by the host immune cells and their injury plays a major role in rejection. Here we demonstrate that exposure to hypoxia, which is prevalent in ischemic transplanted organs, is sufficient to elevate both membranal and soluble expression of HLA-DR in human ECs, and to enhance T-cell cytokine secretion.

Hypoxia alone elevated ECs surface expression of HLA-DR in both HUVEC and EaHy926 cells. Therefore, due to the low number of HUVEC in each preparation and the high variability between preparations, we continued exploring this phenomenon in EaHy926 cells, which are currently used in many ECs studies [20]. The main regulatory checkpoint for HLA-DR is considered transcriptional, and was mostly investigated *in vitro* in IFNγ-induced professional APCs [12,13]. However, hypoxia did not affect the amount of HLA-DR mRNA in cultured ECs, suggesting basal transcription of small amounts of HLA-DR mRNA even in normoxic ECs. The difference between the constant levels of HLA-DR mRNA and its elevated protein expression in hypoxia suggested that hypoxia regulates HLA-DR post-transcriptionally or post-translationally. This is in accordance with our previous findings of post-transcriptional or post-translational hypoxic regulation of other immunologically related proteins in monocytes/macrophages, such as reduced CD80 expression [21], loss of iNOS activity [22], and dimin-

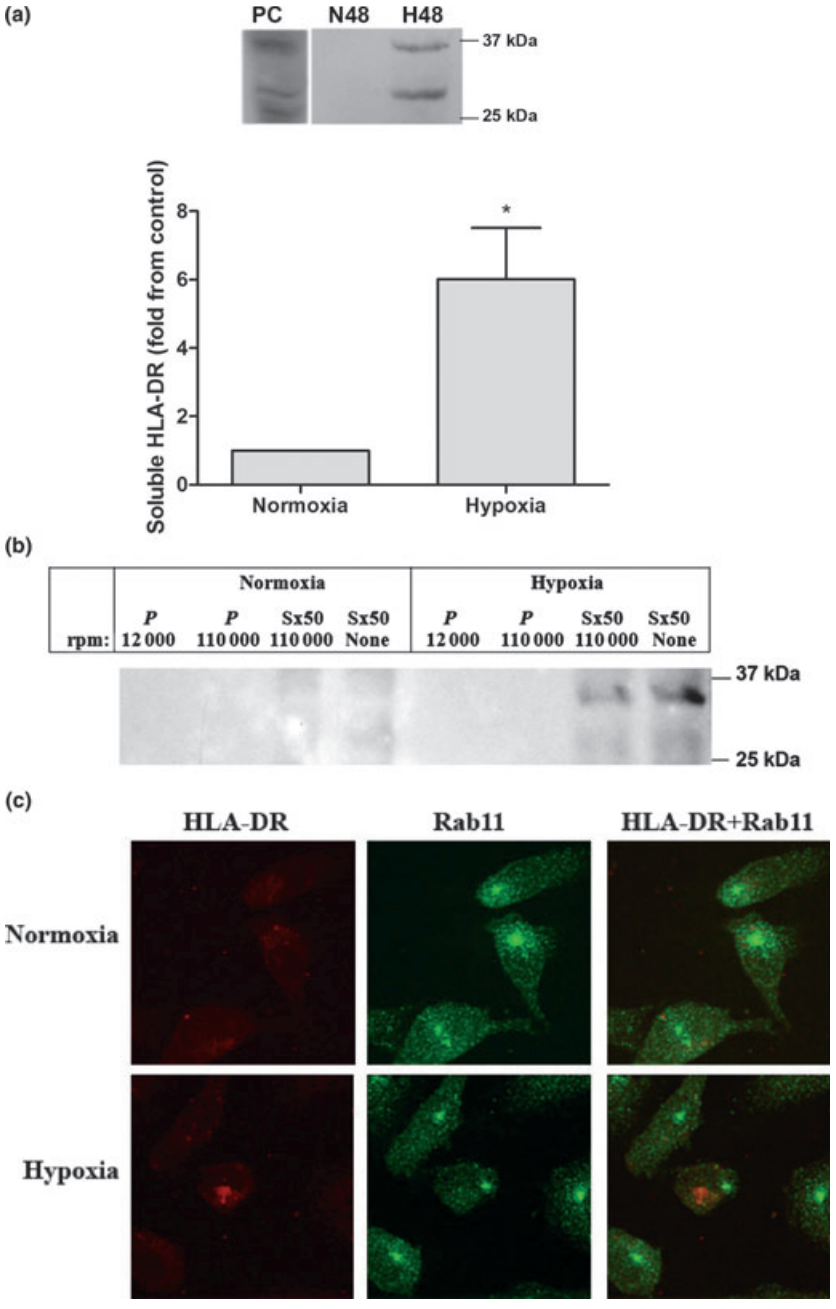

Figure 3 Hypoxia increases soluble HLA-DR secretion, not by exosomes. 10^6 EaHy926 cells were incubated in normoxia and hypoxia for 48 h. (a) Supernatants were collected, concentrated (×20) and subjected to Western blot analysis (n = 5). PC, cellular extract of the U937 monocytic-like cells. (b) Collected supernatants were centrifuged first at 6516 ***g*** and then at 547 501 ***g*** to pellet the exosomes, and expression of HLA-DR in the pellets or in the concentrated (×50) supernatants was detected by Western blot analysis (n = 4). (c) Confocal microscopy reveals induced HLA-DR expression in some EaHy926 cells (red fluorescence), that was not co-localized with the exosomal marker Rab11 (green fluorescence). Magnification ×150.

ished MMP-9 secretion [16]. First we looked for changes in the intracellular amounts of HLA-DR, which could reflect altered trafficking, but only a minor elevation was measured, consistent with the weak fluorescent detection of HLA-DR in many of the hypoxic cells.

In triggered professional and nonprofessional APCs (including ECs), membranal HLA-DR is continuously recycled to the early endosomes [23] and late endosomes/lysosomes [24]. However, using confocal microscopy, we could not clearly demonstrate co-localization of HLA-DR with early (Rab5) and late (Rab7) endosomes in hypoxic ECs, because of the masking effect of the dominant green fluorescence of the Rab vesicle markers, which obscured the weak red HLA-DR fluorescence (data not shown). The mechanisms regulating trafficking of HLA-DR to and from the plasma membrane are yet not fully elucidated, but involvement of actin fibers is well documented [17,25]. We showed that disruption of actin polarization using cytochalazin B, dose-dependently elevated surface HLA-DR expression in normoxic ECs, reaching the hyp-

Table 1. Effect of protease inhibitors on HLA-DR secretion from EaHy926 cells.

Inhibitor (final concentrations)	Target	Fold	*P*-value
None		1.000	
Aprotinin (5 μg/ml)	Serine proteases	1.053 ± 0.09	NS
Leupeptin (10 μg/ml)	Serine and cysteine proteases	1.103 ± 0.09	NS
Pepstatin (0.5 μM)	Aspartic proteases	1.040 ± 0.22	NS
Phenanthroline (0.5 μM)	Metalloproteinases	0.927 ± 0.12	NS
Bafilomycin A1 (0.5 nM)	Lysosomal (vacuolar H^+ ATPase)	0.796 ± 0.05	0.0055

10^6 EaHy926 cells were incubated in hypoxia with the inhibitors and the change in the secreted HLA-DR relative to the nontreated cells was detected by Western blots (n = 4).
NS, nonsignificant.

oxic levels. This may suggest that hypoxia rearranges actin fibers [16,26], so that it selectively interferes with HLA-DR recycling into the endosomal compartments, but not with the transport to the plasma membrane, leading to its accumulation on the cell surface. Hypoxia-induced influences on the translation of HLA-DR were not evaluated in this study. Thus, we could speculate that enhanced translation, possibly mediated by the hypoxia-induced alleviation of translational repression by microRNAs, could coexist with changes in intracellular trafficking. This hypothesis merits investigation.

Hypoxia also induced soluble HLA-DR in the supernatants. In professional APC HLA-DR is secreted via exosomal or nonexosomal transport [18]. Although exosomes are secreted from ECs [27,28], they do not contain HLA-DR, as we showed by ultracentrifugation, and by the lack of co-localization with the exosomal marker Rab11 [18,19,29,30]. We also showed that alternative splicing, a common mechanism to secrete transmembranal proteins [21,31], is not utilized. However, HLA-DR secretion from hypoxic ECs was inhibited by bafilomycin A1, which prevents lysosome acidification. Thus, secretion of HLA-DR from hypoxic ECs may involve a lysosomal protease, which is activated in the acidic hypoxic lysosome [32]. Such a protease probably cleaves HLA-DR protein close to the lysosomal membrane, as we did not detect a significant change in the molecular weight of the secreted protein. Circulating, nonexosomal HLA-DR was already found [30], and implicated in the pathogenesis of transplant rejection [33–35]. A beneficial role for soluble HLA-DR was also demonstrated, as it inhibited ECs injury mediated by anti-HLA-DR alloantibodies [36]. As hypoxic ECs survived the addition of antibodies to HLA-DR (data not shown), we speculate that secretion of soluble HLA-DR, at least in these hypoxic culture conditions reduces endothelial injury.

Recognition of MHC class II by $CD4^+$ T cells is the main initiator of allograft rejection [37]. Therefore, we evaluated the influence of environmental (membranal and soluble) HLA-DR molecules on $CD4^+$ T-cell cytokine production in an allogeneic EC/T-cell co-cultures subjected to hypoxia. We found that in normoxia ECs induced both IL-2 and IL-10 secretion from $CD4^+$ T cells, while hypoxia enhanced IL-2 and lowered IL-10 secretion. Survival of transplanted organs was observed to be correlated with high concentrations of the anti-inflammatory Th2 cytokine IL-10, reduced production of the pro-inflammatory Th1 cytokine IL-2, and elevated numbers of T regulatory cells [38–40]. Moreover, up-regulation of IL-10 provided cytoprotection and improved survival of transplanted kidneys [41]. Thus, hypoxia seems to alter

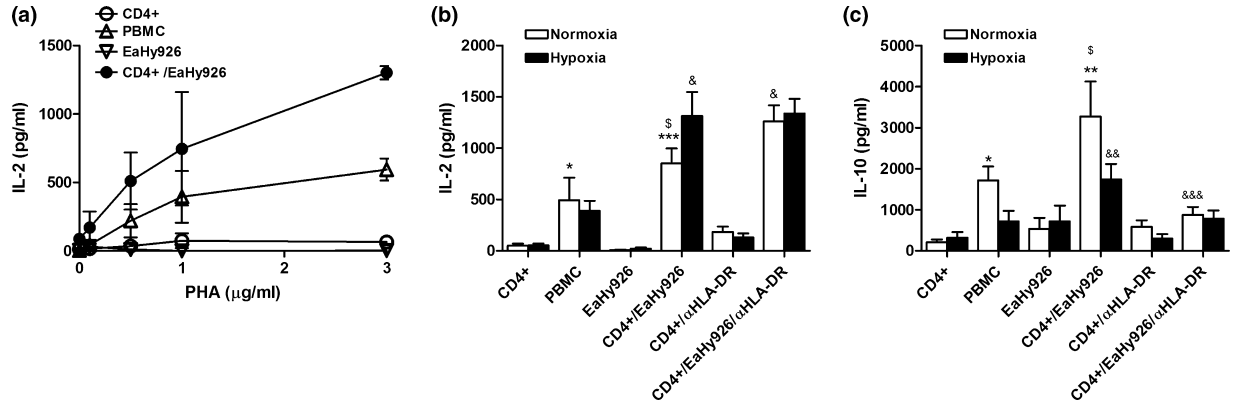

Figure 4 Hypoxia increases the activation of allogeneic $CD4^+$ T cells. (a) Increasing amounts of PHA were added to $CD4^+$ T cells, EaHy926 cells, their co-culture or PBMC, and secretion of IL-2 was measured (n = 5). Allogeneic $CD4^+$ T cells were cultured alone, with non-T cells (PBMC) or with EaHy926 cells for 48 h in normoxia or hypoxia, with or without addition of monoclonal anti-HLA-DR (when indicated) and secretion of (b) IL-2 (n = 8), and (c) IL-10 (n = 6) was measured in the supernatants.

the balance of T-cell cytokines stimulated by allogeneic ECs toward a pro-rejecting profile. This effect is directly mediated by HLA-DR, as its ligation by antibodies further enhanced IL-2 secretion in normoxia, reaching the hypoxic levels, and markedly reduced IL-10 secretion.

It was already found that ligation of membranal MHC class II molecules initiates signaling in the presenting cells themselves. This signaling was observed to induce or prevent death in monocytes and activated B cells, depending on the localization of these molecules within or outside lipid rafts [42–44]. In ECs, ligation of membranal HLA-DR by allo-antibodies resulted in ECs injury [45], which was prevented through specific signaling pathways [46]. Although effects of HLA-DR ligation on cell death were demonstrated, its influences on cytokine production were not yet studied. As our co-cultures consisted of different cell types, each capable of expressing and secreting HLA-DR to which anti-HLA-DR could bind, it was impossible to assess the individual contribution of HLA-DR ligation of each cell type to the final outcome of cytokine secretion.

Clinically, organs do not suffer sustained exposure to hypoxia, as most are transplanted within 24 h and are further protected by hypothermia. Although there are differences between the clinical scenario and our *in vitro* setting and although we did not test the effects of hypothermia, our findings provide a possible mechanism that highlights the role of ECs in this phenomenon, and at least partially explain why ischemic/hypoxic injury is so closely associated with chronic graft rejection.

Authorship

NL: designed the research and wrote the manuscript. LWC: performed the experiments. HB: assisted in writing the manuscript. MAR: designed the research, collected data, and wrote the manuscript.

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