Antioxidative treatment inhibits the release of thrombogenic tissue factor from irradiation- and cytokine-induced endothelial cells

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Received 18 May 2006; received in revised form 18 December 2006; accepted 21 December 2006
Available online 30 December 2006
Time for primary review 25 days

Abstract

Objectives: The aim of this study was to investigate the effect of the antioxidants pyrrolidine dithiocarbamate (PDTC) and N-acetylcysteine (NAC) on the ionizing radiation (IR)- and tumor necrosis factor-α (TNF-α) induced tissue factor (TF) expression and its release from human umbilical vein endothelial cells (HUVECs).

Methods: HUVECs were irradiated with a single dose of either 5 Gy or 10 Gy and stimulated with TNF-α (10 ng/mL) in the presence or absence of PDTC and NAC, respectively. Quantitative real-time PCR, ELISA, and TF activity measurements were performed, including TF activity in the supernatant. Apoptosis was detected by flow cytometric active caspase-3 measurement and formation of reactive oxygen species (ROS) by chemiluminescence.

Results: We demonstrated a thus far uninvestigated persistent induction of TF expression in HUVECs after treatment with IR and TNF-α. Combined stimulation with IR and TNF-α led to an immense shedding of microparticle-associated TF which was positively correlated with apoptosis and ROS formation. Antioxidative pre-treatment reduced not only apoptosis and ROS formation, but also the release of thrombogenic microparticles.

Conclusions: Antioxidative treatment inhibited apoptosis and shedding of microparticles, thereby reducing thrombogenicity. Thus, antioxidants may help to prevent late thrombosis after antiproliferative treatment when used in combination with anticoagulants.

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Keywords: Tissue factor; Cytokines; Apoptosis; Antioxidants; Pyrrolidine dithiocarbamate; N-acetylcysteine

1. Introduction

Antiproliferative therapies such as application of ionizing radiation (IR) or treatment with chemotherapeutics such as sirolimus are noted to be associated with organ thrombosis and fibrosis [1–3]. In cardiology, delayed re-endothelialization, prolonged fibrin deposition, and platelet recruitment to stent struts have been discussed as potential causes of late post IR thrombosis [4]. IR activates the coagulation system via de novo synthesis of tissue factor (TF) in endothelial cells [5]. However, IR-induced TF expression has not been linked to the occurrence of late thrombosis yet. TF, the primary physiological initiator of coagulation, induces thrombus formation by activation of Factor VII, resulting in activation of Factors IX and X. TF circulates at high levels in the blood of patients with...
coronary artery disease, thereby increasing the blood prothrombogenicity [6–9]. In addition to IR, stimuli such as endotoxins, tumor necrosis factor-α (TNF-α) and other inflammatory cytokines potentiate TF expression and induce the release of TF-bearing microparticles (MPs) of various origin [10–12]. We have recently shown that procoagulant soluble TF generated by alternative splicing is released from endothelial cells in response to TNF-α [13,14]. Blood-borne TNF-α is not only detectable in acute coronary syndrome [15], but also after treatment with IR [16]. Thus, thrombogenic effects of IR may be aggravated by inflammatory agents.

In addition, both IR and TNF-α can induce apoptosis [17]. Association of apoptosis with the expression of TF and an increase in procoagulability has been reported [18]. IR-induced apoptosis and enhanced coagulation are both associated with shifts in redox/thiol balance and increased vascular oxidant stress [19–21]. Moreover, pathways linked to apoptosis are in part regulated by reactive oxygen species (ROS) [22], which can be inhibited by antioxidants and/or antioxidant enzymes [23]. Alterations in cellular redox potential lead to activation of transcription factors, such as nuclear factor kappa B (NFśB) [23], known to be associated with TF expression [24]. Furthermore, expression of TF has been reported to be redox-sensitive [25]. Various antioxidants have not only been shown to regress radiation-induced tissue damage [26] but also to reverse impairment of endothelium-dependent dilation in patients with coronary artery disease [27], and to alter the endothelial hemostatic balance in a beneficial way [28].

We postulated that IR may contribute to the thrombogenicity of endothelial cells by increasing cellular TF expression and releasing TF-bearing MPs as well as soluble TF into the extracellular space. We also examined the release of MP-associated TF induced by IR in the presence of inflammatory cytokines. Furthermore, we investigated whether the treatment with the antioxidants pyrrolidine dithiocarbamate (PDTC) and N-acetylcysteine (NAC) affects the hemostatic balance of endothelial cells by altering IR- and cytokine-induced TF release.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) and endothelial cell culture medium were purchased from PromoCell (Heidelberg, Germany). HUVECs were maintained in endothelial cell growth medium (containing 5% fetal calf serum) in cell culture flasks coated with 0.1% gelatine at 37 °C in a humidified incubator (5% CO2, 95% air). Cells were subsequently treated with a single IR dose of either 5 Gy or 10 Gy. IR was generated by a linear accelerator (Varian Clinac 600 CD, Varian Medical Systems Germany GmbH, Darmstadt, Germany) with maximum photon energy of 6 MeV. Cell cultures were irradiated in a water equivalent environment by a 25 × 25 cm² photon field. With this set-up, the dose homogeneity in the cell culture media is approximately 100%. Following irradiation, cells were maintained in growth medium for 1, 3, 7 and 15 days and later subjected to further analysis. In other flasks, HUVECs were pre-treated with the antioxidant PDTC (100 μM, Sigma-Aldrich, Schnelldorf, Germany) or NAC (5 mM, Sigma-Aldrich) one hour before IR. Continuously, PDTC or NAC were added to the cell culture medium every 24 h until further experiments were conducted. Pre-treatment with PDTC and NAC was performed to analyze their effects on IR-induced thrombogenicity of endothelial cells. Of note, neither PDTC nor NAC reduces the viability of endothelial cells [29].

2.3. Stimulation of HUVECs with tumor necrosis factor-α

To assess the impact of inflammation on irradiated endothelial cells with or without PDTC- or NAC-pre-treatment, HUVECs were serum-starved for 2 h and subsequently stimulated with TNF-α (10 ng/mL, Sigma-Aldrich) for 8 h on day 7 after IR. Cells and supernatants were then analyzed.

2.4. TF isoform specific real-time PCR (TaqMan®)

The impact of IR on the endothelial TF mRNA expression was examined by real-time PCR using full-length TF, alternatively spliced human TF (asHTF), and GAPDH specific primers and probes as previously described [30].

2.5. TF ELISA

To quantify the total TF protein content (i.e. full-length TF and asHTF) in irradiated and control cells, cells were subjected to ELISA according to manufacturer’s instructions (Imubind Tissue Factor ELISA Kit, American Diagnostica Inc., Pfungstadt, Germany).

2.6. Measurement of TF activity

Measurement of TF activity was performed as previously described [13]. Briefly, HUVECs were washed twice with PBS and, incubated in HEPES buffer containing 0.1 M N-octyl-β-d-glycopyranoside. After addition of Factor VIIa, Factor X, and Ca²⁺, TF-dependent Factor Xa generation was measured at 405 nm using a chromogenic Factor Xa substrate. TF activity units were assessed using a standard curve.

To assess non-cell-bound procoagulability, cell culture medium was replaced with basal medium on days 3, 7 and 15 after IR. To determine procoagulant activity, supernatants of irradiated and control cells were collected after 8 h. To differentiate between the procoagulability of the soluble TF isoform and that of MP-associated TF, MPs were isolated.
from samples by ultra-centrifugation as previously described [13]. To ensure that measured activity was TF-dependent, control samples were incubated with anti-TF-antibodies prior to the addition of Factor VIIa and then treated as described above.

2.7. Detection of IR-induced ROS formation by lucigenin-enhanced chemiluminescence

The impact of IR and PDTC on the release of ROS from HUVECs was estimated using a lucigenin chemiluminescence assay. Briefly, cells were resuspended in 0.5 M glycine-NaOH buffer, pH 9.0. After addition of lucigenin (50 μM, Fluka/Sigma-Aldrich) chemiluminescence was immediately measured in relative light units (RLU) for 30 s using the Orion I microplate luminometer (Berthold Detection Systems, Pforzheim, Germany). Specific extracellular ROS detection was validated by the complete abolition of the lucigenin signal with cell-impermeable superoxide dismutase (Sigma-Aldrich).

2.8. Flow cytometry

To analyze apoptosis post IR and cytokine treatment, flow cytometric analysis of active caspase-3 stained cells was performed. Irradiated and control cells were maintained as described above and analyzed 7 days post irradiation. Briefly, 2.5 × 10^5 cells were fixed with 3.7% paraformaldehyde and additionally permeabilized with 0.1% Triton X-100. Washed cells were incubated for 1 h at room temperature (RT) with PBS supplemented with goat serum (5% v/v) and PE-labelled antibodies against active caspase-3 (Clone C92-605, BD Pharmingen, Heidelberg, Germany). Fluorescence thresholds were set in terms of binding of isotype-matched control antibodies (1.5 μg/mL; Coulter Immunology, Miami, Florida, USA). Measurements were performed on a FACScan (BD Bioscience) and data were collected using CellQuest software (BD Bioscience).
2.9. Statistical analysis

Values were derived as mean ± SEM. All experiments were performed at least four times. To assess statistical significance of differences between groups, Mann–Whitney U-test or ANOVA were used as appropriate. Spearman correlation analysis was used to investigate the relationship of MP-associated TF activity and ROS formation and apoptosis, respectively post IR and cytokine treatment; \( P < 0.05 \) was considered significant.

3. Results

3.1. Ionizing radiation induces cellular TF expression in HUVECs

To investigate the effect of IR on endothelial TF expression, TF activity as well as TF antigen concentration were measured in either untreated or irradiated HUVECs at various time points. Significant increase in cellular TF activity became prominent 3 days after IR with 5 Gy as well as 10 Gy and persisted throughout the duration of the study (>2 weeks), indicating that IR alters cellular thrombogenicity (Fig. 1, Panel A).

Consistent with these findings, TF ELISA revealed significant differences in levels of cellular TF protein starting from day 3 after IR (Fig. 1, Panel B). Compared to the untreated controls, a 5.8 fold increase in TF antigen was observed 7 days after IR with 10 Gy \( (P < 0.05) \), whereas irradiation with 5 Gy resulted in a 4 fold increase \( (P < 0.05; \text{Fig. 1, Panel B}) \).

Quantitative real-time RT-PCR was also performed. Because no significant change in TF protein expression was found on day 1, real-time RT-PCR was not performed at this time point. IR led to an increased expression of full-length TF mRNA in HUVECs (Fig. 2, Panel A). On day 15 after IR, TF/GAPDH mRNA ratios yielded the highest mRNA expression levels (control vs. 5 Gy \( [P < 0.05] \) and control vs. 10 Gy \( [P < 0.0001] \), respectively), pointing to the late induction of TF. IR also induced expression of the mRNA encoding the soluble TF isoform, asHTF, which was significantly elevated on days 7 and 15 after IR with 10 Gy compared to the corresponding controls \( (P < 0.007; \text{Fig. 2, Panel B}) \).

3.2. Effect of inflammatory cytokines on the release of the soluble TF isoform and MP-associated TF after IR

On day 7 post IR, the soluble TF isoform was secreted from HUVECs. This was associated with an increase in procoagulant activity of the supernatant \( (2.70 ± 0.34 \text{ U vs. } 1.71 ± 0.12 \text{ U control supernatant}, P < 0.03, \text{Fig. 3}) \). Cytokine treatment in addition to IR not only impacted cellular TF expression (data not shown) but also triggered the release of asHTF from HUVECs (Fig. 3). In addition, IR-induced procoagulability of the supernatant was even more increased by the release of MP-associated TF: the combination of both IR and TNF-\( \alpha \) enormously boosted the MP-associated procoagulability compared to untreated control (Fig. 4).

3.3. Impact of PDTC and NAC on the IR- and cytokine-induced procoagulability of supernatant and cells

To evaluate the impact of antioxidants on IR- and TNF-\( \alpha \)-induced procoagulability, HUVECs were pre-treated with either PDTC or NAC. Both compounds decreased the IR- and cytokine-induced procoagulability of the MP-free supernatant (Fig. 3). Furthermore, NAC and to a larger extent PDTC significantly reduce the procoagulant activity of MPs measured after combining IR and cytokine treatment (Fig. 4). Contrarily, TF activity of whole cell extracts was increased under pre-treatment with PDTC, both, in non-irradiated and irradiated HUVECs (Fig. 5).
3.4. Association of MP-release, apoptosis, and ROS formation

The release of TF-bearing MPs was associated with apoptosis of endothelial cells, as evident from flow cytometric measurements of cellular active caspase-3 (Fig. 6). Flow cytometric analysis revealed significantly increased active caspase-3 in the irradiated cells, that had been stimulated with TNF-α compared to the untreated control cells (Fig. 6). Both antioxidants reduced IR- and TNF-α-induced apoptosis, as depicted in Fig. 6 by the reduction of active caspase-3. Furthermore, active caspase-3 positively correlated with the TF activity of released MPs ($r^2 = 0.524, P < 0.01, n = 31$).

IR also led to increased ROS levels, which were further elevated by TNF-α (Fig. 7). A positive correlation was found between ROS formation and the release of MP-associated TF ($r^2 = 0.675, P < 0.01, n = 29$). Antioxidative pre-treatment prior to IR and TNF-α stimulation significantly diminished ROS formation. PDTC, but not NAC reduced ROS formation to baseline levels. The reduction in ROS formation was accompanied with a reduced release of thrombogenic MPs from endothelial cells, pointing to an association of ROS and the release of thrombogenic MPs.

4. Discussion

Thus far uninvestigated, this study demonstrated a persistent induction of TF expression in endothelial cells after treatment with IR and TNF-α. We also documented an increased release of MP-associated TF in response to these stimuli that was associated with an increased ROS formation and apoptosis. The combination of both, IR and stimulation with TNF-α led to an immense shedding of MP-associated TF, pointing to endothelial cells to be important modulators of vascular thrombogenicity. Pre-treatment with antioxidants reduced the release of thrombogenic MPs, possibly by suppressing apoptosis and ROS formation.

Cells surviving acute genotoxic stress after IR were shown to display delayed responses that can result in persistent effects such as apoptosis [31] and late thrombosis [32].

Treatment with IR is associated with the occurrence of thrombotic events, possibly by directly increasing the thrombogenicity of irradiated cells. Biosynthesis of TF in endothelial cells is induced by the transcription factors activating protein-1 (AP-1) and NFκB [24], which are also involved in coordinating the cellular response to DNA damage post IR [33]. Several possible underlying mechanisms may account for the TF upregulation observed here. On the one hand, a direct activation of the NFκB pathway by IR as shown by Russell et al. [34] is possible. On the other hand, our data suggest IR to induce the production of ROS, which may serve as second messenger in pathways regulating the NFκB system as previously shown [35,36]. ROS formation may also be triggered by endogenous TNF-α produced in response to IR [37]. The exact mechanism underlying ROS-induced NFκB activation is still unclear.

In addition, thrombogenic MPs released from apoptotic cells after antiproliferative treatment also contributed to the increased thrombogenicity, as documented here. Elevated levels of procoagulant MPs derived from apoptotic endothelial cells did not only circulate in blood of patients with acute
recently reported that procoagulant soluble TF is released from HUVECs in response to TNF-α [13]. Soluble TF also increased the thrombogenicity after IR, evident from this study. However, regarding the degree of measurable procoagulability, soluble TF appeared to play a secondary role for the IR- and cytokine-induced increase in thrombogenicity. Thus, MP-associated TF was found to be the major contributor to extracellular thrombogenicity post IR under inflammatory conditions.

Natural antioxidants such as vitamins A, C, E and carotene suppress the activation of NFκB in vitro and in vivo [41–43]. Although structurally different, both PDTC and NAC are radical scavengers and act as antioxidative agents in viable cells through alterations in ROS metabolism [44,45]. In addition, PDTC has unique reciprocal activities in activating AP-1 and inhibiting NFκB, which are both redox-sensitive [46]. This may explain the induction of cellular TF by PDTC observed here. Furthermore, we found the release of TF-bearing MPs to be reduced after PDTC and to a lesser extent after NAC treatment. Blockade of NFκB activation by the antioxidants PDTC as well as NAC was recently reported to prevent endothelial cells from apoptosis [45,47]. PDTC has further been shown to inhibit IR and TNF-α-induced ROS formation, which also led to attenuation of apoptosis [36,48]. NAC has recently been reported to possess a 2 to 3 times less antioxidative effect than PDTC [49]. This may explain the induction of cellular TF by PDTC observed here. Furthermore, we found the release of TF-bearing MPs to be reduced after PDTC and to a lesser extent after NAC treatment. Blockade of NFκB activation by the antioxidants PDTC as well as NAC was recently reported to prevent endothelial cells from apoptosis [45,47]. PDTC has further been shown to inhibit IR and TNF-α-induced ROS formation, which also led to attenuation of apoptosis [36,48]. NAC has recently been reported to possess a 2 to 3 times less antioxidative effect than PDTC [49]. This may explain the induction of cellular TF by PDTC observed here. Furthermore, we found the release of TF-bearing MPs to be reduced after PDTC and to a lesser extent after NAC treatment. Blockade of NFκB activation by the antioxidants PDTC as well as NAC was recently reported to prevent endothelial cells from apoptosis [45,47]. PDTC has further been shown to inhibit IR and TNF-α-induced ROS formation, which also led to attenuation of apoptosis [36,48]. NAC has recently been reported to possess a 2 to 3 times less antioxidative effect than PDTC [49]. This may explain the induction of cellular TF by PDTC observed here. Furthermore, we found the release of TF-bearing MPs to be reduced after PDTC and to a lesser extent after NAC treatment. Blockade of NFκB activation by the antioxidants PDTC as well as NAC was recently reported to prevent endothelial cells from apoptosis [45,47]. PDTC has further been shown to inhibit IR and TNF-α-induced ROS formation, which also led to attenuation of apoptosis [36,48].

Apoptosis is known to be accompanied by an increased formation of MPs [50]. We showed here that the reduction in ROS formation and apoptosis by antioxidants was associated with a reduced release of TF-bearing MPs. Thus, both antioxidants reduced the thrombogenicity after IR and cytokine treatment, although the extent of the observed reduction seemed to differ depending on the antioxidant used. It was not the aim of this study to directly compare two different antioxidants. However, with respect to the antioxidants’ influence on ROS formation, we can only speculate that the effects of NAC and PDTC on thrombogenicity may be – at least in part – exerted via mechanisms independent of their antioxidative function, as previously suggested [51].

In conclusion, antiproliferative treatment with IR induced a persistent expression of cellular TF and enhanced the release of MP-associated TF, which was further potentiated by an inflammatory cytokine. Pre-treatment with PDTC and NAC reduced the release of thrombogenic MPs, possibly by inhibiting apoptosis and – at least in part – by diminishing ROS formation.

Acknowledgements

This study was supported by grants from the Deutsche Forschungsgemeinschaft (DFG) (Ra-799 3/1 to U.R. and K.P., SFB-TR19 to U.R. and H.-P.S. and GRK 865 to U.R.). Björn Szotowski was a scholarship holder of the DFG-Graduiertenkolleg 865. We thank Ms. Franziska Bleis for her technical assistance.

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