Ionizing radiation-induced E-selectin gene expression and tumor cell adhesion is inhibited by lovastatin and all-trans retinoic acid

Tobias Nübel¹, Wolfgang Dippold², Bernd Kaina¹ and Gerhard Fritz¹,³

¹University of Mainz, Institute of Toxicology, Division of Applied Toxicology, Obere Zahlbacher Str. 67, D-55131 Mainz and
²St Vincenz and Elisabeth Hospital, An der Goldgrube 11, D-55131 Mainz, Germany
³To whom correspondence should be addressed
Email: fritz@mail.uni-mainz.de

E-selectin mediated tumor cell adhesion plays an important role in metastasis. Here we show that ionizing radiation (IR) induces E-selectin gene and protein expression in human endothelial cells at therapeutically relevant dose level. E-selectin expression is accompanied by an increase in the adhesion of human colon carcinoma cells to primary human umbilical vein endothelial cells (HUVEC). The HMG-CoA reductase inhibitor lovastatin impairs IR-stimulated E-selectin expression as analyzed at the level of the protein, mRNA and promoter. Inactivation of Rho GTPases either by use of Clostridium difficile toxin A or by co-expression of dominant-negative Rho blocked IR-induced E-selectin gene induction, indicating Rho GTPases to be essential. Radiation-induced expression of E-selectin was also blocked by all-trans retinoic acid (at-RA), whereas 9-cis retinoic acid was ineffective. Abrogation of IR-stimulated E-selectin expression by lovastatin and at-RA reduced tumor cell adhesion in a dose-dependent manner. Combined treatment with lovastatin and at-RA exerted additive inhibitory effects on radiation-induced E-selectin expression and tumor cell adhesion. Therefore, application of statins at-RA might have clinical impact in protecting against E-selectin-promoted metastasis, which might arise as an unwanted side effect from radiation treatment.

Introduction

Small GTPases of the Rho family play a pivotal role in the regulation of a variety of cellular functions, including the organization of the actin cytoskeleton (1), cell cycle progression (2,3), cell adhesion (4–6), tumor progression (7–9) and gene expression (10,11). Since Ras/Rho GTPases need C-terminal isoprenylation for correct intracellular localization and function, it is assumed that part of the biological effects of HMG-CoA reductase inhibitors (i.e. statins) is due to inhibition of Ras/Rho function (12–14). Recently, we showed that activation of stress inducible signal mechanisms including the stimulation of c-Jun N-terminal kinases/stress activated protein kinases (JNK/SAPK) and NF-κB is impaired by the HMG-CoA reductase inhibitor lovastatin (12,15).

It is well established that the expression of various cell adhesion molecules is regulated by the transcription factor NF-κB (16). NF-κB becomes activated not only by inflammatory cytokines but also by genotoxic agents such as ionizing radiation (IR) and others (17,18). Therefore, it is tempting to speculate that, beside inflammatory cytokines, IR is also able to increase the expression of cell adhesion molecules. In line with this, IR has been demonstrated to induce the expression of ICAM, VCAM and E-selectin (19–21). Notably, IR stimulates E-selectin expression much stronger than the expression of ICAM-1 (22). Inhibition of NF-κB by over-expression of dominant-negative (dn) construct blocks ICAM-1 and E-selectin gene induction by IR (22). Radiation-provoked increase in the expression of cell adhesion molecules was also observed in vivo, both in rodent lung (23) and in human skin (19). An increased expression of cell adhesion molecules on endothelial cells promotes the adhesion of leukocytes and is therefore believed to be the molecular basis of inflammation provoked as a side effect of radiation therapy. In line with this, E-selectin was reported to be essential for the enhanced adhesion of leukocytes to irradiated vascular endothelium (24). The anti-inflammatory effect of low radiation dose (i.e. 0.3–0.6 Gy) was ascribed to the attenuation of cytokine-triggered expression of E-selectin (21,25).

Beside its well-known contribution to inflammatory responses, E-selectin also plays a central role in the process of metastasis by promoting the adhesion of circulating tumor cells to the endothelium (26), which is a prerequisite for their extravasation. The correlation of the expression of E-selectin and tumor-specific expressed ligands for E-selectin [i.e. sialyl-LewisX (sLewX) or sialyl-LewisA] with the metastatic potential of malignant cells has been most convincingly demonstrated for colon carcinoma cells (27–31). Thus, overall, expression of E-selectin can be considered as one important factor promoting metastasis.

Bearing this in mind, we wished to clarify whether radiation treatment is able to affect the level of tumor cell adhesion to the endothelium. To our best knowledge this aspect of a putative side effect of radiation treatment has not yet been considered so far. The present study aimed at characterizing the effect of IR on E-selectin expression and tumor cell adhesion to the endothelium and furthermore addressed the questions as to (i) the involvement of Rho GTPases in the regulation of IR-induced expression of E-selectin and (ii) whether IR-stimulated E-selectin expression and tumor cell adhesion might be blocked by clinically established pharmaceuticals.

Materials and methods

Materials
Lovastatin was purchased from Calbiochem-Novabiochem (Bad Soden, Germany). Oligonucleotides originate from Sigma-Aldrich Fine Chemicals (Taufkirchen, Germany). Clostridium difficile toxin A (ToxA) was a gift from LJust (Institute of Toxicology, Hannover, Germany). 9-cis retinoic acid

Abbreviations: at-RA, all-trans retinoic acid; HUVEC, human umbilical vein endothelial cells; IR, ionizing radiation; 9c-RA, 9-cis retinoic acid; ToxA, Clostridium difficile toxin A.
(9c-RA) and all-trans retinoic acid (at-RA) were purchased from ICN Biomedicals GmbH (Eschwege, Germany). Anti-E-selectin, anti-P-selectin and anti-ERK2 antibodies used in this study were purchased from Santa Cruz Biotechnology (Heidelberg, Germany), antibody directed against ICAM-1 was from R&D Systems GmbH (Wiesbaden-Nordenstadt, Germany). POD-conjugated anti-mouse antibody used for ELISA analyses was obtained from Promega (Mannheim, Germany). Phospho-specific IκBα antibody was purchased from New England Biolabs GmbH (Frankfurt am Main, Germany). For treatment of cells with IR (i.e., γ-rays), a 40 Co source was used. The Titan One Tube RT-PCR Kit originates from Roche Diagnostics GmbH (Mannheim, Germany). For cloning of the E-selectin promoter PowerScript DNA Polymerase was used (PANalytical Biotech GmbH, Aidensbach, Germany). Luciferase activity was determined by use of the Luciferase assay system (Promega). CDNAs and expression vectors for dnRho GTPases (N17Rac and N19RhoB) are described elsewhere (32). dnRhoA (N19Rho) originates from A.Hall (University College London, UK). The cell line EA.hy-926 was obtained from Cora-Jean S.Edgell (University of North Carolina, NC). Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex (BioWhittaker Europe, Verviers, Belgium).

Cell culture conditions
EA.hy-926 cells and human colon carcinoma cells (DLD1) [originating from the American Type Culture Collection (ATCC)] were grown in Dulbecco’s medium containing 10% heat inactivated fetal bovine serum. EA.hy-926 culture medium additionally contains 1% HAT media supplement. HUVEC were cultured using endothelial cell growth media system (EGM-2) from Cambrex (BioWhittaker Europe) containing 2% fetal calf serum according to the manufacturer’s protocol. HUVEC were used in the fourth to sixth passage for experiments.

Western blot analysis
To detect activation of NF-κB signaling pathway, phosphorylation of the inhibitory molecule IκBα was measured by western blot analysis using phospho-specific anti-IκBα antibody (detecting IκBα phosphorylated on Ser32). Fifteen minutes after irradiation, cells were harvested and 30 μg of protein was separated by polyacrylamide gel electrophoresis (10% denaturing gels). After transfer of the proteins to nitrocellulose, membrane was blocked (5% dry milk in PBS/0.1% Tween, 2 h, RT) before overnight incubation with phospho-specific antibody (1:1000) according to the manufacturer’s protocol. After washing and incubation with peroxidase-coupled secondary antibody, phosphorylated IκBα was visualized by chemiluminescence.

ELISA analysis
For quantification of E-selectin protein expression an ELISA-based method was used. To this end, 4 × 106 endothelial cells were seeded into 96-well microtitre plates and grown overnight. Before pre-treatment and stimulation of the cells the medium was changed. About 5 h after stimulation, plates were stored on ice for 10 min. Afterwards the cell layer was washed twice with ice-cold PBS/1% BSA. Cells were incubated with anti-E-selectin antibody for 90 min (1:50 in PBS/1% BSA). After washing (3×), POD-conjugated secondary antibody (1:1000 in PBS/0.1% BSA) was added and incubated for 30 min. All steps were performed on ice. Upon addition of p-nitrophenyl phosphate solution enzymatic reaction was allowed to proceed for 30 min at room temperature before optical density (E 405 nm) was quantified using an ELISA plate reader. Specific binding of the antibody was calculated by subtracting non-specific binding of IgG2a-isotype (E 405 nm). At least three independent experiments (each performed in triplicate) were used for statistical interpretation of the data (mean value ± SD).

RNA expression analysis (RT-PCR)
Total RNA was isolated by use of RNA Purification Kit (Sigma-Aldrich, Germany). Reverse transcription was performed at 50°C for 30 min. For PCR reaction 25 cycles were performed (denaturation: 95°C, 1 min; annealing: 60°C, 2 min; polymerization: 68°C, 2 min). PCR products were separated onto agarose gels and DNA was visualized by ethidium bromide staining. To E-selectin RNA amplification were designed according to the E-selectin mRNA sequence published [NCBI Nucleotide GenBank (accession number: NM-000450) 5‘-primer: 5’-CTCTCTCAGCTCTACATTG-3‘, 3‘-primer: 5’-TCTTCTGCTGTGACACCAC-3‘]. E-selectin PCR product is 383 bp in length. Primers for GAPDH amplification (392 bp PCR product) were as follows: 5‘-primer: 5’-GTCCTACACACACATGGAGAGCT-3‘ and 3‘-primer: 5’-GACGCTTACGAGCTTCTTC-3‘.

Cloning of the E-selectin promoter and reporter gene analysis
Total genomic DNA was isolated from EA.hy-926 cells using the DNeasy® Tissue Kit (Qiagen GmbH, Hilden, Germany). PCR primers for cloning of the human E-selectin promoter were designed according to the E-selectin gene sequence published (33) (5‘-primer: 5’-AGGCTGGTCTTCGATCCCGC-3‘, 3‘-primer: 5’-GACCTCAAGGTTCCTTCCACC-3‘). After amplification by touch down PCR (30 cycles) from 66°C down to 54°C, the 1.24 kb E-selectin promoter fragment was cloned into XhoI/HindIII site of pGL3-Basic Vector (Promega). Successful cloning was confirmed by sequencing. To examine trans-cplational activation of the E-selectin promoter or of NF-κB minimal promoter construct [3x NF-κB-luciferase (34)] subconfluent EA.hy-926 cells or HUVEC were transiently transfected with 2 μg of the corresponding construct. In the case of EA.hy-926 cells, transfection was done by use of the Effectene method (Qiagen GmbH), transfection of HUVEC was performed using ESCORT™III Transfection Reagent (Sigma-Aldrich Fine Chemicals). Eight hours after transfection, cells were pre-treated or not and stimulated after a further incubation period of 16 h. About 12 h after exposure, cells were harvested and luciferase activity was determined according to the manufacturer’s protocol (Promega). Determination of protein concentration of cell extracts was done by the method of Bradford (35). Relative luciferase activity of treated cells was related to that of untreated control, which was set to 1.0.

Analysis of cell adhesion
The adhesion of human colon carcinoma cells (DLD1) to a confluent monolayer of endothelial cells (HUVEC) was studied upon fluorometrical labeling of the tumor cells by calcein AM (excitation wavelength: 495 nm; emission: 517 nm) basically according to the Vybrant cell adhesion assay ( Molecular Probes, Europe, Leiden, The Netherlands). Shortly, colon carcinoma cells were labeled by incubation with 5 μM of calcein AM (Sigma-Aldrich Fine Chemicals) for 30 min at 37°C. Afterwards, 5 × 104 of labeled tumor cells were added to a confluent cell layer of HUVEC, which have been stimulated or not 5 h earlier with γ-rays. Cell attachment was allowed to proceed for 2 h at 37°C. Afterwards, non-attached cells were removed by washing with PBS (3×) at room temperature before fluorescence was determined (Spectra MAX plate reader, Molecular Devices, Albertville, MN). Under basal conditions 10% of DLD1 tumor cells (corresponding to ~5 × 104 cells) adhered to HUVEC. Increase in tumor cell adhesion upon stimulation of HUVEC was calculated in relation to the basal adhesion of tumor cells to non-stimulated HUVEC, which was set to 1.0.

Results
Exposure of primary HUVEC to IR (i.e., γ-rays) results in a dose-dependent increase in the level of E-selectin protein as analyzed by ELISA. IR-stimulated E-selectin protein expression was clearly detectable already at a low, therapeutically relevant dose of 2 Gy (Figure 1A, left panel). Radiation-induced increase in the expression of E-selectin was confirmed on the level of the mRNA (data not shown) and by reporter gene analysis using a 1.24 kb human E-selectin promoter fragment, which has been cloned from endothelial cell line (i.e. EA.hy-926) (Figure 1A, right panel). Sequence analysis confirmed that the cloned E-selectin promoter is identical to that described by others (33). Similar to HUVEC, the endothelial cell line EA.hy-926 responded to IR with a dose-dependent increase in E-selectin protein and gene expression (Figure 1B). E-selectin is believed to be involved in tumor progression by promoting the adhesion of tumor cells to the endothelium (26). Therefore, we wondered whether radiation treatment would lead to an increase in tumor cell adhesion. To our very best knowledge, this has not been demonstrated so far. To this end, we examined the adhesion of fluorescence-labeled colon carcinoma cells (DLD1) to a confluent layer of irradiated versus non-irradiated HUVEC. As illustrated in Figure 2A, exposure of HUVEC to IR leads to an increased adhesion of DLD1 cells to the irradiated endothelial cell layer. Increase in tumor cell adhesion by γ-irradiation was dose-dependent, becoming clearly detectable at a radiation dose of 2–5 Gy and becoming saturated at a radiation dose of 20 Gy (Figure 2B).

If IR-stimulated E-selectin expression is a main reason for the observed increase in tumor cell adhesion, an experimental approach to prevent this effect of radiation treatment would be to inhibit IR-induced E-selectin expression. It is known that
NF-κB is required for E-selectin induction both by TNFα (36) and IR (22). Recently, we have shown that lovastatin, which belongs to the group of clinically highly relevant HMG-CoA reductase inhibitors, impairs UV- and doxorubicin-induced activation of NF-κB, very likely by interfering with the activity of Rho GTPases (15). As shown here, lovastatin also blocks IR-stimulated activation of NF-κB, as indicated by the reduced phosphorylation of IkBα at Ser32 (Figure 3A). At the same time, lovastatin largely attenuated IR-stimulated expression of E-selectin in HUVEC, which was demonstrated both on the level of the protein (by an ELISA-assay) (Figure 3B and C), the mRNA (by RT–PCR analysis) (Figure 3D) and by reporter gene analysis (Figure 3E). Identical results were obtained using the established endothelial cell line EA.hy-926 (data not shown). The inhibitory concentration of lovastatin blocking radiation-induced E-selectin protein expression in vitro by 50% is ~5 μM. Another HMG-CoA reductase inhibitor (i.e. simvastatin) exerted the same inhibitory effect as lovastatin (data not shown). Altogether, the data show that statins are powerful inhibitors of IR-stimulated increase in E-selectin gene and protein expression. This occurs very likely by abrogating the radiation-mediated activation of the transcription factor NF-κB. It should be noted that activation of NF-κB alone is not sufficient for stimulation of E-selectin gene expression. This is concluded from the observation that UV-C light and doxorubicin rather stimulated NF-κB-driven gene expression, as assayed by reporter gene analysis using a NF-κB minimal promoter construct (Figure 4A), yet these agents did not increase E-selectin expression (Figure 4B). Furthermore, maximal TNFα-induced E-selectin expression (obtained with high concentration of TNFα) is further increased upon exposure of the cells to IR (Figure 4C). The data show that the regulation of IR-induced E-selectin expression is complex and different from TNFα-stimulated E-selectin expression.

Rho GTPases are reported as putative targets of statins (12–14). Rho proteins have also been suggested to interfere with the regulation of NF-κB (37) and have been reported to potentiate TNFα-induced E-selectin expression (38). Bearing this in mind, we examined whether specific inhibition of Rho by use of ToxA or by co-expression of dnRho mimics the inhibitory effect of lovastatin on IR-stimulated E-selectin gene expression. As shown in Figure 5, specific inactivation of Rho GTPases using ToxA from C. difficile (39,40) largely blocked radiation-induced increase in E-selectin protein (Figure 5A) and gene expression (Figure 5B). In line with these data, co-expression of N19RhoB or N17Rac inhibited the
IR-induced activation of the E-selectin gene. Co-expression of dnRhoA (N19RhoA) had no effect (Figure 5C). Based on these findings, we suggest that Rho proteins, in particular Rac1 and RhoB, are essential for E-selectin gene induction upon exposure of endothelial cells to IR.

Retinoic acid has been reported to interfere with NF-κB signaling (41,42) and other stress-inducible transcription factors such as AP-1 (43). As shown in Figure 6A, at-RA efficiently blocks radiation-induced activation of NF-κB as analyzed by reporter gene assays. As opposed to at-RA,
Fig. 5. Rho GTPases are involved in the regulation of E-selectin gene expression by IR. (A) HUVEC were left untreated (Con) or were pre-treated overnight with Rho-inactivating clostridial ToxA (175 ng/ml). After pre-treatment period cells were exposed to γ-rays (2 Gy). Five hours after exposure, cells were harvested for analysis of E-selectin expression by ELISA-based method described in Materials and methods. Data shown are mean values ± SD from at least three independent experiments each performed in triplicate. (B) Twenty-four hours after transfection of EA.hy-926 cells with E-selectin promoter luciferase construct, cells were pre-treated or not (Con) with ToxA (175 ng/ml), which specifically inactivates small GTPases of the Rho family (38,39). Thereafter, cells were stimulated with IR (10 Gy). After overnight incubation, cells were harvested for determination of luciferase activity. Data shown are mean values ± SD from at least three independent experiments each performed in triplicate. Identical results were obtained using HUVEC (not shown). (C) HUVEC were transfected with E-selectin promoter luciferase construct together or without expression construct coding for the dnRho GTPase RhoA (dnRhoA), RhoB (dnRhoB) and Rac1 (dnRac). After overnight incubation, cells were irradiated with a low, therapeutically relevant dose (2 Gy). After further incubation period of ~18 h cells were harvested for determination of luciferase activity. Relative luciferase activity in untreated control was set to 1.0. Data shown are mean values ± SD from at least two independent experiments each performed in duplicate. Con, not transfected with dnRho forms.

Fig. 6. at-RA and 9c-RA differently affect radiation-induced NF-κB-regulated gene expression and E-selectin induction. (A) Twenty-four hours after transfection of HUVEC with NF-κB minimal promoter construct cells were exposed to different doses of γ-rays. 9c-RA (2 μM) and at-RA (2 μM) was added immediately after radiation treatment. After incubation period of ~16 h (overnight), cells were harvested for determination of luciferase activity. Data shown are the mean ± SD from three independent experiments each performed in duplicate. (B) 9c-RA (2 μM) and at-RA (2 μM) were added immediately after irradiation of HUVEC (2 Gy) or EA.hy-926 (10 Gy). HUVEC and EA.hy-926 were exposed to different doses because they differently respond to radiation (see dose–response analysis shown in Figure 1). Five hours after exposure, cells were harvested for analysis of E-selectin expression by ELISA-based method as described. Data shown are mean values ± SD from at least three independent experiments each performed in triplicate. (C) Twenty-four hours after transfection of endothelial cells with E-selectin promoter construct, 9c-RA (2 μM) and at-RA (2 μM) were added. Immediately after addition of retinoic acid derivatives, cells were irradiated [HUVEC (2 Gy), EA.hy-926 (10 Gy)]. After overnight incubation, cells were harvested for analysis of luciferase activity, which was set to 1.0 in the non-irradiated controls. Data shown are mean values ± SD from at least two independent experiments each performed in duplicate.
9c-RA was not effective (Figure 6A). Investigating the effect of retinoic acid on E-selectin gene expression, we observed that at-RA abrogated IR-induced E-selectin gene and protein expression, both in HUVEC and EA.hy-926 cells (Figure 6B and C). Under identical experimental conditions 9c-RA had no effect (Figure 6B and C). It should be noted that none of the retinoic acid derivatives used affected the basal level of E-selectin gene expression (Figure 6C). Overall, the data show that the efficiency of different retinoic acid isomers to inhibit NF-κB activation after exposure to IR is paralleled by a blockage of IR-induced E-selectin gene and protein expression. We would like to note that IR did not up-regulate P-selectin protein expression (data not shown). Furthermore, in contrast to E-selectin expression, radiation-induced ICAM-1 protein expression was only marginally attenuated by lovastatin or at-RA (Figure 7). This finding indicates that the inhibitory effect of lovastatin and at-RA is specific for the cell adhesion molecule E-selectin.

Having identified lovastatin and at-RA as highly efficient inhibitors of E-selectin expression after exposure of endothelial cells to IR, the question arises as to whether inhibition of E-selectin expression by these agents is sufficient to attenuate the adhesion of human tumor cells. As shown in Figure 8, pre-treatment of HUVEC with either lovastatin or at-RA did not significantly affect the basal level of tumor cell adhesion. Yet, both compounds strongly reduced the radiation-provoked ~4-fold increase in the adhesion of colon carcinoma cells to

Fig. 7. Comparative analysis of the effects of lovastatin and at-RA on radiation-induced expression of the cell adhesion molecules E-selectin and ICAM-1. HUVEC were pre-treated or not with lovastatin (10 μM, overnight). at-RA (2 μM) was added immediately after radiation treatment. Five hours after irradiation (10 Gy), E-selectin and ICAM-1 protein expression was analyzed by ELISA as described in Materials and methods. Data shown are mean values ± SD from one representative experiment performed in triplicate.

Fig. 8. Lovastatin and at-RA impair IR-induced adhesion of human tumor cells to endothelial cells. (A and B) Confluent HUVEC were pre-treated or not with lovastatin (10 μM), at-RA (2 μM) or 9c-RA (2 μM) as described before. Afterwards, cells were irradiated (10 Gy) and adhesion of DLD1 cells was analyzed 5 h later. Data shown under (B) are mean values ± SD from two independent experiments each performed in quadruplicate. Shown is the relative adhesion to non-irradiated HUVEC. Under (A), a representative illustration of the inhibitory effect of lovastatin and at-RA on IR-stimulated tumor cell adhesion is shown. (C) Effect of different doses of lovastatin (0.1–10 μM) on IR-induced (10 Gy) adhesion of DLD1 cells to HUVEC. Data shown are the mean ± SD from two independent experiments each performed in quadruplicate. Shown is the relative adhesion to non-irradiated HUVEC (basal adhesion) was set to 1.0. (D) Effect of different doses of at-RA (0.5–5 μM) on IR-induced (10 Gy) adhesion of DLD1 cells to HUVEC. Data shown are the mean ± SD from two independent experiments each performed in quadruplicate. Basal adhesion to non-irradiated HUVEC was set to 1.0.
A possible strategy for avoiding these side effects of radiation therapy would be the pharmacological inhibition of radiation-induced expression of E-selectin. Regarding the regulation of the E-selectin gene, NF-κB is known to be essential for its transcriptional activation both by γ-rays and the inflammatory cytokine TNFα (22,33,36,49). Recently we found that UV-C- and doxorubicin-induced activation of NF-κB is blocked by the HMG-CoA reductase inhibitor lovastatin (15). By measuring Ser32 phosphorylation of IκBα, we were able to confirm the hypothesis that lovastatin also attenuates activation of NF-κB by IR. In line with this, we observed that lovastatin largely blocks IR-induced expression of the E-selectin gene. As can be expected under these conditions, E-selectin protein expression was also blocked. Although UV-C light and doxorubicin activated NF-κB very efficiently, these agents did not augment E-selectin expression. This indicates that activation of NF-κB is rather a necessary but not a sufficient component for E-selectin induction by IR. Thus, the regulation of E-selectin gene expression upon exposure of endothelial cells to TNFα or IR is obviously different.

Addressing the question as to the molecular mechanisms underlying radiation-induced E-selectin expression and its inhibition by lovastatin, we analyzed whether Rho GTPases are involved. We focused on Rho GTPases because these isoprenylated regulatory proteins are reported as targets for statins (12,13,15,50) and furthermore are described to be involved in the regulation of NF-κB (37) and to potentiate E-selectin expression upon TNFα exposure (38). We observed that pre-treatment of endothelial cells with C. difficile ToxA, which causes specific inactivation of Rho GTPases (39,40), abrogated E-selectin gene and protein induction provoked by γ-irradiation. Furthermore, as analyzed by reporter gene assays, co-expression of N19RhoB and N17Rac1, but not of N19RhoA, also abrogated radiation-induced increase in E-selectin gene expression. It is important to note that the basal level of E-selectin expression was neither affected by ToxA nor by expression of dnRho GTPases. Altogether, these data show that Rho GTPases, in particular RhoB and Rac1, are essential for radiation-stimulated increase in the expression of the E-selectin gene. This has not been demonstrated before. In a previous report we showed that also the TNFα-triggered E-selectin gene and protein expression is impaired by statins as well as by co-expression of dnRhoA (51). As opposed to IR, TNFα-induced E-selectin expression was inhibited by dnRhoA (51), supporting the hypothesis that the regulation of E-selectin gene induction upon exposure of endothelial cells to TNFα and IR is different.

Besides lovastatin, we also investigated the influence of retinoic acid derivatives on E-selectin induction by IR, as retinoic acid receptors are known to exert pleiotropic effects on stress-induced gene expression (52). We observed that at-RA efficiently abrogates E-selectin expression by IR. 9c-RA had no effect. Interestingly, stimulation of E-selectin expression by TNFα was sensitive to 9c-RA (data not shown), which is in line with another report (53), whereas at-RA had no effect (data not shown). The different efficiencies of 9c-RA and at-RA to inhibit TNFα and IR-induced E-selectin expression, respectively, supports the view that the mechanisms involved in the regulation of E-selectin gene expression by inflammatory cytokines and IR are different. It is known that at-RA acts as a specific ligand for nuclear RAR receptor whereas 9c-RA additionally binds to RXR type receptors (54,55). Bearing this in mind, we suggest that RAR interferes with IR-induced E-selectin gene expression, whereas RXR specifically interacts with RAR.
with E-selectin induction stimulated by TNFα. Previously, an inhibitory effect of retinoic acid has been reported on NF-κB-dependent iNOS expression provoked by cytokine treatment, although the molecular mechanism remained unclear (56). Recent data indicate that the inhibitory effect of retinoic acid although the molecular mechanism remained unclear (56). On the other hand, at-RA has been described to interfere also with AP-1-dependent gene expression (43,58,59). Since, in addition to an NF-κB binding site, the E-selectin promoter also contains an AP-1 binding element, it appears possible that at-RA-mediated inhibition of radiation-induced AP-1 activation is partially involved in the observed attenuation of E-selectin expression. Thus, although reduction in radiation-stimulated increase in E-selectin expression by at-RA can be assumed to be due to inhibition of NF-κB and/or AP-1, the exact mechanisms involved remain to be determined and will be the subject of forthcoming studies.

Having shown that lovastatin and at-RA impair IR-induced E-selectin expression, the question remained to be answered was whether inhibition of E-selectin expression is sufficient to reduce IR-stimulated tumor cell adhesion. The data obtained show that this is indeed the case. If applied at higher doses, lovastatin (10 μM) and at-RA (2 μM) reduced IR-stimulated tumor cell adhesion by 70–90%. 9c-RA neither affected E-selectin expression nor tumor cell adhesion. Thus, inhibition of IR-induced E-selectin expression was paralleled by a reduced adhesion of tumor cells to the endothel. Combined treatment with lovastatin (10 μM) and at-RA (1 μM) completely blocked both E-selectin expression and tumor cell adhesion. Notably, lovastatin and at-RA exerted only marginal inhibitory effects on radiation-induced ICAM-1 expression. Thus, together with published data (26), our findings support the view that, upon irradiation of human endothelial cells, it is mainly the induction of E-selectin, which contributes to a rise in the adhesion of DLD1 cells. This hypothesis gains further support by our recent observation that lovastatin also attenuates TNFα-driven E-selectin expression and tumor cell adhesion without affecting ICAM-1 induction (51). Of course, despite all of this evidence, we cannot completely rule out the possibility that other radiation-inducible factors, which exert similar functions as E-selectin and are also sensitive to tumor cell-specific functions promoting cell–cell adhesion.

Regarding the question as to the nature of the tumor-specifically expressed ligands for E-selectin, it has been shown that colon carcinoma cells bind to E-selectin mainly via sLewX (29,60,61). Therefore, it appears very likely that it is sLewX on the side of the DLD1 cells, which interacts with E-selectin thereby increasing their adhesion to HUVEC. We also observed a radiation-stimulated increase in the adhesion of mammary carcinoma cells (MCF-7) to HUVEC, which could also be blocked by lovastatin (data not shown). Yet, it should be noted that adhesion of MCF-7 cells to HUVEC is much weaker (~50%) than that observed for DLD1 cells (data not shown). This finding is in line with a previous report suggesting that sLewX is not majorly involved in endothelial adhesion of MCF-7 cells (62). There is evidence that the basal expression of E-selectin might be enhanced in tumor cells, in particular in prostate cancer cells (63). With respect to DLD1 cells, we detected only a very low basal level of E-selectin protein expression, which was slightly below that of non-stimulated HUVEC (data not shown).

E-selectin expression in DLD1 cells was found to be inducible ~2-fold upon irradiation (data not shown). Yet, noteworthy, this is a very weak effect as compared with the ~20-fold increase of E-selectin expression observed after exposure of HUVEC. Further, it should be noted that cellular responses on the level of tumor cells, which might influence their adhesive properties, are not relevant in our experimental setting, since we exclusively irradiated HUVEC. Nevertheless, bearing in mind that under therapeutic conditions in patients both endothelial and tumor cells will be exposed to γ-rays, it will be an important subject of our future studies to characterize radiation-inducible tumor cell-specific functions promoting cell–cell adhesion.

Statins have recently been reported to inhibit metastasis in vivo (i.e. in mouse xenograft system) (64,65), therefore being suggested as antitumor drugs (66). Our data support this view, suggesting that inhibition of E-selectin-mediated tumor cell adhesion contributes to their anti-metastatic potency. HMG-CoA reductase inhibitors are clinically well established and frequently used drugs in the therapy of hypercholesterolaemia. They are also reported to show anti-inflammatory effects (67–69). It is tempting to speculate that inhibition of cytokine-triggered E-selectin expression by statins, leading to a reduced adhesion of leukocytes, might be involved here. Thus, it would be an interesting topic of forthcoming studies to analyze whether statins might be useful to soften cytokine- and radiation-induced inflammatory reactions.

Overall, our data indicate that radiation therapy might bear the potential to promote metastasis by up-regulation of Rho-regulated E-selectin expression and subsequent increase in tumor cell adhesion. This unwanted side effect might be alleviated by statins and at-RA. The findings provide a firm basis for future in vivo studies and clinical trials to elucidate the clinical impact of the data.

Acknowledgements

We would like to thank I.Just and A.Hall for providing C.difficile ToxA and dnRhoA, respectively. This work was supported by the Deutsche Forschungsgemeinschaft (Fr 1241/3-1) and the Wilhelm Sander-Stiftung (AZ: 99.014.1).

References


Received November 13, 2003; revised February 17, 2004; accepted February 20, 2004