

Sustained Inflammation Due to Nuclear Factor-Kappa B Activation in Irradiated Human Arteries

Martin Halle, MD, PhD,*† Anders Gabrielsen, MD, PhD,†‡ Gabrielle Paulsson-Berne, PhD,†
Caroline Gahm, MD, PhD,§ Hanna E. Agardh, BA,† Filip Farnebo, MD, PhD,*
Per Tornvall, MD, PhD†‡
Stockholm, Sweden

Objectives	The aim of this study was to investigate gene expression networks related to cardiovascular disease in radiated human arteries.
Background	Recent epidemiological studies have shown that radiotherapy is associated with cardiovascular disease years after treatment. However, the molecular mechanisms underlying late effects of radiation are poorly described.
Methods	Arterial biopsies from radiated and nonradiated human conduit arteries, from the same patient, were simultaneously harvested during microvascular free tissue transfer for cancer-reconstruction in 13 patients, 4 to 500 weeks from radiation treatment. Radiated and nonradiated arteries were compared, with Affymetrix (Santa Clara, California) microarrays on a subset of the material to generate candidate genes. A Taqman (Applied Biosystems, Foster City, California) low-density array of 45 selected genes was designed for analysis of the whole material.
Results	Thirteen genes were synchronously expressed in all patients ($p = 0.0015$), including CCL8, CCL3, CXCL2, DUSP5, FGFR2, HMOX1, HOXA9, IL-6, MMP-1, PTX3, RDH10, SOD2, and TNFAIP3. A majority of differentially regulated genes related to the nuclear factor-kappa B (NF- κ B) signaling pathway and were dysregulated even years after radiation. The NF- κ B activation was confirmed by immunohistochemistry and immunofluorescence.
Conclusions	In the present study, we found sustained inflammation due to NF- κ B activation in human radiated arteries. The results are supported by previous in vitro findings suggesting that deoxyribonucleic acid injury, after radiation, activates NF- κ B. We also suggest that HOXA9 might be involved in the regulation of NF- κ B activation. The observed sustained inflammatory response can explain cardiovascular disease years after radiation. (J Am Coll Cardiol 2010;55:1227–36) © 2010 by the American College of Cardiology Foundation

Radiation-induced vasculopathy has been associated with treatment of lymphomas and breast cancer as well as head and neck cancer. However, the incidence of ischemic heart disease and stroke does not increase until years after treatment (1,2). Radiation therapy has until recently been widely used for prophylaxis against restenosis after coronary angioplasty but limited by late restenosis and vascular occlusion (3). Furthermore, surgical wounds within previously irradiated tissues are subject to vascular alterations associated with increased incidence of post-operative complications, includ-

ing microvascular occlusion and delayed wound-healing (4). The adverse effects of radiation on tissues can be divided into acute (early), usually occurring within 4 to 6 weeks after irradiation, and late effects, which might be evident months or even years after irradiation, indicating an ongoing pro-

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gressive process (5). The evidence of late adverse effects mainly comes from epidemiological studies (1–5), whereas previous experimental studies have focused on acute effects, mainly in cell culture experiments (6–9) and animal models (10–14). Therefore, it is of particular interest to study long-term biological effects of radiotherapy on the vasculature in humans.

The high radiation sensitivity of the vasculature has previously mainly been linked to endothelial dysfunction

From the *Department of Molecular Medicine and Surgery, Section of Reconstructive Plastic Surgery, †Department of Medicine, Center for Molecular Medicine, ‡Department of Medicine, Section of Cardiology, and the §Department of ORL, Head and Neck Surgery, Karolinska Institute, Stockholm, Sweden. This study was supported by the Swedish Heart-Lung Foundation.

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Abbreviations and Acronyms

cDNA = complementary deoxyribonucleic acid
IL = interleukin
MMP = matrix metalloproteinase
NF-κB = nuclear factor-kappa B
PBS = phosphate-buffered saline
RNA = ribonucleic acid
TLDA = Taqman low-density array
Tris = tris(hydroxymethyl)aminomethane

(6,7,11). Previous in vitro studies suggest that radiation induces endothelial activation (15) characterized by activation of the transcription factor nuclear factor-kappa B (NF-κB) (13,16), resulting in alterations in adhesion molecule expression (17,18) and cytokine and chemokine production (8). The activated endothelium is prone to atherosclerosis (19) and has prothrombotic properties, by promoting leukocyte- or platelet-endothelial cell adherence (17,20), leukocyte infiltration into tissue (18,21), and thrombus formation (22). Sugihara et al. (23) have shown that—ex vivo—nitric oxide-mediated,

endothelial-dependent relaxation is impaired in human cervical arteries 4 to 6 weeks after irradiation. Because released nitric oxide relaxes vascular smooth muscle and inhibits platelet aggregation, thereby preventing the occurrence of ischemia and vascular occlusion, it has been suggested that endothelial dysfunction contributes to vascular abnormalities in irradiated tissues. However, there is a paucity of studies in humans that describes gene expression alterations in arteries exposed to therapeutic doses of radiation. Furthermore, the long-term alterations of human arteries after radiotherapy are unknown. We have studied gene expression in human conduit arteries after therapeutic doses of radiation. During autologous microvascular free tissue transfers, irradiated arterial biopsies were harvested at the site of cancer reconstruction simultaneously with nonirradiated arteries from free tissue transfers. Therefore we could study the effects of radiation on gene expression in human arteries with an internal control excluding the influence of confounding interindividual factors. Radiation injury within a large time-frame from exposure was investigated to get insight into factors determining progressive chronic vasculopathy in irradiated arteries.

Methods

Human tissue specimens. Thirteen pairs of arterial biopsies were harvested during head and neck cancer reconstruction with microvascular free tissue transfer in 13 preoperatively irradiated patients. The age of the patients ranged from 46 to 77 years with a mean of 58.5 ± 9.0 years. Dose and time-point for radiation were unknown for 2 patients. In 11 patients, the total pre-operative dose of radiation averaged 60.4 Gy (range 50 to 68 Gy), whereas the median time elapsed from termination of radiotherapy to harvest of biopsy was 30 weeks (range 4 to 500 weeks) (Table 1). Before performing microvascular anastomosis, biopsies were harvested from the irradiated cervical donor artery and from the nonirradiated recipient artery of the transferred tissue. Biopsies were freed from surrounding tissue and surgical material under a dissection microscope. Care was taken to ensure that the endothelium was not damaged during tissue preparation. Immediately after excision, biopsies were placed in RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany), frozen, and stored at -80°C until RNA extraction. For analysis of histology and immunohistochemistry, irradiated and nonirradiated arteries from 3 patients were harvested. The age of the patients ranged from 53 to 64 years with a total pre-operative radiation dose of 64 Gy, with a range of 3 to 5 years elapsed from termination of radiotherapy to harvest. Biopsies were fixed in 10% formalin and embedded in paraffin until immunohistochemistry. The study was approved by the Ethical Committee of Stockholm and was performed in agreement with institutional guidelines and the principles of the Declaration of Helsinki.

RNA extraction. Extraction of RNA was performed with the RNeasy Mini kit (Qiagen) including an on-column DNase digestion step. The RNA quality was analyzed by microcapillary electrophoresis with an Agilent Bioanalyzer (Agilent, Palo Alto, California), whereas the amount of RNA was determined by ultraviolet spectrophotometry with a NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Scientific NanoDrop, Wilmington, Delaware).

Table 1 Demographic and Clinical Characteristics

Age (yrs)/Sex	RT Dose (Gy)	Time After RT (weeks)	Tissue Transfer	Current Smoking	CVD
60/M	64	500	Forearm	No	Hypertension
77/F	54	4.5	Fibula	Yes	
46/M	64	5	Forearm	Yes	
68/F	64	45	Forearm	No	
60/M	50	6.5	Forearm	No	
48/M	64	150	Fibula	No	
59/M	68	30	Fibula	No	Past CVL
67/M	N/A	N/A	Fibula	No	Hypertension
47/F	N/A	N/A	Forearm	No	
50/F	54	4	Forearm	Yes	
49/F	64	20	Forearm	No	Hypertension
59/M	54	7	Forearm	Yes	
50/M	64	91	Forearm	No	

CVD = cardiovascular disease; CVL = cerebrovascular lesion; RT = radiotherapy.

Complementary deoxyribonucleic acid (cDNA) was synthesized from total RNA with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, California). The RNA and cDNA were stored at -80°C .

Gene expression profiling. Gene expression profiling was performed with Affymetrix Human Genome U133 2.0 Plus oligonucleotide microarrays (Affymetrix, Santa Clara, California). Due to a limited supply of RNA from the human biopsies, there was only sufficient RNA to perform 6 microarrays consisting of radiated and nonradiated arteries from 3 patients. Data generated from Affymetrix arrays were used to identify clusters of altered gene expression in radiated arteries compared with nonradiated arteries from the same patient. Highly differentially expressed genes were subjected to enrichment testing with Gene Ontology Tree Machine (24). For each gene ontology term, a hypergeometric test was used to compare observed and expected number of regulated genes. Terms with a significant overrepresentation of observed genes were selected for further investigation. Raw signal intensities were normalized with both the GCOS algorithm (Affymetrix) and the robust multiarray averaging algorithm (25). After both methods of normalization, genes above and below a common threshold were selected for confirmation. Differential expression in selected genes was confirmed by real-time polymerase chain reaction by using a Taqman low-density array (TLDA) (Applied Biosystems, Foster City, California) with a 48-

gene configuration, to analyze 45 candidate genes and 3 housekeeping genes. The selection of 3 housekeeping genes was performed after using a Taqman Endogenous Control Plate on 3 samples. The cDNA from arterial biopsies were run down all 8 channels of 10 identical TLDA from the same production batch. Data derived across these TLDA were normalized against the housekeeping gene 18S showing negligible variation between the microarrays. Relative quantification values were generated from the TLDA analysis performed in triplicates.

Immunohistochemistry. Radiated and nonradiated arteries were immediately fixed in formalin in the operation theatre and kept overnight for embedding in paraffin and sectioning the following day. The $10\text{-}\mu\text{m}$ cut sections were formalin fixed and boiled in tris(hydroxymethyl)amino-methane (Tris)-ethylenediaminetetraacetic acid buffer at 750 W and further heated for 20 min at 360 W to unmask epitopes. After blocking of endogenous peroxidase (0.5% hydrogen peroxidase in Tris-buffered saline) and unspecific binding (5% sera in Tris-buffered saline)— 30 min , respectively—the sections were incubated with a primary antibody or matched isotype control at 4°C overnight. The following primary antibodies were used; rabbit anti-human p65 (GeneTex diluted $1:400$) (GeneTex, Irvine, California) to detect NF-κB activity, monoclonal mouse anti-human CD68 (DAKO diluted $1:800$) (DAKO, Carpinteria, California) for analysis of macrophages, monoclonal rabbit

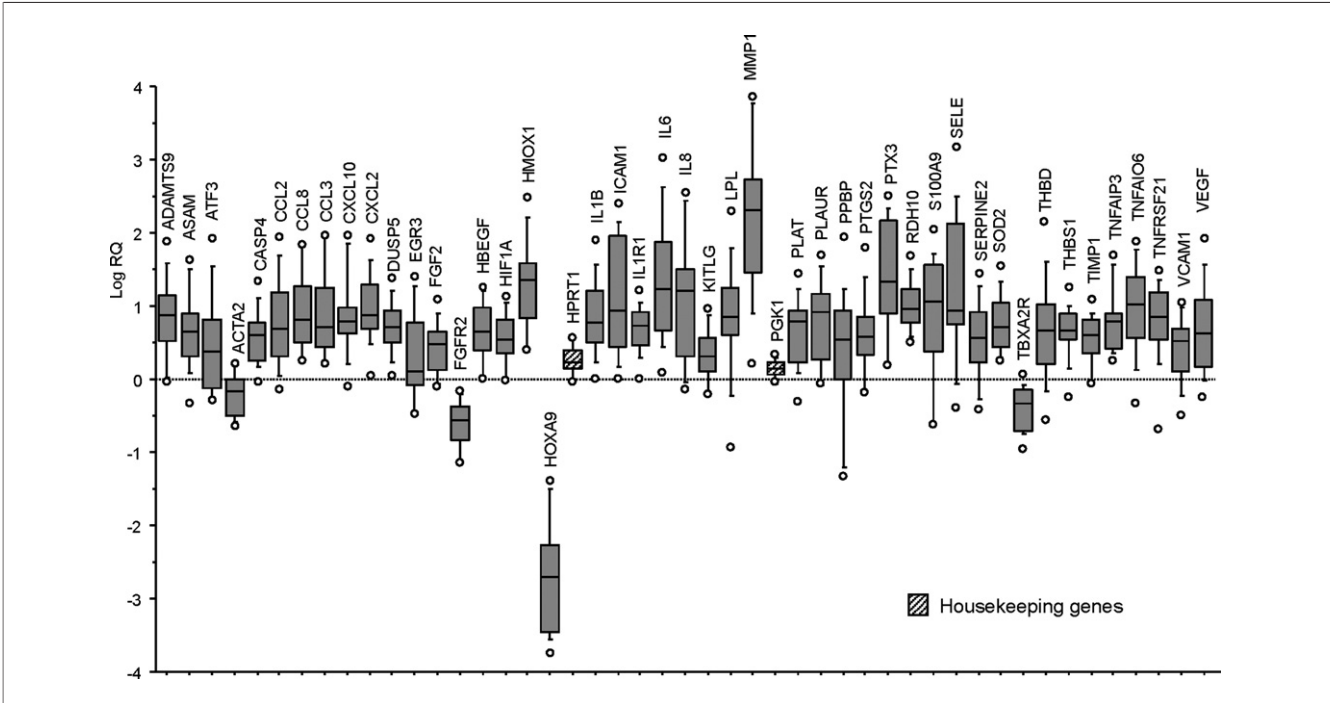


Figure 1 Box Plots of Data for Candidate Genes Derived From Analysis of Irradiated Arteries Compared With Nonirradiated Internal Controls

Box plots of data for candidate genes (shaded) derived from Taqman low-density array analysis of irradiated arteries compared with nonirradiated internal controls for all patients ($n = 13$). Whiskers represent maximum and minimum values that are no more than 1.5 times of the interquartile range. Outliers are indicated if present. Differential gene expression displayed as log-values of relative quantification (RQ). Data were normalized against the housekeeping gene 18S. Two additional housekeeping genes were included as controls (striped).

anti-human CD3 (DAKO diluted 1:800) for analysis of T-cells, monoclonal rabbit anti-human matrix metalloproteinase (MMP)-1 (Genetex diluted 1:25), and monoclonal mouse anti-human von Willebrand factor (DAKO diluted 1:400) to visualize the endothelium. The sections were then incubated with biotinylated goat anti-rabbit or horse anti-mouse immunoglobulin G, followed by avidin-biotin per-

oxidase, and developed with diaminobenzidine. Nuclei were stained with hematoxylin. Two types of controls were used: isotype matched controls and controls without primary antibodies. No positive staining could be observed in any of the controls.

For double labeling, immunofluorescence technique was performed. The tissue sections were deparaffinated, boiled

Table 2 Genes With Abbreviations, Main Functions, and p Values

Gene		Main Function	p Value
18S	18S	Housekeeping	—
ACTA2	Actin, alpha-2, smooth muscle, aorta	Cytoskeleton	0.05462
ADAMTS9	ADAM metalloproteinase with thrombospondin type 1	Remodeling	0.00237
ASAM	Adipocyte-specific adhesion molecule	Adhesion	0.00187
ATF3	Activating transcription factor-3	Intercell. signaling	0.07474
CASP4	Caspase-4, apoptosis-related cysteine peptidase	Apoptosis	0.00187
CCL2	Chemokine (C-C motif) ligand-2	Inflammation	0.00237
CCL3	Chemokine (C-C motif) ligand-3	Inflammation	0.00147
CCL8	Chemokine (C-C motif) ligand-8	Inflammation	0.00147
CXCL10	Chemokine (C-X-C motif) ligand-10	Inflammation	0.00187
CXCL2	Chemokine (C-X-C motif) ligand-2	Inflammation	0.00147
DUSP5	Dual specificity phosphatase-5	Intercell. signaling	0.00147
EGR3	Early growth response-3	Growth	0.08686
FGF2	Fibroblast growth factor-2 (basic)	Growth	0.00577
FGFR2	Fibroblast growth factor receptor-2	Growth	0.00147
HBEGF	Heparin-binding EGF-like growth factor	Growth	0.00298
HIF1A	Hypoxia-inducible factor-1, alpha subunit	Angiogenesis	0.00298
HMOX1	Heme oxygenase (decycling)-1	Inflammation	0.00147
HoxA9	Homeo box-A9	Angiogenesis	0.00147
HPRT1	Hypoxanthine ribosyl transferase	Housekeeping	0.00298
ICAM-1	Intercellular adhesion molecule-1 (CD54)	Adhesion	0.00187
IL1B	Interleukin-1, beta	Inflammation	0.00187
IL1R1	Interleukin-1 receptor, type I	Inflammation	0.00187
IL-6	Interleukin-6 (interferon, beta-2)	Inflammation	0.00147
IL-8	Interleukin-8	Inflammation	0.00237
KITLG	KIT ligand	Regeneration	0.00713
LPL	Lipoprotein lipase	Metabolism	0.00298
MMP-1	Matrix metalloproteinase-1 (interstitial collagenase)	Remodeling	0.00147
PGK	Phosphoglycerokinase	Housekeeping	0.00465
PLAT	Plasminogen activator, tissue	Coagulation	0.00237
PLAUR	Plasminogen activator, urokinase receptor	Coagulation	0.00298
PPBP	Pro-platelet basic protein	Coagulation	0.01591
PTGS2	Prostaglandin-endoperoxide synthase-2	Inflammation	0.00465
PTX3	Pentraxin-related gene, rapidly induced by IL-1-beta	Inflammation	0.00147
RDH10	Retinol dehydrogenase-10 (all-trans)	Inflammation	0.00147
S100A9	S100 calcium binding protein A9 (calgranulin B)	Stress response	0.00298
SELE	Selectin E (endothelial adhesion molecule-1)	Adhesion	0.00237
SERPINE2	Serpin peptidase inhibitor, clade E (nexin, PAI-1)	Coagulation	0.00577
SOD2	Superoxide dismutase-2, mitochondrial	Oxidative stress	0.00147
TBXA2R	Thromboxane A2 receptor	Coagulation	0.00187
THBD	Thrombomodulin	Coagulation	0.00187
THBS1	Thrombospondin-1	Coagulation	0.00373
TIMP1	TIMP metalloproteinase inhibitor-1	Remodeling	0.00187
TNFAIP3	Tumor necrosis factor, alpha-induced protein-3	Inflammation	0.00147
TNFAIP6	Tumor necrosis factor, alpha-induced protein-6	Inflammation	0.00298
TNFRSF21	Tumor necrosis factor rec. superfamily, member 21	Inflammation	0.00187
VCAM-1	Vascular cell adhesion molecule-1	Adhesion	0.00713
VEGF	Vascular endothelial growth factor	Growth	0.00298

in Citrate buffer (LabVision, Thermo Scientific) for 20 min, and then cooled to room temperature for 20 min. After washing in phosphate-buffered saline (PBS), sections were incubated with blocking serum (5% goat serum albumin in PBS) for 30 min at room temperature. Rabbit anti-p65 polyclonal antibodies (GeneTex diluted 1:400) and mouse anti-CD68 (DAKO diluted 1:800) were used as primary antibodies. Negative controls were incubated in 1% bovine serum albumin alone. All sections were incubated with a mixture of the primary antibodies overnight at 4°C. After washing with PBS, bound antibodies were visualized by use of a mixture of indocarbocyanine (Cy3, red color)-conjugated goat anti-rabbit antibody (Jackson Immuno Research Laboratories, Inc., West Grove, Pennsylvania; diluted 1:1,000) and Alexa 488 (green color)-conjugated donkey anti-mouse (Molecular Probes, Eugene, Oregon; diluted 1:500) applied for 60 min at room temperature. After washing with PBS, the nuclei of all sections were counterstained with 4',6-diamidino-2 phenylindole (blue color; Boehringer Ingelheim, Ingelheim am Rhein, Germany; diluted 1:10,000) for 1 min, and sections were then mounted with glycerol: PBS, 2:1. Evaluation of immunofluorescence staining was performed with a Leica DMRB fluorescence microscope with Leica filter cube L4 (Leica, Wetzlar, Germany).

Statistical analysis. Wilcoxon signed rank test was used to test differences between radiated and nonradiated arteries. Due to the limited number of patients and multiple testing, a conservative alpha criterion was used to minimize the risk

of type 1 errors where only gene expression results were considered statistically significant if log-values of relative quantification from all patients differed from 0 with an error rate of at most $p = 0.0015$ to guarantee a family error rate of <0.05 .

Results

The results obtained with Affymetrix Human Genome U133 2.0 Plus oligonucleotide microarrays showed a significant overrepresentation in Gene Ontology terms associated with angiogenesis, coagulation, and inflammation (Online Appendix).

Further results with TLDA analysis showed synchronous regulation (Fig. 1), either up or down for 13 genes: CCL3, CCL8, CXCL2, DUSP5, FGFR2, HMOX1, HOXA9, IL-6, MMP-1, PTX3, RDH10, SOD2, and TNFAIP3 (gene names with abbreviations and p values are displayed in Table 2). The strongest down-regulation was seen for the regulatory homeobox gene HOXA9, whereas the strongest up-regulation was seen for the matrix metalloproteinase MMP-1. The majority of highly expressed genes included chemokines, such as CCL3, CCL8, and CXCL2; interleukins (ILs); and associated genes such as IL-6 and TNFAIP3. Increased expression of the acute phase protein PTX3 was also seen in all samples. The leukocyte adhesion molecules ICAM-1 and SELE, also associated with an acute inflammatory response, were up-regulated in all but 1 sample.

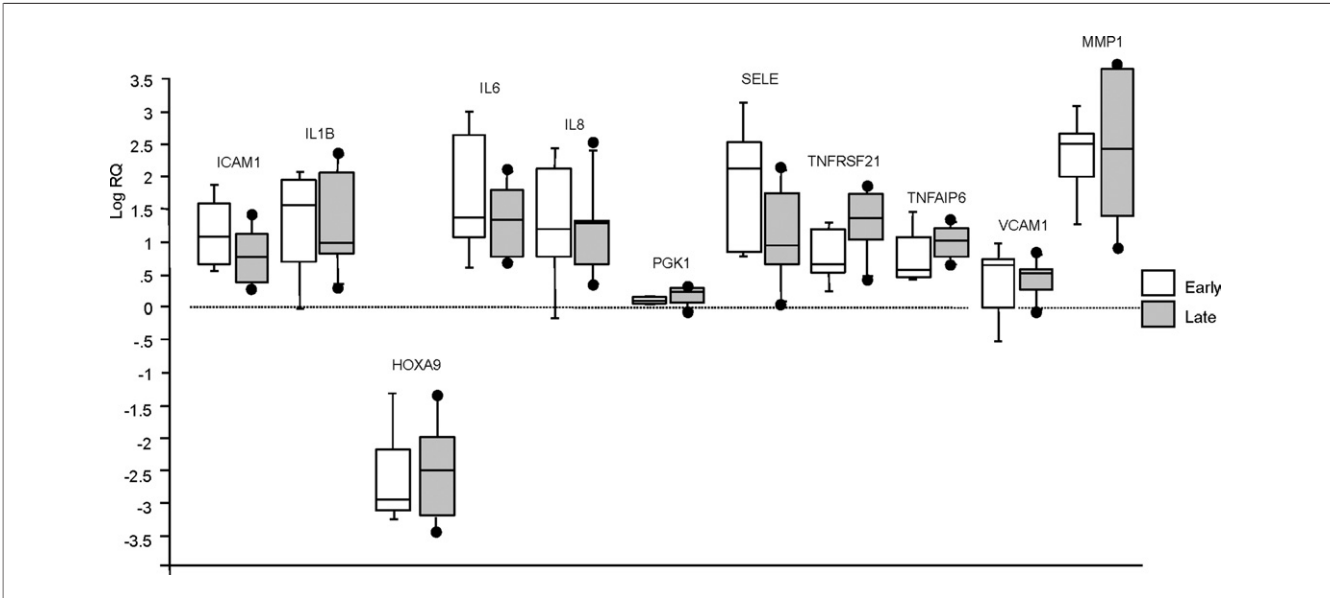


Figure 2 Box Plot of NF-κB Related Genes Derived From Analysis of Irradiated Arteries Compared With Controls

Box plot of nuclear factor-κ B (NF-κB)-related genes derived from Taqman low-density array analysis of irradiated arteries compared with controls for patients where time elapsed from termination of radiotherapy to operation was registered (n = 11). Whiskers represent maximum and minimum values that are no more than 1.5 times of the interquartile range. Outliers are indicated if present. Data were normalized against the housekeeping gene 18S. Selected genes with particular interest for inflammation are shown together with the housekeeping gene PGK1. Patients are divided into 2 groups according to the accepted nomenclature of early (open) and late (shaded) effects of radiotherapy, with time ranging from 4 to 7 weeks and 20 to 500 weeks, respectively. Differential gene expression displayed as log-values of relative quantification (RQ).

Further analysis was performed to detect variations between acute (4 to 7 weeks) and late (20 to 500 weeks) effects of radiotherapy. The CCL2, CCL8, IL1B, ICAM-1, and SELE showed a tendency toward acute high expression and lower late expression. Sustained high expression of inflammatory genes was seen, in particular, for CCL3, CXCL2,

IL-6, IL-8, TNFAIP3, and PTX3. However, due to the limited number of patients in each subgroup, statistical analysis was not considered to be of any value, and the material is instead presented graphically (Fig. 2) (Online Appendix).

Immunohistochemistry, with representative biopsies from 3 patients, showed enhanced nuclear staining for p65

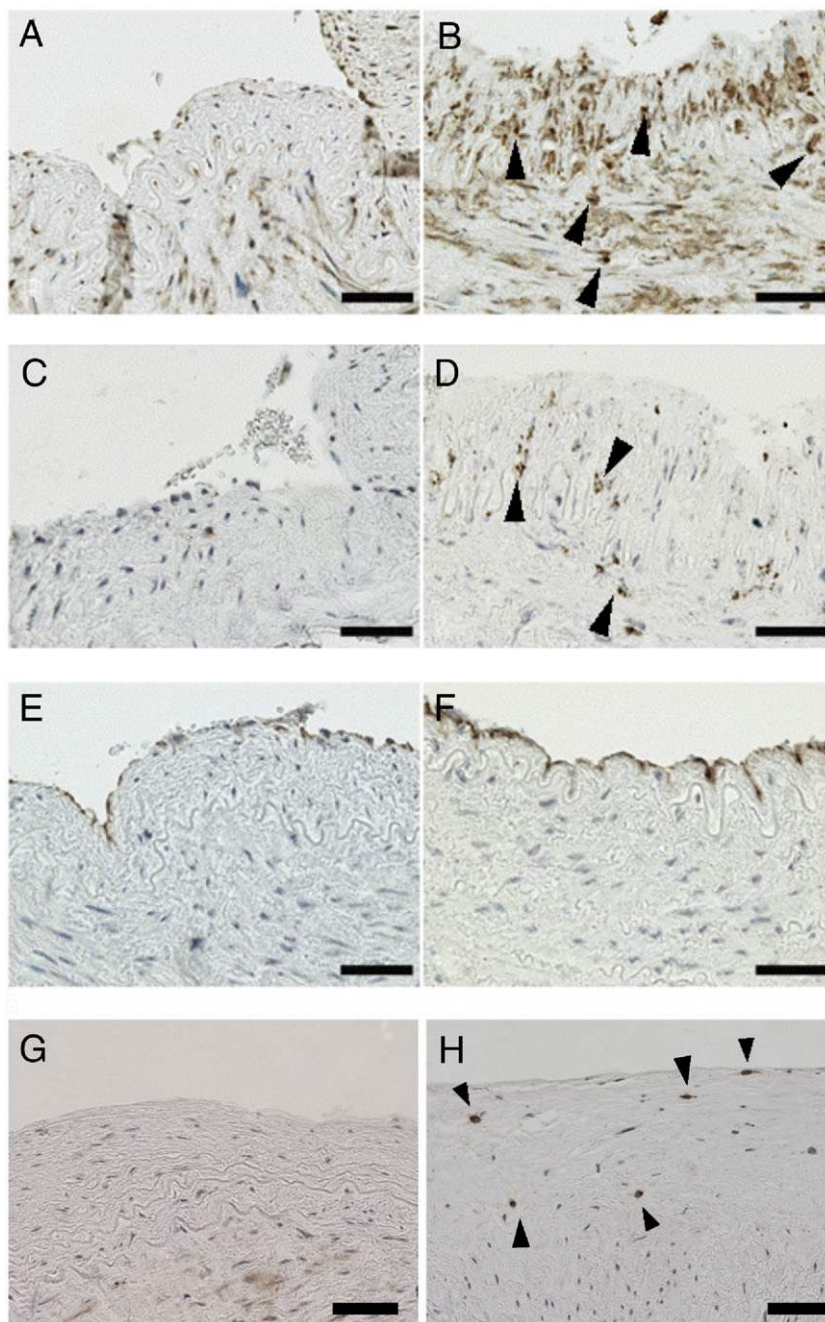


Figure 3

Immunohistochemical Stainings of Nonirradiated Human Radial Forearm Arteries (Controls) and Irradiated Human Cervical Arteries

Irradiated arteries showed positive nuclear factor-kappa B (B), CD68 (macrophages) (D), and CD3 (T-cells) (H) staining compared with controls (A, C, G). Irradiated (F) and control (E) arteries both presented a morphologically intact endothelium shown by staining for von Willebrand factor. Images from a patient, radiated 4 years before harvest of biopsies, are representative for stainings of control and irradiated arteries in 3 patients. Bar = 50 μ m.

in radiated arteries compared with nonradiated arteries, even when more than 3 years had elapsed between last radiotherapy session and surgery. Invasion of T-cells and macrophages in the arterial wall was seen. The von Willebrand factor-staining showed that the endothelium was morphologically intact, in all patients (Fig. 3). Staining for MMP-1 clearly showed increased protein expression in radiated compared with nonradiated arteries (Fig. 4).

Immunofluorescence showed that the p65 subunit was localized to the nuclei of radiated arteries. On the contrary, nonradiated arteries from the same patient showed p65-staining mostly localized to the cytoplasm (Fig. 5).

Discussion

In the present study, we have used a global gene expression strategy to study the underlying gene network mediating chronic vasculopathy in human conduit arteries after high-dose radiotherapy. By simultaneously harvesting radiated and nonradiated arterial biopsies from the same patient, during autologous microvascular free tissue transfers, we were able to eliminate interindividual differences in gene expression pattern and thereby study the sole effect of radiation.

Initial gene expression profiling by microarrays showed a significant over-representation in Gene Ontology terms associated with angiogenesis, coagulation, and inflammation when data from radiated arteries were compared with nonirradiated arteries in paired analysis. Taqman analysis

confirmed, for the first time in man, sustained inflammation in radiated arteries within a large time-frame. We suggest that the sustained inflammatory activity of the arterial wall years after radiotherapy can result in a vascular injury that mimics early arteriosclerosis. Support for this hypothesis is found in the studies by Dorresteijn *et al.* (26) and Darby *et al.* (1) that described cardiovascular events many years after exposure. Neither of these studies could prove an increased morbidity during the first 10 years after radiotherapy, whereas an increased rate of carotid wall thickening (26) and myocardial infarction (1), respectively, was seen after a post-radiotherapy interval of more than 10 years. Further support for this hypothesis is the adverse, long-term effects of intracoronary irradiation, with a delayed and progressive restenotic process (3,27). Deiner *et al.* showed in animals that intracoronary irradiation initially inhibits cell proliferation, but cellular and molecular inflammatory processes are enhanced within the arterial wall by activation of NF- κ B. This proinflammatory effect of radiation has been suggested to be responsible for the observed delayed proliferation and the resulting lumen loss (28). Furthermore, the increased expression of MMP-1 might modulate this process. In fact, secretion of MMP-1 has proved to be dependent on NF- κ B activation in both macrophages (29) and smooth muscle cells (30).

In the present study, we were able to identify alterations in gene expression by studying the effects of irradiation of human arteries as the single factor. The radiated artery with

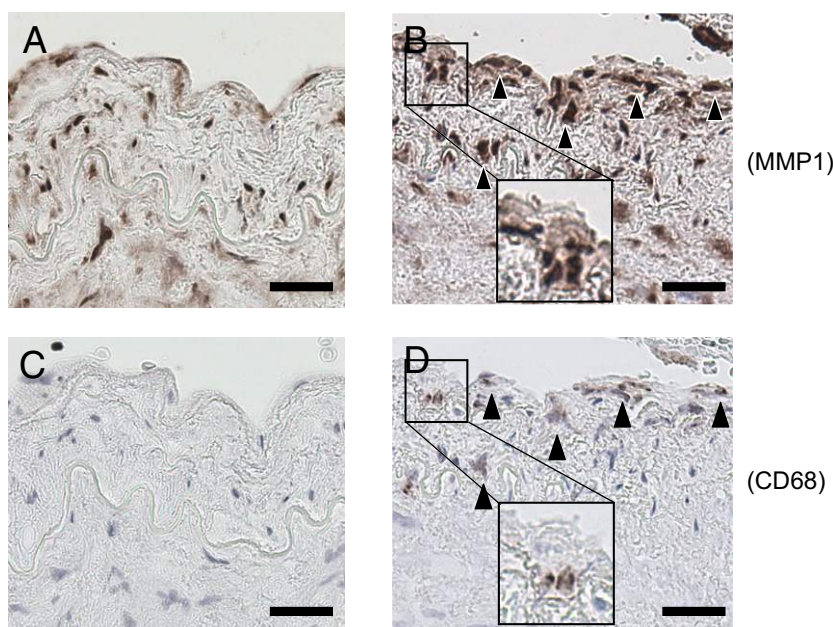


Figure 4 Immunohistochemical Stainings of Nonirradiated Human Radial Forearm Arteries (Controls) and Irradiated Human Cervical Arteries

Increased staining for matrix metalloproteinase (MMP)-1 in radiated arteries (B) compared with controls (A) was confined to macrophages visualized by CD68-staining in consecutive sections of radiated (D) and control (C) arteries, representative of paired biopsies from 3 patients. Bar = 25 μ m.

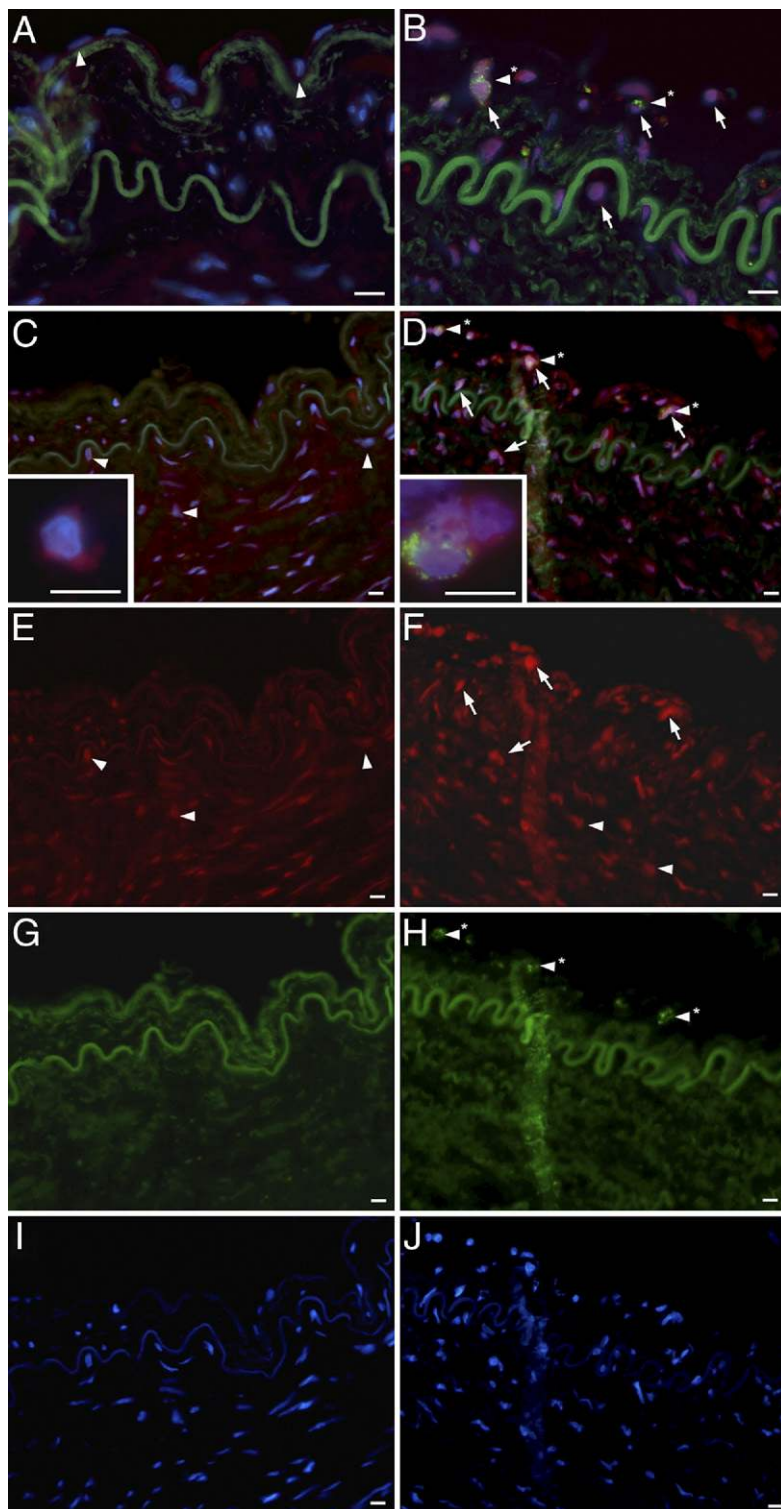


Figure 5 Staining of p65 Subunit Superimposed on Photographs of DAPI Nuclear Staining

Staining of p65 subunit (red fluorescence) superimposed on photographs of 4',6-diamidino-2-phenylindole (DAPI) nuclear staining (blue fluorescence) indicating nuclear factor- κ B (NF- κ B)-activation by nuclear translocation seen with co-localization of p65 and DAPI in the cell nucleus (purple) in irradiated arteries (**B, D**) compared with non-irradiated arteries (**A, C**). Co-staining for CD68 (green) showed that the p65 subunit was localized to the nuclei in macrophages but only present in radiated arteries. Single channel stainings of the merged photographs (**C and D**) are visualized for nonirradiated (**E, G, I**) and irradiated (**F, H, J**) arteries from the same patient, irradiated 4 years before harvest of biopsies, representative for stainings of nonirradiated and irradiated arteries in 3 patients (arrows = p65 nuclear stains; arrowhead = p65 expression in the cytoplasm; arrowhead asterisk = p65 nuclear staining positive cells co-expressed with CD68 in the cytoplasm). Bar = 10 μ m.

the genetically identical control vessel serves as a study-model of localized chronic arterial inflammation. Previous experimental studies have mainly been performed in vitro with, in many aspects, contradicting results. There is also a paucity of studies that have described effects of clinical doses of radiation within a large time-frame. Therefore it was highly relevant to study global gene expression profiles in humans, to understand the relationship between previous and in many ways fragmented in vitro and in vivo findings. With support from previous epidemiological findings we believe the temporal aspect of radiotherapy to be a key factor. The late effects of radiation develop through complex interacting processes that are yet not well-understood. The response of the endothelium to radiation occurs within hours of exposure and has so far been proved to be sustained for several weeks. It has also been shown that the maintenance of such a response could contribute to the development and progression of late tissue radiation damage, such as fibrosis of skin, bowel, and lungs (5), but there is a paucity of studies of late effects on the arterial wall. Our data indicate an acute as well as a chronic effect on the innate immune response.

It is today generally accepted that atherosclerosis represents a chronic inflammatory disease of the arterial wall in which macrophages plays a critical role (31). A persistent inflammatory response after radiation exposure has been reported previously both in vitro and in vivo but not in humans and only with a time-span up to several weeks for doses up to 20 to 40 Gy (9,12,32,33). As pointed out in the preceding text, our data clearly indicate an innate immune response to irradiation. Immunohistochemistry confirms an activation of NF- κ B signaling. Our data are supported by previous findings suggesting that DNA injury, after radiation, activates NF- κ B (34,35), but the mechanisms remain unclear. Recent studies suggest a regulatory role of the homeobox transcription factor HOXA9 (36–38). However, the published data are contradictory, with Bandyopadhyay et al. (36) showing that HOXA9 acts as an obligate proinflammatory factor by mediating cytokine induction of SELE. In contrast, Trivedi et al. (37,38) recently demonstrated that down-regulation of HOXA9 is an essential event during endothelial activation with expression of leukocyte adhesion molecules such as ICAM-1, VCAM-1 and SELE, suggesting that HOXA9 is involved in maintaining the basal state of endothelial cells. Our findings clearly support the work by Trivedi et al. (37,38), because we identified HOXA9 as 1 of the strongest differentially down-regulated genes together with a sustained activation of the NF- κ B signaling pathway. However, HOXA9 protein expression in adult blood vessels has never been shown, and our gene-expression results with clearly decreased levels were not possible to confirm on a protein level (data not shown). This is not surprising, because both our gene expression data together with results of previous studies show a nearly complete down-regulation from already low levels (38).

Several limitations of this study should be acknowledged. Due to a low frequency of operations in radiated patients, the number of biopsies is low. Furthermore, the biopsies from the neck arteries were small, because the main part often was included in the tumor specimen and the remaining part should serve as recipient arteries on the neck. The lack of material made it impossible to analyze more than 3 pairs by Affymetrix microarrays. We compared expression in arteries from 2 different anatomical regions, instead of having a control group of nonradiated patients, but with arteries from the head-neck region. It was not possible to fully confirm findings on a protein level and impossible to determine the cellular origin of detected expression. Only unspecific staining was obtained for IL-6 with immunohistochemistry on paraffin-embedded sections (data not shown). Antibodies for other cytokines need frozen sections, and this procedure could not be performed due to a lack of this type of material. Further studies with isolated cell fractions and culture techniques will be required to elucidate specific gene expression and protein synthesis on a cellular level.

Taken together, we have studied arterial gene expression by comparing radiated and nonradiated arteries harvested from the same patient at the same time, thereby eliminating interindividual factors. Our results showed a sustained inflammation due to NF- κ B activation. Furthermore, a role for HOXA9 in the regulation of NF- κ B is suggested. We believe that the arterial inflammation seen together with the identification of a consistent and rather uniform pattern of differentially expressed genes between radiated and nonradiated arteries have generated new perspectives for future research on inflammatory alterations in vascular biology.

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Reprint requests and correspondence: Dr. Martin Halle, Reconstructive Plastic Surgery, Karolinska University Hospital, 171 76 Stockholm, Sweden. E-mail: martin.halle@ki.se.

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Key Words: gene expression ■ human arteries ■ inflammation ■ irradiation.

APPENDIX

For supplementary material, please see the online version of this article.