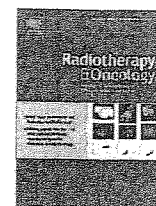




ELSEVIER

Contents lists available at ScienceDirect

Radiotherapy and Oncology

journal homepage: www.thegreenjournal.com

Clinical radiobiology

Novel insights into pathological changes in muscular arteries of radiotherapy patients

Nicola S. Russell^{a,*}, Saske Hoving^{b,1}, Sylvia Heeneman^c, J. Joris Hage^d, Leonie A.E. Woerdeman^d, Remco de Bree^e, Peter J.F.M. Lohuis^f, Ludi Smeele^f, Jack Cleutjens^c, Addy Valenkamp^e, Lucille D.A. Dorresteijn^g, Otilia Dalesio^h, Mat J. Daemen^c, Fiona A. Stewart^b

^a Department of Radiotherapy, The Netherlands Cancer Institute, Amsterdam, The Netherlands^b Division of Experimental Therapy, The Netherlands Cancer Institute, Amsterdam, The Netherlands^c Cardiovascular Research Institute, Maastricht, The Netherlands^d Department of Plastic and Reconstructive Surgery, The Netherlands Cancer Institute, Amsterdam, The Netherlands^e Department of Otolaryngology/Head and Neck Surgery, VU University Medical Center, Amsterdam, The Netherlands^f Department of Head and Neck Oncology and Surgery, The Netherlands Cancer Institute, The Netherlands^g Department of Neurology, Radboud University, The Netherlands^h The Netherlands Cancer Institute, Amsterdam, The Netherlands

ARTICLE INFO

Article history:

Received 27 February 2009

Received in revised form 20 May 2009

Accepted 25 May 2009

Available online 21 June 2009

Keywords:

Radiotherapy
Arterial pathology
Cardiovascular
Late effects

ABSTRACT

Background and purpose: Vascular disease is increased after radiotherapy and is an important determinant of late treatment-induced morbidity and excess mortality. This study evaluates the nature of underlying pathologic changes occurring in medium-sized muscular arteries following irradiation.

Materials and methods: Biopsies of irradiated medium-sized arteries and unirradiated control arteries were taken from 147 patients undergoing reconstructive surgery with a vascularised free flap following treatment for head and neck (H&N) or breast cancer (BC). Relative intimal thickening was derived from the ratio of the thickness of the intima to the thickness of the media (IMR) on histological sections. Proteoglycan, collagen and inflammatory cell content were also scored.

Results: Intimal thickness was significantly increased in irradiated vessels: in the H&N group the IMR was 1.5-fold greater without correction for the control artery ($p = 0.018$); in the BC group the IMR increased 1.4-fold after correction for the control artery ($p = 0.056$) at a mean of 4 years following irradiation. There was an increase in the proteoglycan content of the intima of the irradiated IMA vessels, from 65% to 73% ($p = 0.024$). Inflammatory cell content was increased in the intima of the irradiated H&N vessels ($p = 0.014$).

Conclusions: Radiation-induced vascular pathology differs quantitatively and qualitatively from age-related atherosclerosis.

© 2009 Elsevier Ireland Ltd. All rights reserved. Radiotherapy and Oncology 92 (2009) 477–483

Due to earlier detection and better treatments, increasing numbers of cancer patients achieve long-term survival following treatment. There are an estimated 100 million cancer survivors world-wide, of whom about half will have been treated with radiotherapy. Health and quality of life issues in cancer survivors have received increasing attention in recent years and research in this field has been declared a priority by the National Cancer Institute NCI and ASCO [1,2]. Vascular disease is one of the most important determinants of late morbidity and mortality after irradiation for malignancy. Cohort studies have demonstrated a clear increased

risk of vascular disease, e.g. stroke and cardiovascular disease, more than 10 years after radiotherapy for Hodgkin's lymphoma, breast cancer, head and neck (H&N) cancer and others [3–17]. The cure rate for early-stage Hodgkin's lymphoma, which has a peak incidence in the second and third decades of life approaches 90% following radiotherapy. Patients are, therefore, at risk of developing long-term treatment-related complications. The risk of coronary heart disease is strongly increased compared to the age and sex matched general population, with a standardised incidence ratio of 3.6 for myocardial infarction and 4.0 for angina pectoris. Breast cancer survivors are more prevalent in the general population, and a third of patients are under the age of 50 years at diagnosis. Long-term follow-up studies of breast cancer patient cohorts have shown a standardised incidence ratio of 1.23 for myocardial infarction and 1.30 for angina pectoris for irradiated patients compared to the general female population. Long-term

* Corresponding author. Address: Department of Radiotherapy, The Netherlands Cancer Institute – Antoni van Leeuwenhoek Hospital, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

E-mail address: n.russell@nki.nl (N.S. Russell).

¹ These authors contributed equally to this work.

follow-up of radiotherapy trials for early breast cancer has shown a 4% excess risk of non-breast cancer deaths in irradiated patient compared to unirradiated patients at 20–30 years, mostly due to cardiovascular causes. Irradiation to the neck has been shown to increase the risk of ischemic stroke by 5-fold at 5 years. Carotid angiography of such cases reveals luminal narrowing at the level of the previous radiation portals. Previous irradiation is also a risk factor for graft failure after reconstructive surgery [18,19].

The assumed pathogenesis is an acceleration or induction of atherosclerosis in major arteries located in the irradiated field, but data from studies on human tissue are scarce, and mostly from post-mortem studies of end-stage disease [20–22]. New insights into the aetiology of changes that occur in irradiated arteries are needed to develop preventative intervention strategies. We therefore conducted a histopathological study in muscular arteries in order to characterize and quantify vascular changes within 10 years after radiotherapy, i.e. before they become symptomatic. The study was conducted on vessels from H&N cancer and breast cancer patients, as patients treated for these diseases with radiotherapy have a well-documented increased risk of vascular complications. A large number of patients were included and so we could compare radiation-induced pathology with changes in unirradiated control vessels.

Materials and methods

Recipient arteries for free flap reconstructions were biopsied from patients undergoing a resection of a tumour in the H&N region or breast reconstruction following a mastectomy for breast cancer. In irradiated patients, the biopsied artery had been within the radiation fields. The irradiated biopsies were compared to biopsies from two types of control arteries; the unirradiated donor vessels from the same patient, which allowed us to control for systemic vascular changes; and with the same type of recipient artery from unirradiated patients undergoing a similar free flap reconstruction procedure (Fig. 1).

Patients undergoing reconstructive surgery at The Netherlands Cancer Institute or the VU University Medical Center, Amsterdam were included in the study. The study complied with the declaration of Helsinki and was approved by the Ethics Committees of both hospitals. Patients gave their written informed consent at The Netherlands Cancer Institute before inclusion in the study but this was not required by the VU University Medical Centre Ethical Committee. The first group of patients comprised 91 patients with H&N cancer who underwent such extensive resections for a primary tumour or a recurrence that reconstruction with a free vascularised myo- or fascio-cutaneous flap was required to close the defect. A free radial forearm flap was generally used, and a micro-vascular anastomosis formed between the radial artery and one of the branches of the external carotid artery, most frequently the facial artery. In irradiated patients, this artery was within the radiotherapy planning target volume for the primary tumour and/or lymph node metastases. The second group comprised 56 patients who had previously undergone a mastectomy for breast cancer and were admitted for a breast reconstruction using a free flap technique with a perforator adipocutaneous free flap from the lower abdominal wall. A microsurgical anastomosis was created between the deep inferior epigastric artery and the ipsilateral internal mammary artery, which lie in the full dose region if patients had been irradiated to the internal mammary lymph node chain. A 5 mm biopsy from the free end of both vessels used for the anastomosis was obtained for the study.

Detailed clinical data were collected for each patient including parameters such as age, sex, oncological diagnosis and treatment, time since any irradiation, chemotherapy and/or hormonal ther-

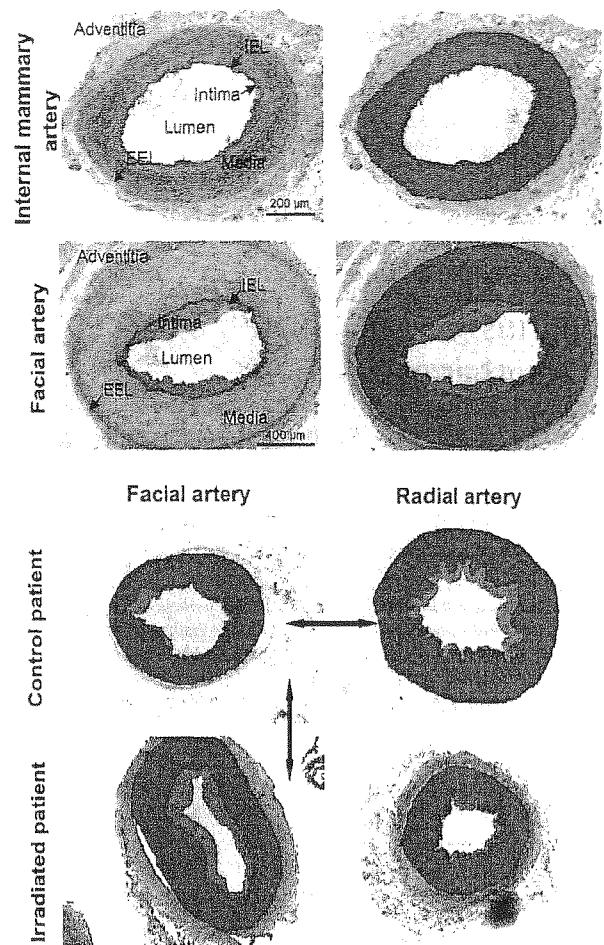


Fig. 1. Histological arterial cross-sections. Top 4 panels: left, Lawson's stain for the elastic laminae (IEL, internal elastic lamina; EEL, external elastic lamina); right, block colouring of the intima (grey) and media (pink) to allow automated image analysis of cross-sectional surface areas. Lower 4 panels: comparison of irradiated and control arteries from the H&N cancer patient group.

apy. The position of the radiation target volume and the dose in relation to the biopsied artery in the irradiated patient groups was obtained from the radiation planning data. Data were collected on vascular risk factors, including body mass index (BMI), smoking history, medication and co-morbidity; in particular endocrine, metabolic or vascular diseases (coronary vascular disease, cerebrovascular disease and/or peripheral vascular disease).

The arterial biopsies were fixed in 1% paraformaldehyde for 48 h and embedded in paraffin before serial transverse sectioning. Sections of 4 μ m were stained with haematoxylin and eosin. For measurement of the intima and media, Lawson staining (a modified Verhoeff-von Giessen staining) was used to delineate the internal and external elastic laminae. The cross-sectional surface area for the intima was defined as the surface area covered in the sections between the endothelium and the internal elastic lamina, and for the media was defined as the surface area between the internal elastic lamina and the external elastic lamina at the adventitia (Fig. 1). Morphometric parameters were analysed using a microscope coupled to a computerized morphometry system (Leica Qwin V3, Leica, Rijswijk, The Netherlands). The degree of intimal thickening for each vessel was calculated from the ratio of the cross-sectional surface area of the intima to that of the media (intima-media ratio IMR). The ratio was used to correct for variations in artery size. All measurements were made, without knowledge of the treatment group, by one investigator. Intra-observer variation was less than 10%, as determined by repeat measurements on six slides.

We also compared changes in the interstitial matrix and inflammatory cell infiltration between the irradiated and unirradiated control arteries. The total collagen content was detected by Sirius Red staining. Sections were pre-treated with 0.2% phosphomolybdic acid for 5 min and stained for 90 min in 0.1% Sirius Red in saturated aqueous picric acid. Sections were then treated with 0.01 M HCl for 2 min. Alcian Blue staining was used for histological assessment of the total proteoglycan content. Sections were placed in a 1% solution of Alcian Blue in 3% acetic acid (pH 2.5) for 30 min. Cell nuclei were counterstained for 5 min with Kernechtrot. Four fields from each tissue section of 4 μ m thickness were photographed and analysed using a microscope coupled to a computerized morphometry system (Leica Qwin V3, Leica, The Netherlands). Sirius Red or Alcian Blue stained material from each field was selected and the number of red or blue pixels was determined. The sum of stained material of four sections was then divided by the total amount of tissue in these sections to calculate the percentage collagen or proteoglycan content. All these measurements were conducted within one experiment to avoid inter-experimental variation.

Immuno-histochemical staining was performed to assess the presence of an inflammatory cell response. Representative sections were stained for markers of macrophages (CD68 dilution 1:100; Dako, ITK diagnostics b.v, Uithoorn, The Netherlands) and leukocyte common antigen (CD45; Dako, 1:500). Sections were de-paraffinised and blocked with 0.3% hydrogen peroxide. Antigen retrieval was performed by pepsin digestion (CD68 only) and the sections were incubated with primary antibodies. Binding of the primary antibody was detected using a horseradish-peroxidase-conjugated secondary antibody. Diaminobenzidine (DAB) was used as substrate for localization and the sections were counterstained with haematoxylin. The number of CD68 and CD45 positive cells adhering to the endothelium, in the intima and in the media was recorded for each section, by one observer (Fig. 4).

For the statistical analysis, a log transformation was performed on the data to normalize the distribution. For each comparison, Levine's test for equality of variance was performed. Depending on whether equality of variance could be assumed, the appropriate two-tailed *t*-test for equality of means was performed on the respective data-sets. A *p*-value of 0.05 was considered to be statistically significant. Two sets of comparisons were made for the IMR measurements. First, the IMR measurements from the same artery type (neck artery or internal mammary artery) were compared between the irradiated and the unirradiated patients for each disease group. Second, to take account of variations in underlying vascular pathology between patients, we calculated the ratio of the IMR of the neck artery or internal mammary artery versus the IMR of the free flap artery. The "IMR ratios" for irradiated and unirradiated patients were then compared. The association between different factors and the IMR was investigated by means of a generalised linear model with the log of the IMR measurements from the neck or internal mammary arteries as dependent variable, the log of the IMR of the flap artery as covariate and the factors of interest as fixed factors or covariates depending on the type of variable. The following factors were analysed: radiation, age, gender, BMI, smoking, pack years, alcohol use, diabetes, hypertension, hyperlipidemia, hypothyroidism, coronary vascular disease, cerebrovascular disease and peripheral vascular disease. Missing information on certain clinical parameters in some patients were treated as missing data and were omitted from the analysis for that specific parameter. For the assessment of inflammatory cell infiltration, the absolute numbers of cells per section were analysed. Also the relative cell density per section was calculated by correcting for the circumference of the intima, the surface area of the intima and media, respectively. Statistical analyses were performed using SPSS VERSION 15.0.

Results

Table 1 shows the main characteristics of the patients in the study. There was no statistical difference in age, BMI or number of pack years smoked between the irradiated and control (non-irradiated) groups for the H&N and breast cancer groups, respectively. For the irradiated H&N cancer group the interval between the irradiation and the resection with reconstruction was either a number of months, in the case of residual or recurrent disease after primary radiotherapy, or a number of years, due to the development of second primary tumour within the irradiated field (range 3 months–26 years). For the breast cancer patients the range was 8 months–19 years. As a group, the H&N cancer patients had more co-morbidity including pre-existent vascular disease.

For the IMR measurements, we collected data on a total of 91 H&N patients and for 70 patients biopsies were of sufficient quality to perform reliable IMR measurements. IMR analysis was performed on 25 irradiated neck vessels, 45 unirradiated neck vessels and 54 donor vessels. There was a significant 1.5-fold increase in the mean IMR for irradiated compared to that for unirradiated neck arteries (0.173; SD = 0.113 vs. 0.118; SD = 0.065, *p* = 0.018, Table 2). There was a statistical association between the IMR of the neck artery and the IMR of the corresponding radial artery from the same patient, confirming the relevance of using this artery as a control. When the IMR of the radial artery was used as a covariate in an analysis of covariance including radiotherapy and the IMR of the radial artery, the increase in the IMR after radiotherapy was no longer significant (*p* = 0.121; Fig. 2 and Table 2). The variance in the IMR of the radial artery was quite wide, with a mean value of 0.103 and a standard deviation (SD) of 0.057, illustrating the heterogeneity of the underlying vascular pathology in the group. None of the other factors tested for a possible co-association with IMR value (as listed in the section on statistics) was found to have a significant effect.

In the breast cancer group, a total of 56 patients were included in the study, 27 in the irradiated group and 31 in the non-irradiated group, with 3 patients in both groups, due to bilateral reconstructions following irradiation to one side. The biopsies of two control inferior epigastric arteries were of too poor quality to perform reliable IMR measurements. There was no significant increase in the mean absolute IMR in the irradiated compared to that in the unirradiated internal mammary arteries (0.071; SD = 0.043 vs.

Table 1
Patient and treatment characteristics.

	Head and neck		Breast	
	Control (<i>n</i> = 45)	RT (<i>n</i> = 25)	Control (<i>n</i> = 31)	RT (<i>n</i> = 27)
Age (years)	57 ± 10	54 ± 12	47 ± 8	46 ± 8
Smoking (pack years)	32 ± 23	26 ± 18	4 ± 7	5 ± 13
BMI	24 ± 4	25 ± 7	28 ± 4	27 ± 5
FU (years)	–	4 ± 7	–	3 ± 4
Dose (Gy)	–	66 ± 7	–	49 ± 3
Gender: Male/Female	69/31%	64/36%	0/100%	0/100%
Alcohol abuse	26%*	15%*	0%	0%
Coronary vascular disease	9%	16%	0%	0%
Cerebrovascular disease	7%	0%	0%	0%
Peripheral vascular disease	7%	0%	0%	0%
Diabetes	11%	16%	0%	0%
Hypertension	14%*	21%*	13%	19%
Hyperlipidaemia	11%*	15%*	0%	4%
Hypothyroidism	11%*	0%*	10%	4%
Hyperthyroidism	0%*	0%*	3%	0%
Chemotherapy	4%	32%	61%	93%
Hormone therapy	0%*	0%	45%	52%

Average ± SD.

* Not known for all patients.

Table 2

Results of the measurements of intimal thickening, expressed as the intima: media ratio (IMR) and after correction for the IMR of the control artery from the same patient; the percentage proteoglycan content, the percentage collagen content; and the number of inflammatory cells adhering to the endothelium, in the intima or in the media. CD45: cells staining positive for CD45, common leukocyte antigen; CD68: cells staining positive for CD68, a macrophage marker.

	Head and neck		Breast	
	Control	RT	Control	RT
Intima:media ratio (IMR) ^a	0.118 ± 0.065	0.173 ± 0.113*	0.063 ± 0.034	0.071 ± 0.043
IMR/IMR flap artery ^a	1.27 ± 0.51	1.86 ± 1.36	1.90 ± 1.21	2.61 ± 1.65
Proteoglycan content (%)^a				
Intima	61.1 ± 15.0	68.2 ± 14.4	65.1 ± 15.8	73.4 ± 10.8*
Media	19.4 ± 10.0	25.6 ± 15.0	60.8 ± 19.4	69.3 ± 7.9
Collagen content (%)^a				
Intima	2.7 ± 4.5	2.6 ± 4.9	2.5 ± 4.4	1.8 ± 2.9
Media	5.1 ± 6.2	4.2 ± 5.1	6.3 ± 6.0	6.1 ± 5.0
CD45^b				
Adhering	0.9 (0-6)	1.6 (0-8)	2.6 (0-15)	2.6 (0-25)
Intima	1.3 (0-6)	6.7 (0-29)*	5.2 (0-42)	7.8 (0-80)
Media	0.1 (0-1)	0 (0)	0.6 (0-4)	0.7 (0-4)
CD68^b				
Adhering	4.7 (0-39)	5.1 (0-44)	5.2 (0-50)	3.1 (0-19)
Intima	10.2 (0-165)	12.4 (0-62)	38.0 (0-300)	18.5 (0-194)
Media	1.5 (0-21)	0.6 (0-4)	10.0 (0-58)	5.3 (0-32)

^a Average ± SD.

^b Average (range).

* $p < 0.05$ compared with control.

0.063; SD = 0.034, $p = 0.38$, Table 2). However, after correction for the IMR of the donor artery the relative difference showed a trend to an increase in the irradiated patients (IMR ratio = 2.61; SD = 1.65 vs. 1.90; SD = 1.21, $p = 0.056$). Of note, the absolute IMR in the unirradiated control neck arteries from the H&N cancer patients (mean value: 0.118) was about twice the value for the IMR of the unirradiated control internal mammary arteries in the breast can-

cer patients (mean value; 0.063), $p < 0.0001$ (Fig. 2). Also the IMR from the free flap artery was greater in the radial artery from the H&N group compared to the deep inferior epigastric artery from the breast cancer patients (mean value 0.103 vs. 0.038, $p < 0.0001$). These observations can be explained by the different types of arteries, but a greater general intimal thickening in the H&N group may also contribute.

For both patient groups there was a non-significant effect in time, leading to an increased IMR with increasing follow-up time after radiation, but the numbers were too small for a detailed analysis.

The proteoglycan content of the intima of the internal mammary arteries was statistically significantly different between unirradiated and irradiated vessels (mean 65%; SD = 16% vs. 73%; SD = 11%, $p = 0.024$). There was a trend to an increase in the media from 61% (SD = 19%) in unirradiated vessels to 69% (SD = 8%) in irradiated vessels, $p = 0.060$. In the neck arteries, there was also a trend to an increased proteoglycan content of the intima (61%; SD = 15% vs. 68%; SD = 14%, $p = 0.064$), and of the media of irradiated compared to the content of unirradiated vessels (19%; SD = 10% vs. 26%; SD = 15%, $p = 0.097$). There were no differences in the collagen content of the intima or the media of either patient group (Fig. 3).

We also observed differences in the degree of inflammatory cell infiltrate, as detailed in Table 2. The number of cells per cross-section staining positive for CD45, a marker for leukocytes was significantly increased in the intima in irradiated neck arteries of H&N cancer patients, $p = 0.007$. The mean number of CD45⁺ cells per section was 1.3 (range 0-6) in unirradiated vessels compared to a mean of 6.7 (range 0-29) in irradiated vessels. As the intima area was greater in the irradiated group, we also corrected the level of CD45⁺ cell infiltration by dividing the value by the intima area on the cross-section. The increased level of CD45⁺ cell infiltration in the intima remained significantly higher in the irradiated group compared to the unirradiated group ($p = 0.014$). In the irradiated internal mammary arteries, there was a wide variation in the number of CD45⁺ cells in the intima; the range was 0-42 (mean 5.2) in unirradiated vessels, and 0-80 (mean 7.8) in irradiated vessels, and

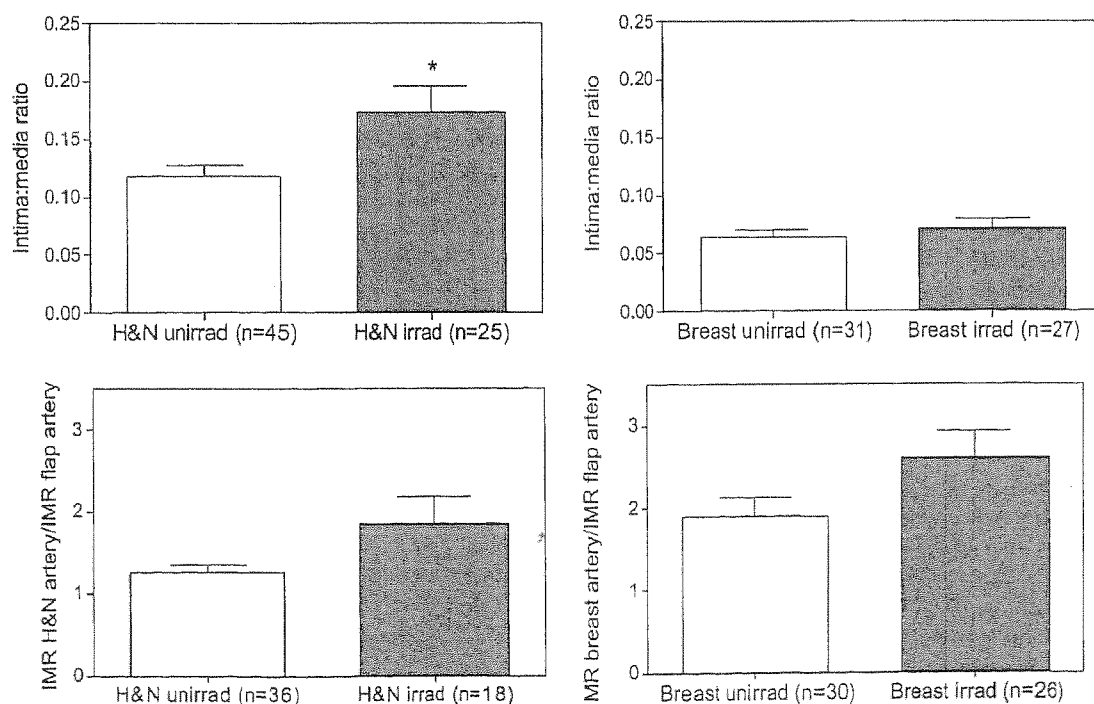


Fig. 2. Top panels: comparison of the intima-media ratio (IMR) between irradiated and unirradiated patients. Lower panels: IMR corrected with the IMR of the free flap artery (IMR ratio). Error bars = standard error of the mean.

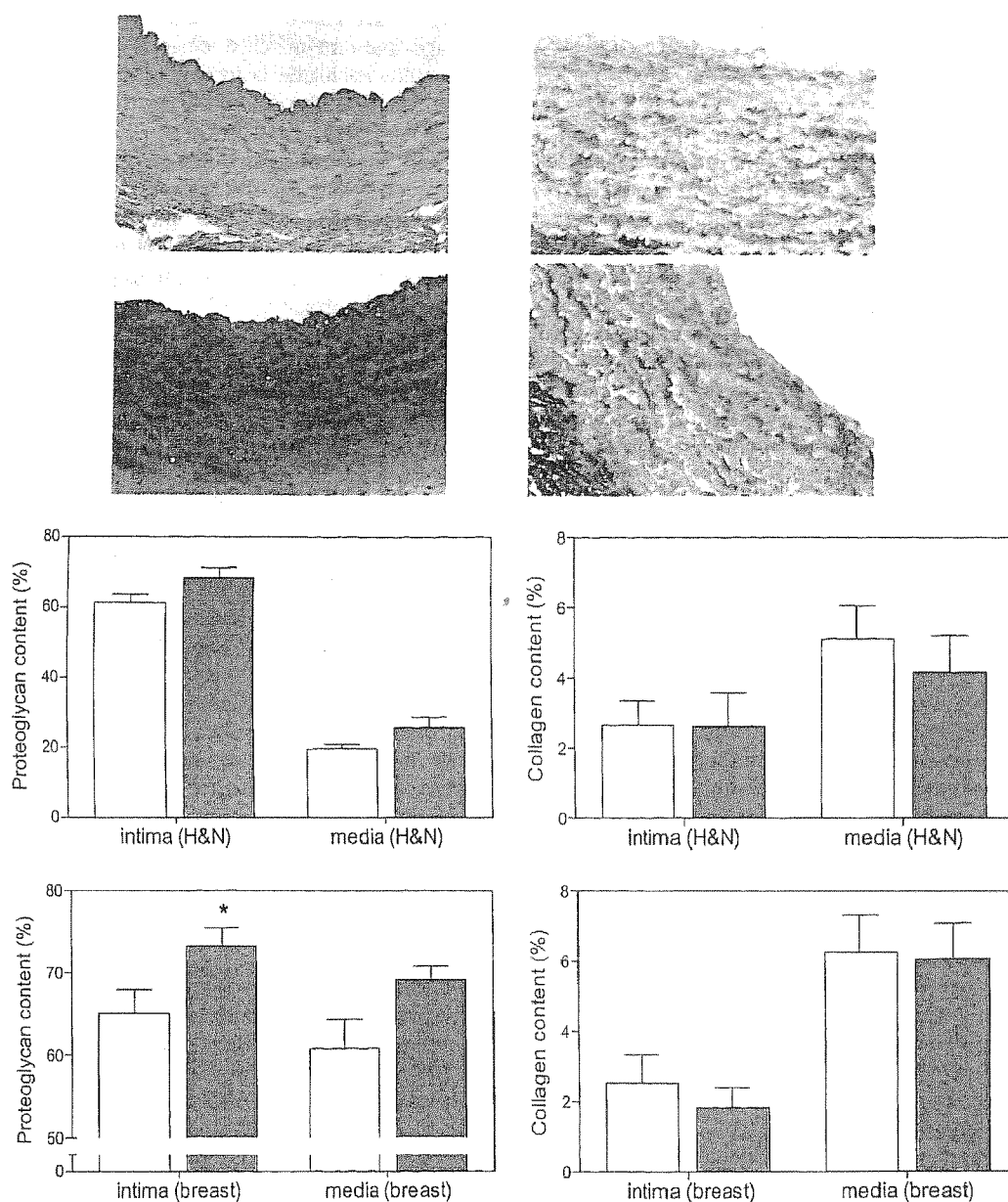


Fig. 3. Left panels: analysis of proteoglycan content; right panels: analysis of collagen content. Upper panels: histological staining 20x objective; above irradiated arteries, below unirradiated arteries; lower panels: histograms of percentage proteoglycan or collagen content in control arteries (light shading) and in irradiated arteries (dark shading) for the intima and media. Error bars = standard error of the mean.

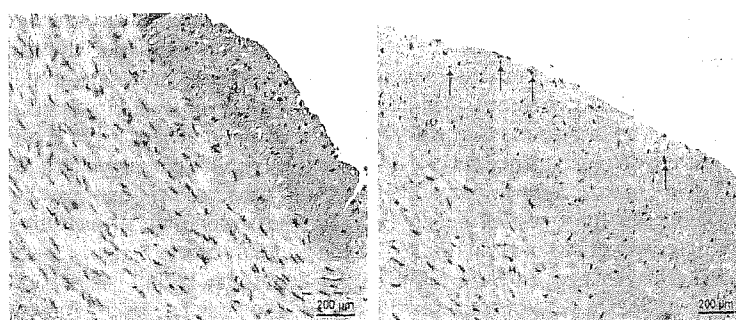


Fig. 4. Immunohistochemistry staining for CD45⁺ cells (leukocytes) in a section of the facial artery. Left panel, unirradiated; right panel, irradiated. Arrows, CD45 positive cells.

this difference was not significant, $p = 0.16$. There were no significant differences observed in the other parameters of inflammatory cell infiltration, including CD45 and CD68 positively staining cells attached to the endothelial layer, or macrophage uptake in the

intima or media in either patient group before or after correction for surface area. There was a noticeable difference between the H&N and breast patient groups in the infiltration of macrophages (CD68⁺ cells) in the intima of unirradiated arteries: mean 10.2 cells

(range 0–165) in the neck arteries and mean 38.0 cells (range 0–300) in the internal mammary arteries ($p = 0.035$).

Discussion

In this study, we analysed arteries from two patient groups at risk of developing late vascular sequelae following irradiation. To our knowledge, this is the largest systematic clinical–pathological study of vascular changes following irradiation. An important aspect of this study is that we were able to use unirradiated vessels to correct for intra- and inter-patient variation in underlying vascular pathology. The results show that radiation induces intimal thickening, proteoglycan deposition and inflammatory cell infiltrate, but with different effects depending on the vessel type.

Intimal thickening can also be measured with clinical imaging techniques. In carotid arteries, the intima media thickness (IMT) is considered a marker of early atherosclerosis development [23,24]. Other authors have evaluated the sub-clinical changes occurring in the carotid arteries of irradiated patients using ultrasound and recorded results consistent with our histological findings. Magnetic resonance imaging of the carotid artery following radiation has shown luminal narrowing [3,25] and an increased IMT in the first 12 months, with further increases in the second year [3,26]. These changes were much more rapid than would be expected on the basis of age-related atherosclerosis. However, the IMT measures the intimal thickening in one radial direction, which can vary considerably across the arterial cross-section (see Fig. 1) and is subject to variations in measurement technique. The IMR measurement used in the present study incorporates information from the whole cross-section of the vessel.

Animal models of radiation-induced atherosclerosis, in which the experimental conditions can obviously be much more controlled than in an observational human study, have shown that radiation accelerates the development of atherosclerotic plaques and predisposes to an inflammatory plaque phenotype prone to haemorrhage, as well as increasing the total plaque burden relative to age-matched animals [27,28]. An increase in inflammatory cell infiltration was observed in the plaques of irradiated vessels in animal models. The pattern of inflammatory cell infiltration seen in the present study (an increase in leukocytes but not of macrophages) could be related to the fact that the vessels studied did not exhibit overt atherosclerosis; no increase in macrophage infiltration was seen and there were no atherosclerotic plaques. The lack of overt atherosclerotic plaques may also explain why no effect on collagen content was observed. In animal models of radiation-induced atherosclerosis, the collagen content is lower in irradiated plaques [28].

The increased deposition of proteoglycans observed after radiation is an interesting finding and not previously described. In the development of age-related atherosclerosis, lipoproteins are transported or leaked through damaged endothelium and are able to bind to proteoglycans. These complexes exhibit an increased susceptibility to oxidation and are important in atherosclerosis plaque development. Proteoglycans are produced by both macrophages and smooth muscle cells [29,30]. Studies have shown that the type of proteoglycans present differs between atherosclerosis-prone and atherosclerosis-resistant artery types [31]. Whether changes in the arterial proteoglycan content are related to the increased atherosclerosis risk following irradiation remains to be investigated.

The choice of the type of vessels biopsied and patient selection for this study can be discussed. The internal mammary artery is known to be rather resistant to atherosclerotic changes, and indeed it is the vessel of choice for coronary artery bypass grafting. Possibly this is related to the predominant proteoglycan type in this vessel [31]. Another feature of this study is that the internal mammary artery biopsies were from breast cancer patients, who have been

shown to have a lower risk of cardiovascular disease than the general age- and time-matched female population [13], possibly due to common etiological factors for breast cancer and reduced cardiovascular disease, respectively. Further, patients were only accepted for breast reconstructive surgery if they stopped smoking and were not overweight, so the group was rather homogeneous for low cardiovascular risk factors. This is reflected in the low IMR values for the unirradiated control vessels in the breast cancer patient group (compared to the H&N group). Even so, we still observe an effect of radiation in the irradiated internal mammary arteries, with a trend to an increase in IMR and a significant increase in proteoglycan deposition, thus strongly suggesting that radiation is an independent causal factor. In contrast, in the H&N patient group there was a greater statistical variance in the IMR data, suggesting that there are additional factors that also contribute to the degree of vessel pathology in these patients. More patients in the H&N group had documented pre-existing cardiovascular disease, and increased level of risk factors (e.g. smoking, male gender, as listed in Table 1). In some of the unirradiated patients there was also thickening of the IMR, as in for example the control vessels in Fig. 1, suggesting that in this group radiation is not the only cause of intimal thickening, but rather that it augments pre-existing pathology. The large variance in IMR values in the control vessels from the H&N group might explain why the difference in IMR between irradiated and control patients was no longer significant when the IMR ratio was calculated. Of relevance is the different disease status between the two patient groups. Active disease was the indication for surgery for all the H&N cancer patients, whereas the breast cancer patients were all in remission at the time of surgery.

One of the unresolved aspects of radiation-induced vascular damage is the long-time interval between irradiation and the occurrence of clinical vascular events, such as ischemic stroke and myocardial infarction. The greatest effect on mortality is seen after 10 years and the incidence curve increases in steepness after 15 years and longer [3,5,6]. However, there are earlier sub-clinical changes which can be observed, such as the intima thickening of the carotid determined by ultrasound following neck irradiation after only 1–2 years follow-up [12]. In the present study, we were able to evaluate the histological changes after shorter (up to 2 years) and longer follow-up periods. A clinical trial is currently underway for patients who receive neck irradiation. Patients are randomised to receive atorvastatin or not during radiotherapy and for 2 years thereafter. In this study, the changes in IMT of the irradiated carotid are measured with ultrasound prospectively with time after radiotherapy.

In conclusion, this study shows that changes consistent with the early stages of the development of atherosclerosis occur within a few years after irradiation of medium-sized arteries. Increases in intima thickness, proteoglycan deposition, and inflammatory cell infiltrate were observed in vessels normally resistant to atherosclerosis; to our knowledge, this is the first report of such changes. In the H&N patient group, which has a high prevalence of known risk factors for atherosclerosis, we observed an augmenting effect of radiotherapy on intimal thickening and inflammatory cell content. In the breast cancer patients, the changes in the media (proteoglycan deposition) seem to be related to the radiation exposure alone, as no other risk factors are present. Further studies are needed to investigate more detailed aspects of radiation-induced vascular pathology compared to those of age-related atherosclerosis. For example, certain subtypes of proteoglycan and collagen may be differentially increased or decreased following radiation. Characterisation of the radiation-induced pathways involved such as cytokine activation (including TGF- β , I-CAM-1, thrombomodulin, etc.) may help to develop a targeted secondary intervention strategy.

The long-term vascular effects of radiotherapy can have a significant impact on the morbidity and mortality of cancer patients and cancer survivors. Clinicians of all disciplines with previously irradiated patients in their care need to be aware of these long-term vascular consequences. With this study we have aimed to gain some insight of the pathophysiology, but further basic and clinical research is needed to improve long-term patient outcome.

Acknowledgements

We thank Dr. Michiel van den Brekel for additional arterial biopsies, and Mrs. Diedie van Dinten for administrative support.

This work was supported by the Dutch Cancer Society (NKB/KWF) Grant No. NKI-2005 3373.

References

- [1] Rowland JH, Hewitt M, Ganz PA. Cancer survivorship: a new challenge in delivering quality cancer care. *J Clin Oncol* 2006;24:5101–4.
- [2] Oeffinger KC, McCabe MS. Models for delivering survivorship care. *J Clin Oncol* 2006;24:5117–24.
- [3] Favourable and unfavourable effects on long-term survival of radiotherapy for early breast cancer: an overview of the randomised trials. *Early Breast Cancer Trialists' Collaborative Group. Lancet* 2000;355:1757–70.
- [4] Adams MJ, Lipsitz SR, Colan SD, Tarbell NJ, Treves ST, Diller L, et al. Cardiovascular status in long-term survivors of Hodgkin's disease treated with chest radiotherapy. *J Clin Oncol* 2004;22:3139–48.
- [5] Aleman BM, van den Belt-Dusebout AW, Klokman WJ, Van't Veer MB, Bartelink H, van Leeuwen FE. Long-term cause-specific mortality of patients treated for Hodgkin's disease. *J Clin Oncol* 2003;21:3431–9.
- [6] Aleman BM, van den Belt-Dusebout AW, De Bruin ML, 't Veer MB, Baaijens MH, de Boer JP, et al. Late cardiotoxicity after treatment for Hodgkin's lymphoma. *Blood* 2007;109:1878–86.
- [7] Bowers DC, McNeil DE, Liu Y, Yasui Y, Stovall M, Gurney JG, et al. Stroke as a late treatment effect of Hodgkin's Disease: a report from the Childhood Cancer Survivor Study. *J Clin Oncol* 2005;23:6508–15.
- [8] Bowers DC, Liu Y, Leisenring W, McNeil E, Stovall M, Gurney JG, et al. Late-occurring stroke among long-term survivors of childhood leukemia and brain tumors: a report from the Childhood Cancer Survivor Study. *J Clin Oncol* 2006;24:5277–82.
- [9] Clarke M, Collins R, Darby S, Davies C, Elphinstone P, Evans E, et al. Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005;366:2087–106.
- [10] Darby S, McGale P, Peto R, Granath F, Hall P, Ekborn A. Mortality from cardiovascular disease more than 10 years after radiotherapy for breast cancer: nationwide cohort study of 90,000 Swedish women. *BMJ* 2003;326:256–7.
- [11] Dorresteijn LD, Kappelle AC, Boogerd W, Klokman WJ, Balm AJ, Keus RB, et al. Increased risk of ischemic stroke after radiotherapy on the neck in patients younger than 60 years. *J Clin Oncol* 2002;20:282–8.
- [12] Dorresteijn LD, Kappelle AC, Scholz NM, Munneke M, Scholma JT, Balm AJ, et al. Increased carotid wall thickening after radiotherapy on the neck. *Eur J Cancer* 2005;41:1026–30.
- [13] Hoening MJ, Aleman BM, van Rosmalen AJ, Kuenen MA, Klijn JG, van Leeuwen FE. Cause-specific mortality in long-term survivors of breast cancer: a 25-year follow-up study. *Int J Radiat Oncol Biol Phys* 2006;64:1081–91.
- [14] Hoening MJ, Botma A, Aleman BM, Baaijens MH, Bartelink H, Klijn JG, et al. Long-term risk of cardiovascular disease in 10-year survivors of breast cancer. *J Natl Cancer Inst* 2007;99:365–75.
- [15] Jaggi R, Griffith KA, Koelling T, Roberts R, Pierce LJ. Stroke rates and risk factors in patients treated with radiation therapy for early-stage breast cancer. *J Clin Oncol* 2006;24:2779–85.
- [16] Jaggi R, Griffith KA, Koelling T, Roberts R, Pierce LJ. Rates of myocardial infarction and coronary artery disease and risk factors in patients treated with radiation therapy for early-stage breast cancer. *Cancer* 2007;109:650–7.
- [17] Swerdlow AJ, Higgins CD, Smith P, Cunningham D, Hancock BW, Horwich A, et al. Myocardial infarction mortality risk after treatment for Hodgkin disease: a collaborative British cohort study. *J Natl Cancer Inst* 2007;99:206–14.
- [18] de Bree R, Quak JJ, Kummer JA, Simsek S, Leemans CR. Severe atherosclerosis of the radial artery in a free radial forearm flap precluding its use. *Oral Oncol* 2004;40:99–102.
- [19] Gill PS, Hunt JP, Guerra AB, Dellacroce FJ, Sullivan SK, Boraski J, et al. A 10-year retrospective review of 758 DIEP flaps for breast reconstruction. *Plast Reconstr Surg* 2004;113:1153–60.
- [20] Atkinson JL, Sundt Jr TM, Dale AJ, Cascino TL, Nichols DA. Radiation-associated atheromatous disease of the cervical carotid artery: report of seven cases and review of the literature. *Neurosurgery* 1989;24:171–8.
- [21] Fonkalsrud EW, Sanchez M, Zerubavel R, Mahoney A. Serial changes in arterial structure following radiation therapy. *Surg Gynecol Obstet* 1977;145:395–400.
- [22] Virmani R, Farb A, Carter AJ, Jones RM. Pathology of radiation-induced coronary artery disease in human and pig. *Cardiovasc Radiat Med* 1999;1:98–101.
- [23] O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL, Wolfson Jr SK. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. *Cardiovascular Health Study Collaborative Research Group. N Engl J Med* 1999;340:14–22.
- [24] Bots ML, Hoes AW, Koudstaal PJ, Hofman A, Grobbee DE. Common carotid intima-media thickness and risk of stroke and myocardial infarction: the Rotterdam Study. *Circulation* 1997;96:1432–7.
- [25] Cheng SW, Ting AC, Ho P, Wu LL. Accelerated progression of carotid stenosis in patients with previous external neck irradiation. *J Vasc Med Biol* 2004;16:409–15.
- [26] Muzaffar K, Collins SL, Labropoulos N, Baker WH. A prospective study of the effects of irradiation on the carotid artery. *Laryngoscope* 2000;110:1811–4.
- [27] Stewart FA, Heeneman S, Te Poele J, Kruse J, Russell NS, Gijbels M, et al. Ionizing radiation accelerates the development of atherosclerotic lesions in ApoE^{-/-} mice and predisposes to an inflammatory plaque phenotype prone to hemorrhage. *Am J Pathol* 2006;168:649–58.
- [28] Hoving S, Heeneman S, Gijbels MJ, Te Poele JA, Russell NS, Daemen MJ, et al. Single-dose and fractionated irradiation promote initiation and progression of atherosclerosis and induce an inflammatory plaque phenotype in ApoE^{-/-} mice. *Int J Radiat Oncol Biol Phys* 2008;71:848–57.
- [29] Boren J, Olin K, Lee I, Chait A, Wight TN, Innerarity TL, et al. Identification of the principal proteoglycan-binding site in LDL. A single-point mutation in apoB100 severely affects proteoglycan interaction without affecting LDL receptor binding. *J Clin Invest* 1998;101:2658–64.
- [30] Khalil MF, Wagner WD, Goldberg IJ. Molecular interactions leading to lipoprotein retention and the initiation of atherosclerosis. *Arterioscler Thromb Vasc Biol* 2004;24:2211–8.
- [31] Talusan P, Bedri S, Yang S, Kattapuram T, Silva N, Roughley PJ, et al. Analysis of intimal proteoglycans in atherosclerosis-prone and atherosclerosis-resistant human arteries by mass spectrometry. *Mol Cell Proteomics* 2005;4:1350–7.