

INVESTIGATIVE REPORT

Radiation Therapy Induces Tenascin Expression and Angiogenesis in Human Skin

RIITTA RIEKKI¹, ARJA JUKKOLA², AARNE OIKARINEN¹ and MATTI KALLIOINEN³

Departments of ¹Dermatology and ²Oncology, University of Oulu, and ³Department of Pathology, Oulu University Hospital, Oulu, Finland

In analysing radiation-induced connective tissue changes, we studied tenascin expression, elastic fibres, angiogenesis and physio-mechanical properties in irradiated and contralateral healthy skin of radiotherapy-treated breast cancer patients. Skin biopsies were obtained from a radiotherapy-treated skin area and a corresponding non-treated skin area. Haematoxylin-eosin and Verhoeff stainings as well as immunohistochemical stainings for tenascin and factor VIII were performed. Epidermal and total skin thickness, together with the amount of elastic tissue calculated by computerized digital image analysis, were measured. Suction blisters were induced on both skin areas. Transepidermal water loss was analysed. Skin elasticity was also measured. Tenascin expression was found to be increased in irradiated human skin. In haematoxylin-eosin and factor VIII-stained sections, an increase in the number of blood vessels was detected. Although skin stiffness measured by an elastometer was increased in irradiated skin, no marked difference in the elastic fibres could be found between treated and non-treated skin. The increased tenascin expression could be due to activation of cytokines as a result of irradiation. An increase in angiogenesis could be caused by an activation of angiogenetic factors by irradiation or due to direct radiation damage on blood vessel walls. Our findings suggest that the effects of irradiation tend to accumulate in the dermal parts of skin. The higher skin stiffness values measured by elastometer in irradiated skin could be due to an accumulation of dermal connective tissue as a result of irradiation. Key words: angiogenesis; human skin; irradiation; tenascin.

(Accepted August 17, 2001.)

Acta Derm Venereol 2001; 81: 329–333.

Riitta Riekkö, Department of Dermatology, University of Oulu, Kajaanintie 50, FIN-90220 Oulu, Finland.
E-mail: riitta.riekki@oulu.fi

Radiation therapy used to treat cancer also produces damage to adjacent tissues. In skin, the effects of irradiation on connective tissue can be readily investigated.

In earlier studies, radiation-induced clinical and histopathological changes in skin have been described. These include fibrosis, blood vessel damage and telangiectasia (1, 2). Recently, the molecular events underlying radiation-induced fibrogenesis have been partially characterized. As a mechanism, the activation of growth factors, such as TGF- β , has been suggested (3). Fibrogenesis has been suggested to be analogous to the events of normal tissue repair (4). However, the processes leading to fibrosis remain to be solved in detail. Information on the molecular mechanisms of skin connective tissue fibrosis induced by radiotherapy would be necessary for finding means to prevent or treat such conditions.

Tenascin is a large extracellular matrix glycoprotein. In fetal tissues it has been associated with morphogenesis. Its expression in most adult tissues is restricted or absent (5–7). In skin, tenascin is associated with wound healing, melanocytic tumors and premalignant and malignant lesions (8–11). In a previous study, tenascin expression was found to be increased in pig cutaneous tissue after high-dose gamma radiation (12). However, tenascin expression in X-irradiated human skin has not been previously studied.

The recoil properties of skin are largely due to elastin, although the amount of this protein in dermal connective tissue is low compared to that of collagen. A modification of collagen synthesis as a result of irradiation has previously been established in both animal models and human skin (13–15). Yet, elastic fibres in X-irradiated human skin have not been previously analysed.

In the present study, we analysed for the first time the distribution of tenascin expression in X-irradiated human skin and the effect of irradiation on dermal elastic fibres and angiogenesis. The histological findings and physio-mechanical properties of radiation-damaged skin were compared to those of non-treated skin.

MATERIALS AND METHODS

Subjects

Twenty women were randomly chosen who had been treated for breast cancer with mastectomy or breast-conserving surgery and radiation therapy (Table I). The irradiated skin area was compared with the contralateral non-treated skin area, and the subjects thus acted as their own controls. Patients with a condition or medication affecting collagen synthesis were omitted from the study. Four of the subjects had received adjuvant therapy. Three had been treated with chemotherapy and one with hormone therapy.

Written informed consent was obtained from all the subjects and the study was approved by the Ethics Committee of the Faculty of Medicine and by Oulu University Hospital.

Histology and immunohistochemistry

A 4-mm biopsy was obtained under local anaesthesia from the irradiated skin area (at least 2 cm from the operation scar) and the contralateral non-treated skin area. Skin samples of 18 subjects were available for analysis. The specimens were fixed in 10% buffered formalin, embedded in paraffin and cut into 4- μ m sections. Haematoxylin-eosin (HE) and Verhoeff stainings as well as immunohistochemical stainings for tenascin and endothelial cells were performed.

Monoclonal antitenascin 143DB7C8 (Locus, Helsinki, Finland) and polyclonal anti-human von Willebrand factor (factor VIII, FVIII) (Dako A/S, Denmark) were used. The sections for immunohistochemistry were pretreated with pepsin digestion for factor VIII. No pretreatment was given for antitenascin. The immunohistochemical stainings were performed using an ABC/HRP kit (Dako A/S,

Table I. Clinical data of the 20 subjects

Age (years)	Time from treatment (months)	Clinical staging (TNM)*					Therapy		Energy (MeV)
		T1N0M0	T1N1M0	T2N0M0	T2N1M0	T1NXM0	A R Dose (Gy)		
42–75 (mean 55)	10–96 (mean 26)	9	2	5	2	1	5 15 30–56 (mean 52)	6–10x (mean 7) 5–12e (mean 9)	

TNM-staging – T: tumour size; N: involvement of the regional lymph nodes; M: metastasis.

A: breast ablation; R: breast resection; x: photon energy; e: electron energy.

*Data on TNM-grading of one of the patients not available.

Glostrup, Denmark) in accordance with the manufacturer's instructions. For negative controls, the antibody was substituted with phosphate-buffered saline (PBS) solution.

The amount of immunohistochemical staining for tenascin in the sections was graded visually under a light microscope (Olympus BH-2, Japan) and given a score from 1 to 3. The scoring was used for statistical analysis.

Skin blood vessels in the FVIII-stained sections were counted under a light microscope (Olympus BH-2, Japan). A 20 × objective was used. The number of blood vessels was counted from both the upper and the lower dermis of the section. The acquired sum of blood vessels was divided by the number of fields counted from each part of a section, and the mean was used for calculations.

Blinded procedures were used in examining the sections.

Suction blisters and physical parameters

Suction blisters were induced in the irradiated skin area and the contralateral healthy skin area using a disposable suction blister device Dermovac® (Ventipress Oy, Lappeenranta, Finland) in accordance with the method of Kiistala (16). Procollagen propeptides were determined from suction blister fluid as described previously (15). Also skin thickness was measured using a 20 MHz ultrasound scanner.

Water evaporation of both examined skin areas of a subject was measured as basal transepidermal water loss (TEWL) using an evaporimeter (EPI, Servomed, Stockholm, Sweden) prior to the induction of suction blisters.

Skin elasticity was measured with a DermaLab® unit (Cortex Technology, Hadsund, Denmark) in accordance with the manufacturer's instructions (17, 18). This device measures the stiffening of the skin by registering the power of vacuum needed to pull the skin for a certain distance. Three measurements were made from both irradiated and non-treated skin, and the mean was used for calculations.

Computerized digital image analysis

Verhoeff staining was performed to visualize elastic fibres. The proportional area of elastic fibres was measured using computerized morphometric analysis. A Nikon EFD 3 (Japan) light microscope with a 40 × E Plan objective was used. The video signal was analysed with a MCID-M4 image analysis system (Imaging Research Inc.). Six fields were analysed from each section, three from the upper (papillary) dermis and three from the lower (reticular) dermis. The proportional area of elastic fibres in the scan area was calculated. The thickness of epidermis was measured from 10 separate sites of each section.

Statistics

Statistical evaluation was performed using the paired samples *t*-test. For tenascin immunostaining, a non-parametric Wilcoxon signed ranks test was employed. SPSS for windows was used for the calculations.

RESULTS

Histology and immunohistochemistry

Collagen bundles and elastin fibres visualized by HE or Verhoeff stainings did not show any marked difference between

irradiated and non-irradiated skin samples. Epidermal thickness was similar in both radiotherapy-treated and healthy skin samples (Table II).

In non-treated skin, the immunohistochemical reaction for antitenascin antibody was expressed primarily as a narrow band in the papillary dermis beneath the epidermis, in the blood vessel walls and adjacent to the cutaneous appendices. Expression of tenascin was not seen in epidermis, lower dermis or subcutaneous fat (Fig. 1). In irradiated skin, the staining for tenascin beneath the epidermis was more intense, thicker and continuous. Immunohistochemical staining showed a marked increase in tenascin expression in irradiated skin ($p = 0.026$).

In irradiated skin samples, an increase in the number of blood vessels in comparison with healthy skin was found (Fig. 2). The mean number of blood vessels was 19.2 in the upper dermis of irradiated skin and 14.7 in the upper dermis of non-treated skin. In the lower dermis, the corresponding figures were 8.2 in irradiated skin and 6.4 in non-treated skin (Fig. 3). In the upper dermis of irradiated skin, the number of blood vessels was markedly increased in FVIII-stained sections compared with non-treated skin ($p = 0.010$). In the lower dermis of irradiated skin, the number of blood vessels was also increased in irradiated skin samples, although the difference was not statistically significant ($p = 0.223$). In both HE and FVIII-stained sections, there appeared to be a greater number of large blood vessels in irradiated skin compared to non-irradiated skin.

Computerized digital image analysis

The mean thickness of the epidermis was 45.9 µm in irradiated skin and 43.1 µm in non-treated skin ($p = 0.355$). In the upper (papillary) dermis, the proportional area of elastic fibres was 3.3% in irradiated skin and 3.1% in non-treated skin ($p = 0.805$). In the lower (reticular) dermis, the proportional area of elastic fibres was 5.6% in irradiated skin and 5.9% in non-treated skin ($p = 0.617$) (Table II). There was no marked difference in the morphology of elastic fibres between irradiated and non-irradiated skin.

Physical parameters

The mean basal TEWL was 7 g/m² h in both treated and non-treated skin. The skin stiffness value was higher in the irradiated skin area (mean 15.1 MPa) than in the corresponding healthy skin area (mean 13.4 MPa) ($p = 0.026$) (Table II). There was also an increase of skin thickness in irradiated skin ($p = 0.004$) (Table II).

The clinical skin reactions among the subjects included

Table II. Mean epidermal thickness, skin thickness, proportional area of elastic fibres in skin biopsies and skin elasticity of irradiated skin area and contralateral non-treated skin area of the 20 patients (SD)

Epidermis (μm)		Skin thickness (mm)		Elastic fibres ¹ (%)				Elasticity (Mpa)	
I	H	I	H	I	I	H	H	I	H
				<i>pd</i>	<i>rd</i>	<i>pd</i>	<i>rd</i>		
45.9*	43.1	1.88*	1.63	3.3	5.6	3.1	5.9	15.1	13.4
(13.1)	(6.7)	(0.35)	(0.21)	(0.02)	(0.02)	(0.01)	(0.02)	(5.6)	(4.7)

I: irradiated skin area; H: corresponding healthy skin area; Pd: papillary dermis; Rd: reticular dermis.

¹Only 18 patients were examined with respect to elastic fibres and epidermal thickness.

* $p = 0.004$ for difference versus healthy skin.

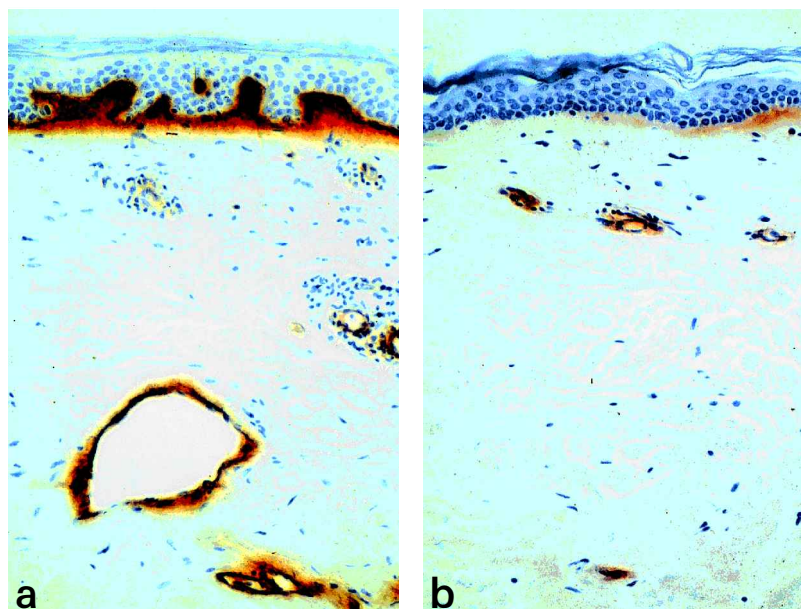


Fig. 1. Immunohistochemical staining for tenascin. Tenascin expression is increased in irradiated skin (A) compared to non-treated skin (B).

induration, telangiectasia and pigmentation of the irradiation-treated skin area.

DISCUSSION

In this study, tenascin expression was studied immunohistochemically in irradiated and normal human skin. For the first time, an increase of tenascin expression in X-irradiated human skin was demonstrated.

The role of tenascin *in vivo* is as yet not clear. Although the expression of tenascin in normal adult human skin is generally low (7), tenascin expression has previously been found to be increased under certain pathological conditions, such as scleroderma (19), hyperproliferative skin diseases (20), tumours (11) and wound healing (8).

The increased tenascin expression in irradiated skin could be due to the activation of cytokines as a result of irradiation. Transforming growth factor β (TGF- β) is a cytokine that has been shown to increase deposition of tenascin by fibroblasts (21). In several animal models, ionizing radiation has been shown to induce TGF- β , which has been implicated as a central cytokine in both tissue repair and fibrotic conditions (13, 22, 23).

Especially at early stages of wound healing, tenascin accumulates into the upper dermis. The fact that tenascin deposition was observed in irradiated skin even several years after treatment confirms the hypothesis that irradiation-induced damage is analogous to long-lasting wound healing.

As a side effect of irradiation, telangiectasiae are found frequently (1), and in chronic radiodermatitis blood vessel damage has been described (24). In the present study, the number of blood vessels was increased in both the upper and lower dermis of irradiated skin in comparison with non-treated skin. Also, there appeared to be more large-calibre blood vessels in the dermis of irradiated skin. The present findings could be due to the activation of angiogenic factors, such as TGF- β , as a result of irradiation or due to the direct damage of vascular walls by irradiation.

There was no difference in the proportional area or morphological appearance of elastic fibres between X-irradiated and non-treated skin. The reason for the higher values measured by elastometer in treated skin could be an accumulation of connective tissue in irradiated skin, as demonstrated previously (15). The increased amount of collagen in irradiated skin could be due to the activation of growth factors, such as TGF- β (3, 4). Also, we have previously found skin type I collagen cross-linking

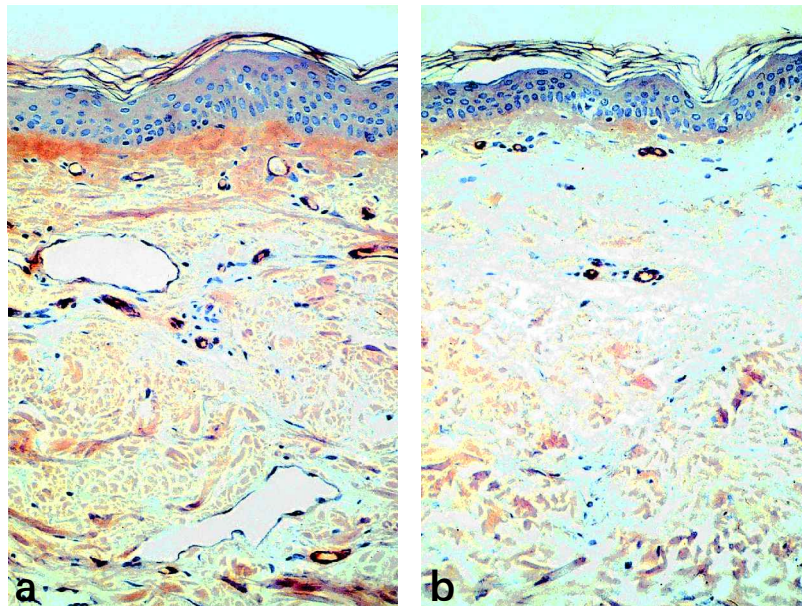


Fig. 2. Immunohistochemical staining for blood vessels (F VIII). The number and diameter of blood vessels is increased in irradiated skin (A) compared to non-treated skin (B).

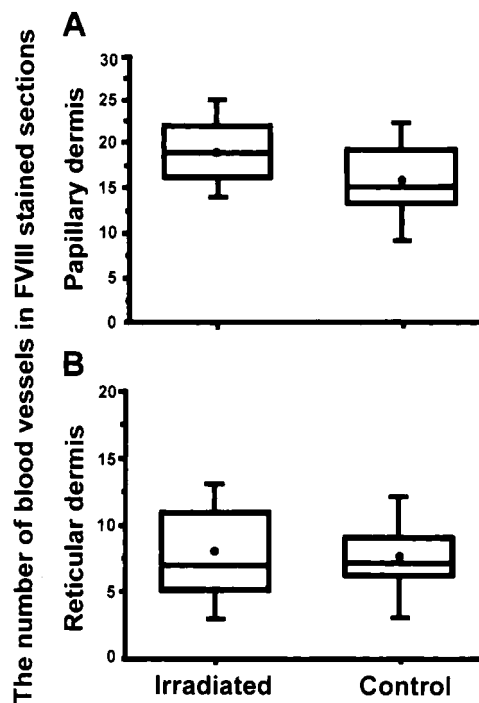


Fig. 3. Number of blood vessels in FVIII-stained sections in irradiated skin and non-treated control skin. Papillary dermis (A) and reticular dermis (B). The bottom of the box marks the 25th centile and the top of the box the 75th centile (the limits within which 50% of the values fall). The dots in the box marks the mean and the horizontal line the median. The vertical lines represent the ranges.

to be increased as a result of radiotherapy in these same patients (25). A modulation of collagen cross-linking could also contribute to the tensile properties of skin in these subjects.

The integrity of epidermis measured by TEWL was not changed as a result of X-irradiation. Measurement of epi-

dermal thickness by computerized image analysis showed no marked difference between irradiated and non-irradiated skin. Both epidermal thickness and epidermal function also seemed to be unaffected by radiation therapy. However, in our previous work, both skin thickness and skin collagen synthesis were found to be increased as a result of radiotherapy. The data have been published in a separate article (15). These findings suggest that the effect of X-irradiation is more visible in the dermal than in the epidermal parts of skin.

In conclusion, these findings indicate increased tenascin expression in irradiated skin similar to that seen in wound healing. Slightly enhanced angiogenesis in irradiated skin was also detected. Epidermis and dermal elastic fibres were found to be unaffected by irradiation, but an increase in the skin stiffness values measured by an elastometer was detected, implicating modification of dermal connective tissue components other than elastin as a result of irradiation.

ACKNOWLEDGEMENTS

This study was supported by a research grant from Oulu University Hospital. We acknowledge the expert technical assistance of Ms Riitta Vuento and Ms Soili Manninen.

REFERENCES

1. Bentzen MS, Overgaard M. Clinical radiobiology and normal-tissue morbidity after breast cancer treatment. *Adv Radiat Biol* 1994; 18: 25–51.
2. Elder D, Elenitsas R, Johnson B, Jaworsky C. Disorders associated with physical agents. In: Elder D, Elenitsas R, Jaworsky C, Johnson B, eds. *Lever's histopathology of the skin*. Philadelphia: Lippincott-Raven, 1997: 311–316.
3. Canney PA, Dean S. Transforming growth factor beta: a promotor of late connective tissue injury following radiotherapy? *Br J Radiol* 1990; 63: 620–623.
4. Border W, Noble N. Mechanisms of disease: transforming growth factor (beta) in tissue fibrosis. *N Engl J Med* 1994; 331: 1286–1292.

5. Chiquet M, Fambrough DM. Chick myotendinous antigen I. A monoclonal antibody as a marker for tendon and muscle morphogenesis. *J Cell Biol* 1984; 98: 1926–1936.
6. Chiquet-Ehrismann R, Mackie EJ, Pearson CA, Sakakura T. Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. *Cell* 1986; 47: 131–139.
7. Lightner VA, Gumkowski F, Bigner DD, Erickson HP. Tenascin/Hexabrachion in human skin: biochemical identification and localization by light and electron microscopy. *J Cell Biol* 1989; 108: 2483–2493.
8. Latijnhouwers M, Bergers M, Van Bergen BH, Spruijt KI, Andriessen MP, Schalkwijk J. Tenascin expression during wound healing in human skin. *J Pathol* 1996; 178: 30–35.
9. Tuominen H, Pöllänen R, Kallioinen M. Multicellular origin of tenascin in skin tumors – an *in situ* hybridization study. *J Cutan Pathol* 1997; 24: 590–596.
10. Stamp GWH. Tenascin distribution in basal cell carcinomas. *J Pathol* 1989; 159: 225–229.
11. Koukoulis GK, Gould VE, Bhattacharyya A, Gould JE, Howedy AA, Virtanen I. Tenascin in normal, reactive, hyperplastic and neoplastic tissues: biologic and pathologic implications. *Hum Pathol* 1991; 22: 636–643.
12. Geffrotin C, Tricaud Y, Crechet F, Castelli M, Lefaix J-L, Vaiman M. Unlike tenascin-x, tenascin-c is highly up-regulated in pig cutaneous and underlying muscle tissue developing fibrosis after necrosis induced by very high-dose gamma radiation. *Radiat Res* 1998; 147: 472–481.
13. Barcellos-Hoff MH. Radiation-induced transforming growth factor β and subsequent extracellular matrix reorganization in murine mammary gland. *Cancer Res* 1993; 53: 3880–3886.
14. Autio P, Saarto T, Tenhunen M, Elomaa I, Risteli J, Lahtinen T. Demonstration of increased collagen synthesis in irradiated human skin *in vivo*. *Br J Cancer* 1998; 77: 2331–2335.
15. Riekkilä R, Jukkola A, Sassi M-L, Höyhtyä M, Kallioinen M, Risteli J, et al. Modulation of skin collagen metabolism by irradiation: collagen synthesis is increased in irradiated human skin. *Br J Dermatol* 2000; 142: 874–880.
16. Kiistala U. Suction blister device for separation of viable epidermis from dermis. *J Invest Dermatol* 1968; 50: 129–137.
17. Barel AO, Lambrecht R, Clarys P. Mechanical function of the skin: state of the art. In: Elsner P, Barel AO, Berardesca E, Gabard B, Serup J, eds. *Skin bioengineering*. Basel (Switzerland): S. Karger AG, 1998: 69–83.
18. Gniadecka M, Serup J. Suction chamber method for measurement of skin mechanical properties: the Dermaflex®. In: Serup J, Jemec GBE, eds. *Handbook of non-invasive methods and the skin*. Boca Raton: CRC Press, Inc. 1995: 329–334.
19. Lacour JP, Vitetta A, Chiquet-Ehrismann R, Pisani A, Ortonne JP. Increased expression of tenascin in the dermis in scleroderma. *Br J Dermatol* 1992; 127: 328–334.
20. Schalkwijk J, Van Vlijmen I, Oosterling B, et al. Tenascin expression in hyperproliferative skin diseases. *Br J Dermatol* 1991; 124: 13–20.
21. Gentilhomme E, Neveux Y, Lebeau J, Desmouliere A, Bergier J, Schmitt E, et al. Modulation of a fibrotic process induced by transforming growth factor beta-1 in dermal equivalents. *Cell Biol Toxic* 1999; 15: 229–238.
22. Martin M, Lefaix J-L, Pinton P, Crechet F, Daburon F. Temporal modulation of TGF- β and β actin gene expression in pig skin and muscular fibrosis after ionizing radiation. *Radiat Res* 1993; 134: 63–70.
23. Martin, P. Wound healing – aiming for perfect skin regeneration. *Science* 1997; 276: 75–81.
24. Spittle MF. Radiotherapy and reactions to ionizing radiation. In: Champion RH, Burton JL, Burns DA, Breathnach SM, eds. *Rook/Wilkinson/Ebling: Textbook of dermatology*, 6th ed. London: Blackwell Science, 1998: 3565–3571.
25. Sassi M-L, Jukkola A, Riekkilä R, et al. Type I collagen turnover and cross-linking are increased in irradiated skin of breast cancer patients. *Radiother oncol* 2001; 58: 17–23.