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Type I collagen turnover and cross-linking are increased in irradiated skin of breast cancer patients

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Abstract

Background and purpose: The effects of radiation therapy on the turnover and structure of type I collagen were studied in irradiated and contralateral skin of 18 breast cancer patients without clinically evident fibrosis.

Materials and methods: The rates of on-going type I collagen synthesis and degradation were assessed by the aminoterminal propeptide of type I procollagen (PINP) and by two different assays (ICTP and SP4) for the carboxyterminal telopeptide of type I collagen in the soluble tissue extracts, respectively. Also, TIMP-1, TIMP-2 and the MMP-2/TIMP-2 complex were measured in the tissue extracts. Insoluble skin matrices, containing the cross-linked type I collagen fibres, were heat-denatured and digested with trypsin. Then, the variants of the carboxyterminal telopeptide of type I collagen were separated by high performance liquid chromatography (HPLC). The major histidinohydroxylysinonorleucine (HHL)-cross-linked variant was quantified by the SP4 assay, and the minor pyridinoline analogue (PA)-cross-linked telopeptide was quantified by the ICTP assay.

Results: Both the synthesis and degradation of type I collagen were increased ($r = 0.906$; $P < 0.001$) on the irradiated side, whereas the concentration of the MMP-2/TIMP-2 complex was decreased. In the insoluble tissue digests, the HHL-cross-linked telopeptides of type I collagen, also, when expressed/tissue hydroxyproline, were increased in the irradiated skin. TIMP-1, TIMP-2 or PA-cross-linked telopeptides of type I collagen showed no differences between the two sides.

Conclusions: Radiotherapy induces a long-term increase in the turnover of type I collagen and leads to the accumulation of cross-linked type I collagen in skin.

Keywords: Collagen telopeptide; Degradation; Fibrosis; Procollagen propeptide

1. Introduction

Radiotherapy after partial or radical mastectomy in breast cancer patients is known to reduce the local recurrence rates [7,25]. However, high-dose radiation has been shown to induce acute (e.g. inflammation or pigmentation change) or delayed and partly irreversible (e.g. telangiectasias and breast tenderness) effects on tissues [1,23]. The long-term effects are usually seen more than 6 months after irradiation. In those rare patients having underlying connective tissue disease, e.g. scleroderma [21] or mixed connective tissue disease [13], the treatment may result in considerable fibrosis and retraction of the breast towards the axilla. Other predictive factors for severe skin reactions include weight, breast size, lymphocele aspiration, smoking, age, skin cancer, tumour stage and radiation dose [17].

Type I collagen is the main extracellular matrix component in the skin in addition to type III collagen. In fibrotic conditions, the amount of type I collagen is believed to increase with profound effects on skin structure and function. It has been previously reported that in breast cancer patients, the markers for the synthesis of type I and III collagens were two-fold increased in the suction blister fluid samples from the irradiated skin, compared with those from the contralateral unradiated skin [2].

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases, which together are able to degrade essentially all the components of the extracellular matrix. MMPs function in a parallel and/or cascade-like fashion and their activity is inhibited by non-specific (e.g.

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α2-macroglobulin) and specific tissue inhibitors of metalloproteinases (TIMPs). In human skin, the synthesis of MMPs is induced in response to various cytokines and growth factors and they play a role in wound repair, bullous diseases, dermal fibrosis and tumour cell invasion [9].

The structure of type I collagen in skin differs from that in other locations [14]. The fibrils of type I collagen in skin have a specific cross-link called histidinohydroxylysinonorleucine (HHL) in the carboxyterminal telopeptide region [26]. In addition to HHL, another trivalent cross-link has been described in skin in the same molecular location, which we will here call the pyridinoline analogue (PA) [3,24].

In this study, we developed a simple method for separating these two trivalently cross-linked variants of the carboxyterminal telopeptides of type I collagen in skin biopsies. Their final quantification was based on two different immunoassays. We also assessed the markers of synthesis and degradation of type I collagen in the soluble tissue extracts. Both the synthesis and degradation of type I collagen were increased on the irradiated side, and there was also an accumulation of HHL-cross-linked type I collagen.

2. Materials and methods

2.1. Patients and skin biopsy samples

The patients for this study included 18 women treated by local radiotherapy after ablation (five cases) or resection (13 cases) for breast carcinoma at the Department of Oncology and Radiotherapy, Oulu University Hospital, during the years 1990–1997 (Table 1). From symmetrical sites of the irradiated and contralateral breasts of each patient, 4-mm punch biopsies of the skin were taken under local anaesthesia. The biopsies were immediately frozen and stored at −20°C until analyzed. At the same time, suction blister samples were taken for procollagen propeptide analyses, the results of which have been published separately [19]. The study was carried out in accordance with the provisions of the declaration of Helsinki after permission from the local ethical committee. Informed consent was obtained from each patient.

2.2. Assay procedures

The concentration of aminoterminal propeptide of type I procollagen (PINP [15]; Orion Diagnostica, Espoo, Finland) was analyzed directly in the soluble extracts using 100-μl samples. The concentration of cross-linked carboxyterminal telopeptide of type I collagen (ICTP [20]; Orion) was performed in the extracts and trypsin digests directly or after dilution with the assay buffer, if necessary. The HHL-cross-linked telopeptide variant was assayed essentially similarly to ICTP by an in-house method [22] using a synthetic peptide, SP4 (SAGFDFSFLPQPPQEKY; produced by Neosystem Laboratories, Strasbourg, France), derived from the carboxyterminal telopeptide region of type I collagen as a tracer and standard antigen. The antiserum (#239) used was produced in a rabbit against the divalently cross-linked carboxyterminal telopeptide antigen of human type I collagen. This antiserum also detects those cross-linked telopeptide structures that only contain the telopeptide of one α1 chain of type I collagen (Fig. 1, lower structure). The inter- and intra-assay variations for PINP and

Table 1
Patients and their treatment characteristics

Patient number	Time from RT to biopsy (months)	Age (years)	TNM	Systemic treatment	Operation	RT Total dose (Gy)	Energy[a]	Skin at the time of biopsy[b]
1	96	58	T2N1M0	Hormone	Ablation	30[c]	–	
2	53	54	T1N0M0		Resection	40 + 16[c]	10x/9e	
3	20	53	T1N0M0		Resection	40 + 16[c]	6x/12e	
4	57	50	T1N0M0	Cytostatic	Ablation	40	5e	T
5	24	69	T1N0M0		Resection	40 + 16[c]	6x/12e	Od, Os, T
6	13	51	T1N0M0		Resection	40 + 16[c]	6x/12e	
7	24	49	T1N1M0	Cytostatic	Resection	50	–	
8	37	60	T1NxM0		Resection	50[c]	10e	
9	24	43	T2N0M0		Resection	40 + 16[c]	6x/12e	
10	15	55	T1N0M0		Resection	50	6e	P
11	11	58	T1N0M0		Resection	40 + 16[c]	6x/12e	
12	36	52	T1N0M0		Resection	40 + 16[c]	6x/7e	
13	17	49	T2N0M0		Ablation	50	7e	P
14	19	42	T1N0M0		Resection	40 + 16[c]	6x/5e	
15	10	75	T2N0M0		Ablation	50	7e	P
16	19	47	T2N1M0	Cytostatic	Ablation	50	7e	P
17	12	44	T2N0M0		Resection	40 + 16[c]	6x/12e	Od, Os, P
18	17	60	T2N0M0		Resection	40 + 16[c]	10x/12e	

[a] x, Photon energy; e, electron energy; –, data not available.
[b] Od, oedema; Os, orange skin; P, pigmentation; T, teleangiectasia.
[c] Booster to the scar.

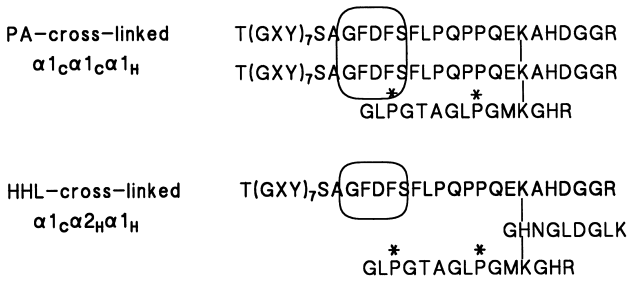

Fig. 1. Comparison of the amino acid sequences of the two differently cross-linked variants of the carboxyterminal telopeptide of type I collagen. The first T (threonine) on the left is after the cleavage site of trypsin. The asterisks above P's indicate hydroxyproline residues. The vertical lines joining the three K's (lysines or hydroxylysines) or two K's and one H (histidine) represent trivalent cross-links, which can be either a PA (upper structure) or HHL (lower structure), respectively. All the N-terminal sequences are based on our own work (at least ten steps) and the rest of the sequences on published data derived from the α1 or α2 chain of the human type I collagen. The immunological determinants detected by the ICTP assay (upper structure) and SP4 assay (lower structure) are approximately indicated by the ovoid boxes.

ICTP varied between 3.1–10.8 and 4.6–10.3%, and between 4.1–7.9 and 2.8–6.2%, respectively.

TIMP-1 and TIMP-2 were measured by enzyme-linked immunosorbent assays (ELISA) using polyclonal antibodies produced in chicken. A peroxidase labelled anti-chicken-IgG was used for detection, and the colour formation was measured at 450 nm. For detection of the MMP-2/TIMP-2 complex, a monoclonal antibody against TIMP-2 and a polyclonal antiserum against MMP-2 were used [16].

2.3. Extraction of the soluble antigens from the skin biopsies

The biopsies were weighed, minced with a scalpel and suspended in 1 ml of phosphate-buffered saline containing 0.04% (w/w) Tween 20 (PBS–Tween), keeping the samples on an ice bath all the time. The homogenized biopsies were centrifuged at 2000 × *g* for 30 min at +4°C (Beckman CS-6KR). The supernatants were separated and analyzed for the concentrations of PINP, SP4, ICTP, TIMP-1, TIMP-2 and the MMP-2/TIMP-2 complex as described above. The results were expressed as the amount of extracted antigen/mg of wet weight of the biopsy (Fig. 2).

2.4. Analysis of the HHL- and PA-cross-linked variants of the carboxyterminal telopeptide of type I collagen in the insoluble matrix

The insoluble tissue residue was lyophilized, suspended in 1 ml of 0.2 M $(NH_4)_2CO_3$, heat-denatured at +70°C for 30 min and digested with trypsin (100 μg; *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated; Worthington Biochemicals, Freehold, NJ) at 37°C. This denaturation and digestion cycle was repeated twice (digestion times being 6, 12 and 6 h). After the final digestion, the residual trypsin activity was destroyed by additional heating at +70°C for 30 min. The minor insoluble residue was removed by centrifugation (2000 × *g* for 30 min at +4°C) and the supernatants were stored at −20°C. We have previously purified the PA- and HHL-cross-linked variants of the carboxyterminal telopeptides of type I collagen and characterized them by slab gel electrophoresis and N-terminal sequence analysis (for methods used see [22]). For assessing the concentrations of these two telopeptide variants, 300-μl samples of the supernatants were separated on a pH stable C_8 high performance liquid chromatography (HPLC) column (Vydac, Hesperia, CA), and each fraction was then analyzed with the SP4 and

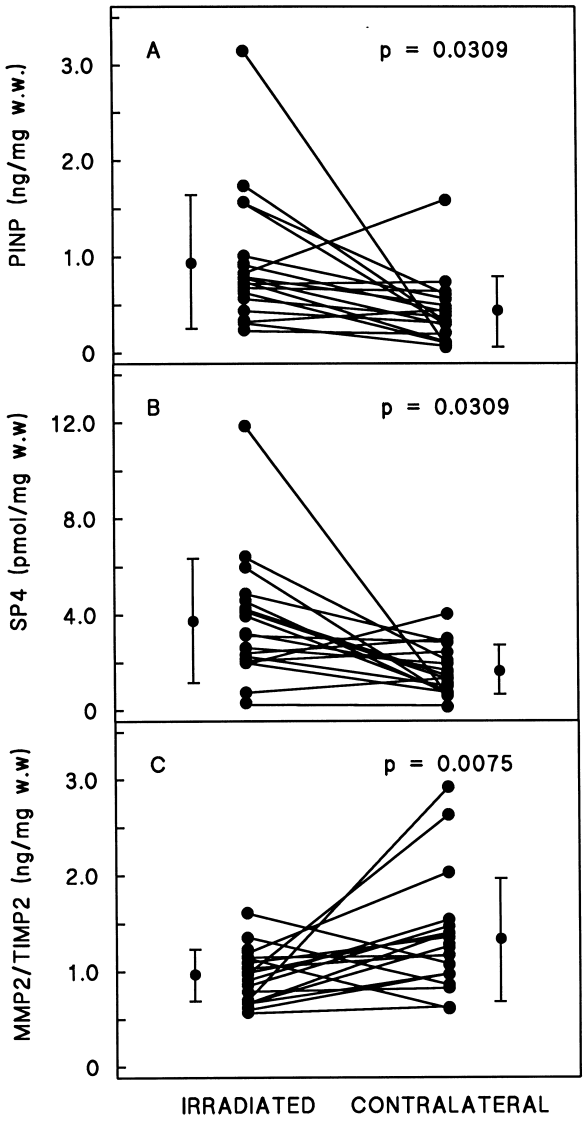

Fig. 2. Concentrations of: (A), PINP; (B), a linear peptide from the carboxyterminal telopeptide region of type I collagen, SP4; and (C), MMP-2/TIMP-2 complexes in the soluble tissue extracts of irradiated and contralateral skin biopsies. Each line joins the results obtained from one individual. The group means ± SDs are given on the left and right sides of the irradiated and contralateral values, respectively. The differences between treated and control sides were all statistically significant (sign test for paired samples). Patient number 5 had the highest concentrations of PINP and SP4, whereas it was patient number 16 who had the reversed findings in these two analytes, compared with the other patients.

ICTP assays (Fig. 3). The fractions containing the HHL- and PA-cross-linked telopeptides were pooled separately from each run, lyophilized and dissolved in 2 ml of PBS–Tween. Then, the concentrations of the two telopeptide variants were analyzed in the pools containing only one variant with the SP4 and ICTP assays, respectively. These results were expressed per the total amount of collagens in the insoluble matrix, which was calculated from the total content of hydroxyproline [10], assuming that it accounts for 12.4% (w/w) of the total amino acids of all the collagenous proteins.

2.5. Statistical analysis

The statistical differences between the concentrations of the analytes in the irradiated and contralateral skin biopsies were tested with the *t*-test for paired samples or the non-parametric sign test for paired samples using SPSS statistical software.

3. Results

3.1. Patients and comparison of the irradiated and contralateral skin biopsies

The mean age of the patients studied was 53.8 ± 8.6 (SD) years. One patient was studied after 8 years, and two after more than 4 years, but the median interval between the radiotherapy and the skin biopsies was 21.7 months (Table 1). The total radiation dose varied from 30 to 56 Gy. Seven of the patients still had skin reactions at the time of the biopsy (Table 1). There was no clear relationship between the skin reactions and the interval after radiotherapy.

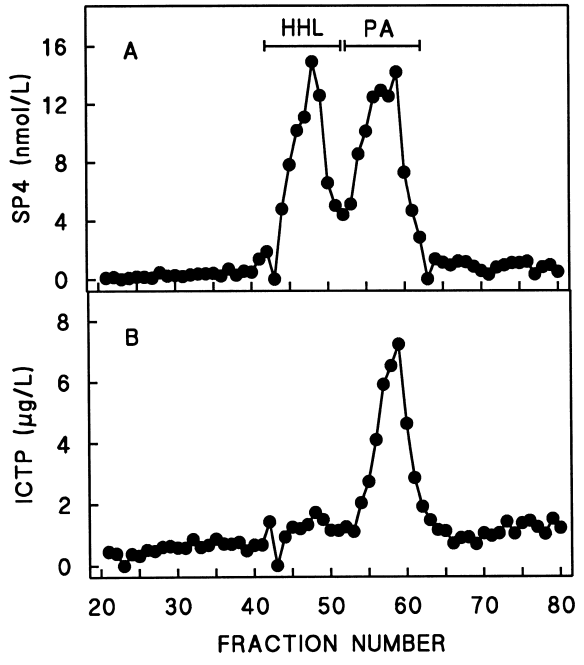

Fig. 3. C_8 reverse phase separation on HPLC of the two variants of the carboxyterminal telopeptide of type I collagen from the trypsin digest of the insoluble skin collagen matrix. The skin sample studied was obtained from breast reduction surgery, and thus contained more subcutis than the bunch biopsies used in the clinical part of this study. In: (A), the upper part, the fractions were measured by the SP4 assay; and (B), the lower part, the same fractions were analyzed by the ICTP assay. The HHL denotes cross-linked variant of the carboxyterminal telopeptide containing only one α1(I)-chain telopeptide, and PA, a variant containing two α1(I)-chain telopeptides. The bars indicate the fractions pooled and lyophilized for further analyses.

The wet weights of the punch biopsies from the irradiated sites were significantly larger than those from the contralateral sites (21.1 ± 6.4 and 16.7 ± 6.5 mg, respectively; $P = 0.031$). The hydroxyproline contents of the biopsies from the irradiated sides were also increased (277 ± 47 and 218 ± 82 µg, respectively; $P = 0.002$). However, there were no differences in the collagen concentrations/wet weight of tissue (14.1 ± 3.9 and 14.1 ± 5.4 µg hydroxyproline/mg wet weight, respectively).

3.2. Soluble metabolites of type I collagen and MMP inhibitors

The concentrations of the markers of both type I procollagen synthesis (PINP assay) and degradation (SP4 assay) were significantly increased in the irradiated sites (Fig. 2). There was no correlation between the concentration of either PINP ($r = 0.033$; ns) or SP4 ($r = -0.037$; ns) in the extract and the interval after the radiotherapy.

However, the correlation between PINP and SP4 concentration was highly significant ($r = 0.906$; $P < 0.001$). The highest concentrations were obtained with patient number 5, who still had several skin reactions 2 years after radiation (Fig. 2). If this patient was omitted from the statistical analyses, there was still a significant difference between the two groups. The patient showing reversed effects was patient number 16.

In contrast to the type I collagen metabolites, the concentrations of the MMP-2/TIMP-2 complexes were significantly lower in the irradiated skin biopsy extracts. No statistically significant differences were found between the irradiated and contralateral sides with respect to the soluble ICTP antigen (0.35 ± 0.18 and 0.24 ± 0.16 ng/mg, respectively), TIMP-1 (0.63 ± 0.20 and 0.81 ± 0.53 ng/mg) or TIMP-2 (0.78 ± 0.23 and 0.90 ± 0.33 ng/mg).

3.3. Insoluble type I collagen

Two differently cross-linked variants of the carboxyterminal telopeptides of type I collagen were separated from the trypsin digests of the insoluble extracellular matrix of the skin by reverse phase chromatography on HPLC (Fig. 3). The first peak contained the HHL-cross-linked telopeptide typical of skin and showed no reaction in the ICTP assay, whereas the other contained the ICTP-related antigen of skin. In fact, a concentration of the HHL-cross-linked telopeptide 72-fold higher than that of the other variant was needed to give a similar inhibition in the ICTP assay (Fig. 4). The skin samples used to characterize the methods came from breast reduction surgery and contained more subcuta-

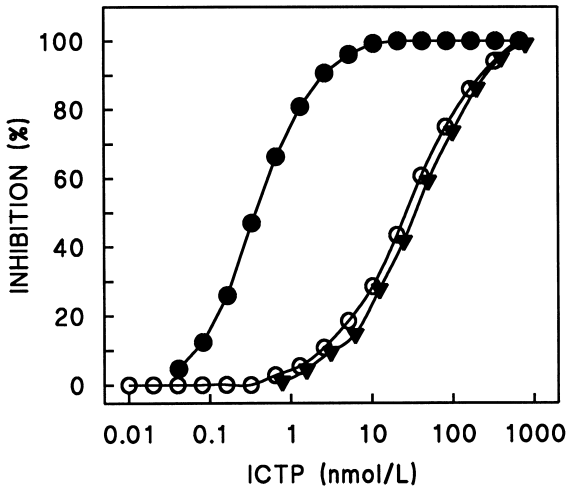

Fig. 4. (●) Cross-reactions of trypsin-generated, trivalent human ICTP; (○), trypsin-generated HHL-cross-linked telopeptide from human skin; and (▲), the synthetic peptide, SP4, in the human ICTP radio-immuno-inhibition assay. Computer analysis of the data gave the following slopes and 50% intercepts of the inhibition curves: human ICTP, 1.188 and 0.38 nmol/l; HHL-cross-linked telopeptide, 1.066 and 27.44 nmol/l; and SP4, 1.116 and 35.92 nmol/l. To obtain the same inhibition as given by trivalent ICTP, 72 times more of the HHL-cross-linked peptide and 95 times more SP4 are needed.

neous tissue than the bunch biopsies. This is a likely reason for the roughly equal amounts of variants, which differs from the situation in the biopsy samples (see below).

A combination of HPLC separation and the SP4 or ICTP assay was used to assess the cross-linking of type I collagen of the biopsies. The average concentration of the HHL-cross-linked variant of type I collagen was higher in the irradiated than in the treated side (323 ± 119 and 188 ± 117 mmol/mol total amount of collagens, respectively; Fig. 5A). A similar, although not statistically significant, difference was noted for the content of the ICTP antigen (0.075 ± 0.064 and 0.048 ± 0.029 mmol/mol, respectively; Fig. 5B). Thus, in the punch biopsies there was 4306 (irradiated side) or 3917 (contralateral side) times more of the HHL- than the PA-ICTP. Irradiation did not change this ratio, nor was there any effect of the interval after the radiation detected on the proportions.

4. Discussion

The main finding of the present study was the increased amount of cross-linked type I collagen on the irradiated side several years after irradiation therapy of breast cancer. However, in contrast to what has been reported on irreversible skin fibrosis [4,18], the type of main cross-link (HHL) was identical to that in normal skin, and thus, the microstructure of the skin collagen fibres with their specific tilt angles was obviously conserved [14]. If the nature of the cross-link had changed, this would also have affected the tissue microarchitecture of the collagen fibres. The minor cross-link (PA) in skin type I collagen was found to be related to the pyridinoline cross-linked ICTP antigen previously characterized from skeletal tissue [20,22]. Its concentration was not significantly increased in our samples.

The amount of HHL-cross-linked type I collagen was increased when expressed/total collagen, measured by hydroxyproline. This may have two explanations. Firstly, it is possible that the amount of HHL-cross-links is increased in type I collagen. However, the SP4 immunoassay would have also been able to detect divalently cross-linked and non-cross-linked variants of the carboxyterminal telopeptide. Another possibility is that the concentration of type I collagen with respect to total collagen is increased. Type I collagen is believed to account for about 70% and type III collagen for about 10–25% of the total collagens in human skin [11]. In this study, we have validated the analysis of the content of type I collagen in human skin. Similar work is underway for the estimation of the content of type III collagen in human skin, for which we have previously validated a procedure to be used in studies on atherosclerotic plagues [5]. In any case, the exact amounts of type I and

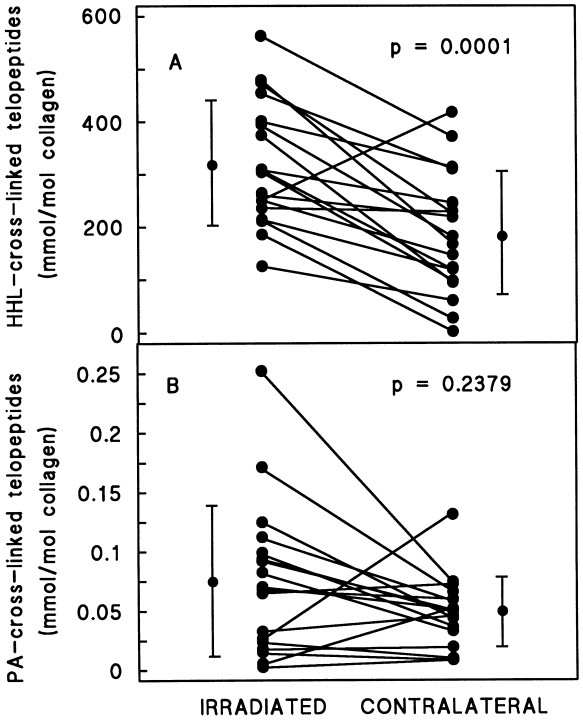

Fig. 5. Concentrations of: (A), HHL-; and (B), PA-cross-linked telopeptides of type I collagen in the insoluble matrices of the irradiated and contralateral skin biopsies. The values are expressed/total amount of collagens calculated from the content of hydroxyproline. Each line joins the results obtained from one individual. The group means ± SDs are given on the left and right sides of the irradiated and contralateral values, respectively. There was a statistically significant difference between the contents of the HHL-cross-linked telopeptides, but not between those of the PA-cross-linked telopeptides (sign test for paired samples). Note the large difference between the scales on the *Y*-axes.

III collagens have been difficult to estimate, and the contributions of the more recently described collagen types (at least types IV, V, VI and VII) are not known. Clearly, further studies are needed regarding the effects of radiation on the metabolism of non-fibrillar collagen types at the tissue level.

During normal ageing, the content of HHL-cross-linked type I collagen increases in skin [27]. Thus, the irradiation-induced change resembles the acceleration of the normal ageing process. Interestingly, the concentration of HHL-cross-linked type I collagen in our study is comparable with the contents reported from the skin of scleroderma patients [8]. In the latter study, the HHL-cross-links were directly analyzed by HPLC after labelling of the skin hydrolysates with 9-fluorenylmethyl chioroformate. The molar ratios were 333 ± 33 and 151 ± 42 mmol HHL/mol collagen in systemic scleroderma skin samples and in the controls, respectively, and those in our results were 323 ± 119 and 187 ± 117 mmol/mol collagen in the irradiated skin and on the contralateral side, respectively. Thus, the immunoassay measuring the telopeptide chains instead of the cross-links gives results comparable with traditional cross-link analyses. It also seems that most of the HHL-cross-links are located in the fibres of type I collagen.

In this study, we found that more than a year after radiation therapy of breast cancer, both the degradation and synthesis of type I collagen are still increased on the irradiated side. The increased synthesis of type I collagen seems to continue several years after radiotherapy [2]. Since there was simultaneously increased collagen degradation, which we found by studying the tissue extract, the increased synthesis does not easily lead to severe fibrosis. Thus, harmful fibrosis is rarely seen in breast cancer patients, unless they have an underlying connective tissue disease, e.g. scleroderma [21] or mixed connective tissue disease [13]. On the other hand, irradiation more usually leads to skin atrophy [1], probably due to greater unbalanced collagen degradation. However, the highly significant correlation ($r = 0.906$; $P < 0.001$) between the markers of type I collagen synthesis (PINP) and degradation (SP4) indicates a good balance in our patients. Since the concentrations of both of these markers was higher than those in serum and there was a difference between the two sides, they must reflect the local balance and cannot, e.g. enter the sample from systemic circulation.

We also found lower concentrations of the MMP-2/TIMP-2 complex in the irradiated skin, although no differences were found in the concentrations of TIMP-1 and TIMP-2. We have previously reported in the same patients, that in the suction blister fluid, there was more of the MMP-2/TIMP-2 complex in the irradiated skin [19]. Thus, the interpretation of these findings is not simple. MMP-2 (gelatinase A) is one of the major collagen degrading MMPs in skin, and it is constitutively expressed in epidermal keratinocytes and dermal fibroblasts [9]. MMP-2 is needed during the prolonged remodelling phase of would healing. Our conclusion is that the collagen degradation capacity and its inhibitors are not changed much, although the overall turnover of type I collagen is increased.

What are the mechanisms inducing these changes in skin collagen? Ionizing radiation induces various growth factors and cytokines, including tumor necrosis factor TNF-α, interleukin-1 (IL-1), fibroblast growth factor FGF and platelet-derived growth factor PDGFα. Transforming growth factor β1 (TGF-β1) has been shown to be involved in immediate skin reactions and also in the late development of skin lesions induced by radiation [12]. In rats, TGF-β1 significantly accelerates soft tissue repair and wound-breaking strength in the irradiated skin [6]. In this respect, the findings in scleroderma are very interesting, since in the presence of an underlying connective tissue disease, the effects of irradiation on skin collagen are aggravated. It would be interesting to study whether radiation-induced damage to skin could be used as an experimental model for scleroderma.

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